UNIVERSITY OF SOUTHAMPTON

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CANCER SCIENCE

EMT induced RIP1 expression defines TRAIL sensitivity in carcinoma cells

by

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ABSTRACT

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EMT INDUCED RIP1 EXPRESSION DEFINES TRAIL SENSITIVITY IN CARCINOMA CELLS

Alhanouf Fahad Alshedi

Despite recent advances in biomarker discovery and therapeutic options, metastasis still stands as the primary reason for cancer-related mortality. This highlights the pressing need for the identification of new targets for the treatment of metastases, and new markers of metastatic capability, to tailor aggressive therapy to higher-risk cases. Metastatic cells often become therapy-resistant, and this necessitates novel therapeutic interventions. Epithelial-Mesenchymal Transition (EMT) is the key cellular process inducing metastatic phenotype in cancers.

Cell culture, western blotting, flow cytometry and microscopy techniques were used in this study. We used naturally epithelial or mesenchymal carcinoma cell lines, TGFβ–induced EMT model and a stable ZEB1 knock-down (Mesenchymal-Epithelial Transition) MET model.

TRAIL (Tumor necrosis factor-related apoptosis-inducing ligand) is known to induce apoptosis in a subset of cancer cells. We hypothesized EMT status of cancer correlates with TRAIL sensitivity. We initially classified a total of 11 (breast and colorectal cancer) cell lines in terms of their EMT status and TRAIL response. Mesenchymal cells responded to TRAIL better than their epithelial counterparts. Proteins that were shown to influence TRAIL response were examined towards their contribution to TRAIL sensitivity and classified according to the EMT status of cell lines. RIP1 expression pattern matched TRAIL sensitive/mesenchymal phenotype. RIP1 holds a critical position in cellular pathways leading to enhanced survival (via NF-κB activation), apoptosis (via death receptor ligation) and necroptosis (via inhibited caspase-8 activity). Our results suggest that RIP1 is actively utilized by the naturally chemo-resistant metastatic cancer cells to gain survival advantage but can be used to direct cellular response from enhanced survival to cell death with TRAIL stimulation.

This study provided evidence that RIP1 is involved in the sensitivity of mesenchymal cells to TRAIL.

Overall, an increased understanding of the survival/apoptosis pathways of metastatic cells will help us devise new potential prognostic biomarkers and therapeutic regimens for cancer therapy.

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Academic Thesis: Declaration of Authorship

I, Alhanouf Alshedi

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

EMT induced RIP1 expression defines TRAIL sensitivity in carcinoma cells

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
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Abbreviations

ATCC	American Type Culture Collections
B	Borderline
BC	Breast Cancer
BCL-2	B-cell lymphoma 2
BSA	Bovine serum albumin
CDH1	E-cadherin
c-FLIP	Cellular flice (fadd-like il-1β-converting enzyme)-inhibitory protein
c-IAP	Cellular Inhibitor of Apoptosis Protein
COX2	Cyclooxygenase
CRC	Colorectal cancer
CSCs	Cancer stem cells
CTNNB	Cadherin-associated protein, Beta
CTNND1	Catenin delta 1
DcR1	Decoy receptor 1
DcR2	Decoy receptor 2
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulphoxide
DRC	Death Receptor Components
DRP1	Dynamin-related protein 1
DRs	Death Receptors
E	Epithelial
E-cad	E-cadherin protein
ECM	Extracellular Matrix
ELAM-1	Endothelial cell leukocyte adhesion molecule-1
EMT	Epithelial–mesenchymal transition
EMT-TFs	EMT- transcription factors
FACS	Fluorescence-activated cell sorting
FADD	Fas Associated Death Domain
FGF	Fibroblast growth factor
FSC	Forward scatter

GSK3β	Glycogen synthase kinase-3β
ICAM-1	Intercellular adhesion molecule-1
IKKα	IκB kinase α
IKKβ	IκB kinase β
IL6	Interleukin-6
IκBα	Inhibitor of nuclear factor kappa B
KD	knock-down
kDa	KiloDalton
M	Mesenchymal
MET	Mesenchymal-epithelial transition
MFI	Mean fluorescence intensity
MLKL	Mixed-lineage kinase-domain like
MLKL	mixed-lineage kinase-domain like
MMP-9	Matrix metalloproteinaase-9
NF- κB	Nuclear Factor –κB
NIK	NF-κB-inducing kinase
NKs	Natural Killer cells
NOS	Neuronal nitric oxide synthase
NR	Non-responders
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffer Saline
PTECs	Proximal tubular epithelial cells
R	Responders
RHD	Rel homology domain
RHIM	Homotypic interaction motif
RIP1	Receptor-interacting serine/threonine-protein 1
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard Error of Mean
shRNA	Short hairpin RNA
SIP1	Smad Interacting Protein 1
siRNA	Small Interfering RNA
SODD	Silence of death domain
SSC	Side scatter

TEMED	Tetramethylethylenediamine
TG2	Transglutaminase 2
TGF-β	Transforming growth factor β
TGF-βRI	TGF-β receptor type I
TGF-βRII	TGF-β receptor type II
TMRE	Tetramethylrhodamine, Ethyl Ester, Perchlorate
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor alpha
TRADD	TNFR1 associated death domain
TRAF2	TNF receptor-associated factor 2
TRAIL/Apo2L	Tumor necrosis factor-related apoptosis-inducing ligand or Apo 2 ligand
VCAM-1	Vascular cell adhesion molecule 1
Vim	Vimentin
WB	Western blotting
WT	Wild type
ZEB1	Zinc finger E-box binding homeobox 2
ZO-1	Zonula occludens-1
α-SMA	Alpha-smooth muscle actin

Chapter 1: Main introduction

1.1Cancer

Cancer is a disease in which an individual mutant cell acquires specific biological features that enable it to divide and grow more vigorously than its neighbouring cells, ultimately allowing it to survive. The selective biological properties include sustaining proliferative signalling, evading growth suppressors**,** resisting cell death**,** enabling replicative immortality**,** inducing angiogenesis**,** and activating invasion and metastasis [2]**.** The first 6 hallmarks of cancer were identified in 2000, these include evading apoptosis, maintaining growth signalling, insensitivity to anti-growth signals, tissue invasion and metastasis, immortality, and sustained angiogenesis [2] **Figure 1**.

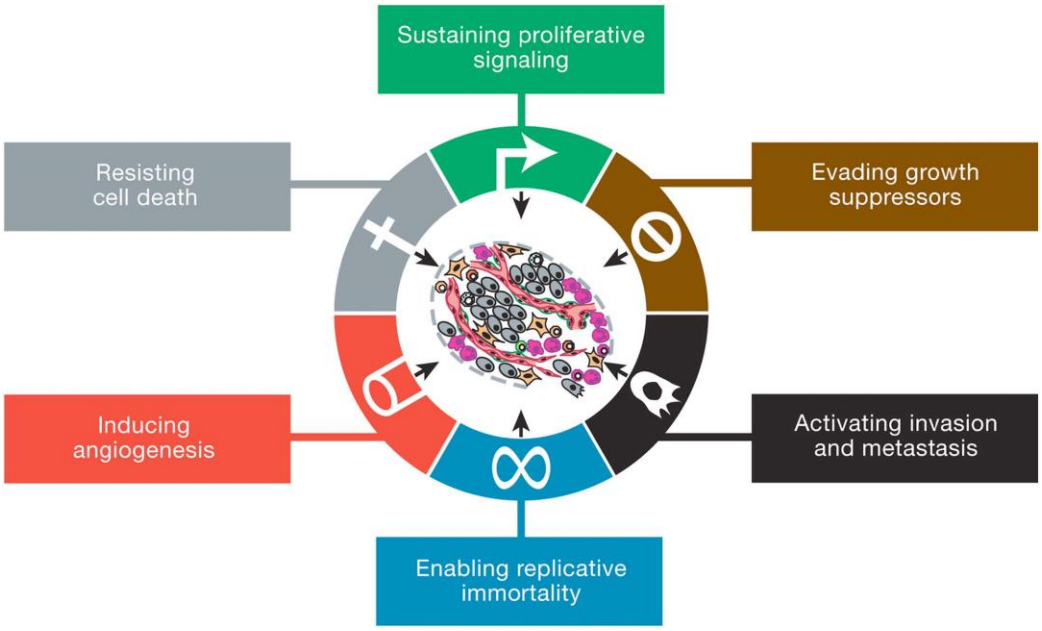


Figure 1. The core hallmarks of cancer established in 2000.
Schematic taken from Hanahan and Weinberg [2].

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths is 2020, according to world health organization, The three most common cases of cancers in 2020 were breast (2.26 million cases), lung (2.21 million cases) and colon and rectum (1.93 million cases). The most common causes of cancer death in 2020 were, lung (1.8 million death), colon and rectum (916 000 deaths) and breast (685 000 deaths) [3]; **see supplementary figure 1**.

1.1.1 Cancer metastasis

The spread of cancer is a multistep process whereby epithelial cells from primary tumours first invade the local basement membrane, the surrounding extracellular matrix (ECM) and the stromal cell layers. They then intravasate into blood and lymphatic vessels, survive in the circulation, escape immune surveillance and populate secondary sites. Next, metastatic cells invade into the microenvironment of distant organs forming micro- metastases, and finally re-start their proliferative programs where they can be clinically detectable [4], [5]; Blockade of any of these steps could result in a complete failure of the whole metastatic process [6]. Cells from most types of carcinomas can detach from the primary tumour and migrate as a group of cells, termed 'collective invasion' or as individual cells. Invasion mechanisms include 'mesenchymal invasion' and 'amoeboid invasion' programs [7]. Although individual cell invasion is not compatible with epithelial tissue architecture due to strong cell-cell adhesion, an embryonic morphogenesis program known as 'epithelial to mesenchymal transition' (EMT) can help explain how cells from an epithelial origin are able to migrate individually [5]. It is recognised that more than 90% of all cancer-related mortalities are as a result of metastasis; accordingly, there is an urgent need to ensure metastasis is prevented [2]. In fact, EMT is believed to be an important process in the conversion of clustered tumour cells into single motile ones that are capable of initiating systemic metastasis [8], [9] . EMT in cancer will be discussed in detail later in this thesis.

1.2 Epithelial-to-Mesenchymal Transition

Epithelial-to-Mesenchymal Transition (EMT) is defined as a critical epigenetic cellular trans-differentiation process that facilitates the change of epithelial cells, adopting a fibroblast-like state [1]. During the 1980s, Hay completed observations on EMT, and was notably recognised as being the first to have used the term now referred to as 'epithelial to mesenchymal transition'[10]. In this regard, the word 'transformation' was replaced with 'transition' in mind of highlighting its transient nature and reflecting the plasticity of the process in converting cells from epithelial to mesenchymal. Importantly, mesenchymal cells can also turn into epithelial cells through a reverse process, which is referred to as 'mesenchymal-epithelial transition' (MET). It can be stated that epithelial cells are acknowledged as having the exceptional capacity to change into mesenchymal cells and accordingly return to their original state [11].

These embryological EMT and MET programs can be also activated in association with tissue repair, fibrosis and cancer progression during adult life. The basic features of embryonic and adult

EMT programs are similar. Many of the signalling pathways and transcription factors which are important during physiological EMT process are also activated during pathological EMT [12]. However, EMT in these different biological settings may differ depending on the biological context and the functional consequence [1]. For that, EMT has been classified into three different subtypes; developmental (Type 1), fibrosis and wound healing (Type 2), and cancer (Type 3) [1]. However, before defining EMT classification (subtypes), it is worth describing the main features of epithelial, mesenchymal and intermediate (metastable) cells, EMT biomarkers and signalling pathways **see figure 2** [13].

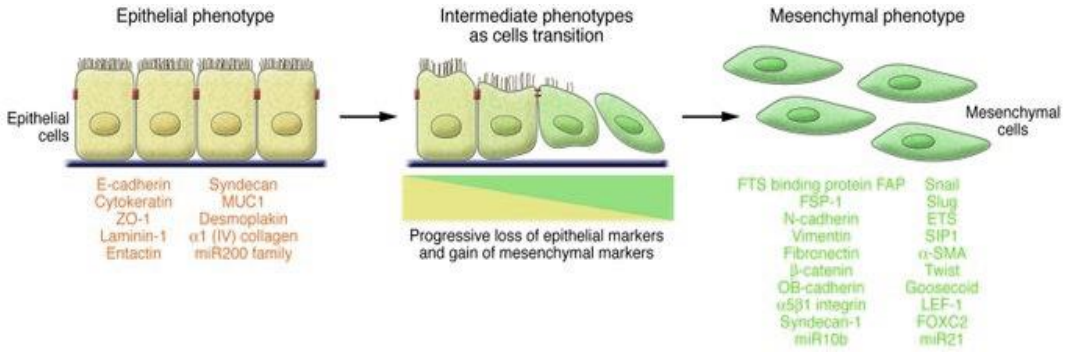


Figure 2. Schematic illustrating the morphological change and protein expression during Epithelial-Mesenchymal Transition (EMT).

EMT is a fundamental biological process, by which epithelial cells lose epithelial features and acquire features similar to mesenchymal cells. Not all cells undergo complete EMT, some cells can have both epithelial and mesenchymal features at the same time, and this called partial EMT with intermediate phenotype. Figure taken from Kalluri and Weinberg (2009) [1].

1.2.1 Epithelial cell phenotype

Epithelial cells are robust tissues that have two biological functions: 1) to support the structure of organs and 2) to aid as effective barriers against pathogens. These vigorous functions require tight association between cells through the assembly of different cell junctions that stabilise the integrity of the tissue. These junctions are occluding junctions (tight junctions), channel-forming junctions (Gap junctions) and anchoring junctions (cell-cell junctions or cell-matrix junctions). Cell-cell junctions are further divided into adherent junctions and desmosomes.

Adherent junctions include trans-membrane cadherin linking two adjacent cells and they are supported by intracellular cytoskeletal actin filaments and intracellular anchor proteins such as β-catenin, α-catenin and p120-catenin. The three well-known cadherins are E-cadherin, which is

present in many types of epithelial cells, N-cadherin that is present in nerve, muscle and lens cells, and P-cadherin which is present on cells in the placenta, epidermis and epithelium [14].

Occluding junctions between squamous epithelial cells serve as selectively permeable barriers, by tight junctions or Zonula occludens that separating the fluid that infuses the tissue on their basal side from fluid with a different chemical composition on their apical side. These junctions also protect molecules from leaking freely across the cell membrane. The major trans-membrane proteins forming occluding junctions are called claudins and occludin [15]. There is also the gap junction (channel-forming junction) which links epithelial cells by creating a pathway that links the cytoplasm of adjacent cells [16].

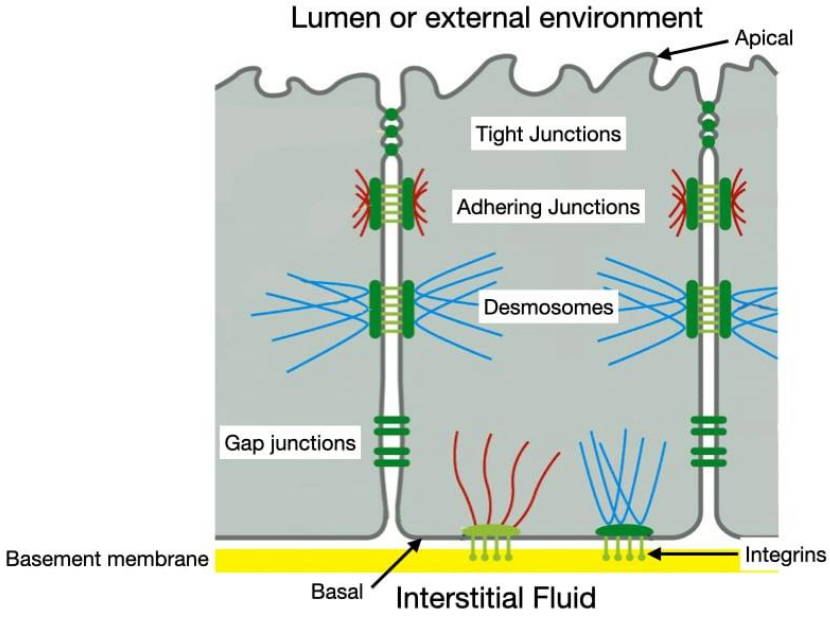


Figure 3. Various junctions in the epithelial cells.
Taken from: [17]

1.2.2 Mesenchymal phenotype

These cells are individual fibroblast cells with a spindle-like shape. Fibroblasts are involved in many processes such as wound healing, scar formation, tissue fibrosis as well as tumour invasion and metastasis during cancer. Fibroblasts secrete high levels of ECM-degrading proteases such as MMP2, MMP3 and MMP9 and abundant quantities of growth factors such as hepatocellular growth factor (HGF), insulin like growth factor (IGF). In culture, fibroblasts are often identified by their spindle-shaped morphology. Mesenchymal carcinoma cells resemble fibroblasts in their shape; elongated with filopodia and displaying a front-back polarity. In addition, they express fibroblasts markers such as vimentin [18] and α-smooth muscle actin (α-SMA) [19].

Nevertheless, both mesenchymal cells and fibroblasts lack specific protein markers, and are considered the least understood cells at the molecular level [20] [1].

1.2.3 Intermediate/borderline phenotype

EMT is a multistep program by which epithelial cells lose their epithelial junctions and acquire mesenchymal cell properties [3]. However, not all cells undergo complete EMT. Instead, some cells undergo partial EMT during development and cancer with cells maintaining an intermediate or a metastable phenotype, which is described by cells possessing some epithelial junctions but simultaneously exhibiting mesenchymal features including invasion, migration and stem-like properties [1], [21], [22]. Huang et al. have classified the EMT status of 43 ovarian cancer cell lines into 4 groups including epithelial, intermediate epithelial, intermediate mesenchymal and mesenchymal [23]. However, until now, intermediate EMT states have not been well characterised, and there is no biomarker/s to specify them. It is proposed that the cells with partial EMT acquire some of the stem-cell like properties [22], [24], although further identifying this subpopulation in EMT would be beneficial. In fact, describing EMT with metastable states could imply a spectrum of heterogeneity instead of only presenting the two extreme ends of the EMT process, and could increase the advantage of more targeted therapies [23].

1.2.1 EMT biomarkers

As previously mentioned, during EMT, epithelial cells gain spindle shape morphology and lose polarity. Such cells also express mesenchymal/fibroblast cell markers and lose epithelial cell adhesion molecules and increase motility [9], [8]. The major EMT biomarkers *in vitro* include cell-surface proteins (E-cadherin, N-cadherin, ZO-1 and integrins), cytoskeletal proteins (α-SMA, vimentin, and β-catenin), extracellular matrix proteins (Collagens, Fibronectin, and Laminin), transcription factors (SNAIL1/2, TWIST1/2, ZEB1/2). The main EMT biomarkers will be discussed in detail with a focus of their expression in cancer.

1.2.1.1 Cell surface Markers of EMT

Down-regulation of E-cadherin is considered a typical marker for EMT in various types of cancers [25-29]. Altered expression of different cadherins (the cadherin switch), have been increasingly used to monitor EMT with N-cadherin and E-cadherin up- and down-regulated, respectively [9]. Zonula occludens (ZO) proteins, comprising ZO-1, -2 and -3, are peripheral proteins which localise at tight junctions of epithelial cells. ZO proteins have a scaffolding function providing the structural basis for various cell-cell junctions [30]. In fact, down-regulation of ZO proteins are reported during the invasion of many tumour types upon EMT activation [31].

Integrins are cell surface adhesion receptor molecules that mediate the attachment of cells to the ECM and upon ligand-binding trigger critical intracellular signalling pathways [32]. A change in the level of expression of different integrins (an integrins switch) often reflects alterations in cell-ECM interactions. For example, in colon carcinoma, cancer cells that have undergone EMT express high levels of αvβ6 (hemidesmosomal) integrins compared to normal colon epithelium and non-invasive cancer cells [33].

1.2.1.2 EMT Cytoskeletal Markers

Vimentin (VIM) is a main component protein of intermediate filaments, which is expressed widely in various cells such as normal fibroblasts, endothelial cells, neuronal precursors, and cells of the hematopoietic lineage. It is known to maintain cellular integrity and is involved in cell migration, motility, and adhesion [34], [35]. When overexpressed in cancer, vimentin correlates with increased tumour growth, invasion and poor prognosis [36], as reported in gastrointestinal tumours, prostate, breast and lung cancers [37-40]. In fact, vimentin has gained significant importance as a marker for EMT [41].

α-SMA is one of the six actin isoforms that is predominantly found in vascular smooth muscle cells and myoepithelial cells. α-SMA has also been found in myofibroblasts which are mainly present in healing wounds and fibrotic tissue [42]. In addition, α-SMA over-expression is associated with EMT in cancer [43], [44].

β-catenin is a cytoplasmic plaque protein which is mainly localised at the membrane of normal epithelial cells. In fact, β-catenin has been used as a marker of EMT in various studies [45], [46].

Cytokeratins are intermediate filament proteins which interact with desmosomes and hemidesmosomes, thus providing mechanical stability and integrity of epithelial cells and tissues. They also collaborate in various cellular processes such as cellular polarity and migration [47]. Loss of keratin expression is another marker of EMT, and it was found to activate EMT features of enhanced motility, invasion and chemo-resistance [48].

1.2.2 EMT-inducing Transcription Factors

Epithelial gene repression and mesenchymal genes induction are coordinated by EMT transcription factors, commonly directing both activation and repression, as highlighted in the work of Mani *et al.* (2008) [49]. The full molecular reprogramming that occurs during EMT is mastered by the following major groups of transcriptional factors: ZEB1/2, SNAIL1/2, TWIST1/2. These factors do not only repress E-cadherin but also to coordinate the whole EMT program [26]

ZEB 1/2: the ZEB family consists of two members (ZEB1; also known as δEF1, and ZEB2; also known as smad-interacting protein 1 (SIP1) which are highly conserved proteins across species [50]. Both ZEB1 (1124 amino acid) and SIP1 (1214 amino acid) proteins contain two clusters of zinc finger domains and one homeodomain. ZEB1 and ZEB2 trigger EMT by direct binding to E-boxes in the E-cadherin (*CDH1*) gene promoter through their zinc finger domains, and as a result repress E-cadherin expression [26],[51]. When EMT is induced, ZEB factors not only transcriptionally repress E-cadherin but also repress other epithelial markers and activate mesenchymal genes [52]. There are many extracellular and intracellular stimuli can induce the activation of ZEB proteins, such as TGF-β [53], [54]. In fact, high expression of these proteins has been found in a wide range of cancers including colorectal, breast, liver, bladder, gastric, prostate, and pancreatic carcinomas which are associated with increased aggressiveness and metastatic capacity [53, 55-58]. ZEB factors have been shown to be a reliable independent predictor of cancer-related survival and response to chemotherapy in many carcinomas [59].

SNAIL1/2: the SNAIL family comprises three members: SNAIL1 (originally known as SNAIL), SNAIL2 (SLUG), and SNAIL3 (Smuc). All of them share a common structure: a highly conserved C-terminal region, containing four to six zinc fingers and a highly conserved N-terminal SNAG domain region. SNAIL1/2 proteins repress E-cadherin expression through binding to the E-boxes in the *CDH1* gene promoter which in turn promotes EMT [60]. Similar to the ZEB family, SNAIL1 and 2 proteins are absent in normal epithelium and their presence is reported to be associated with

aggressive disease and an independent prognostic factor of poor survival in many carcinomas such as breast, colon, liver, gastric, ovary and lung [61-65].

TWIST 1/2: the TWIST family consists of TWIST1 and TWIST2 which share a basic/helix-loop-helix (bHLH) structure; two parallel α-helices joined by a loop required for dimerization [66]. Like ZEB and SNAIL families, TWIST proteins are absent in normal epithelium but are induced in a number of human carcinomas such as breast, liver, prostate and ovarian cancers. TWIST factors are also reported to be independent prognostic factors associated with tumour aggressiveness, recurrence and poor patient survival [67]. However, the upstream signalling pathways of TWIST1 and TWIST2 are less understood compared to ZEB and SNAIL family of EMT-inducers [53].

1.2.3 EMT subtypes

EMT can be categorised into three different subtypes in line with the various biological and biomarker contexts that are seen to take place. EMT associated with embryonic formation, implantation and organ development can be arranged to induce a good degree of diverse cell types; these have mesenchymal phenotypes in common. In this regard, the study of Kalluri & Weinberg suggested that this category of EMT could be considered 'Type 1', whilst 'Type 2' would be seen to represent EMT as linked with the regeneration of tissue, the healing of wounds and organ fibrosis. In the case of neoplastic cells, EMT is referred to as 'Type 3', where neoplastic cells have previously demonstrated epigenetic and genetic changes, especially in the case of genes that have a tendency to show a preference for clonal outgrowth [1]. Importantly, those changes that are detected in cancer cells—notably changes that are genetic in nature—have an impact on both tumour suppressor genes and oncogenes, and further collaborate with EMT regulatory circuits. In turn, this causes a number of results that may differ significantly to those that are recognised in other EMT types.

1.2.3.1 Type 1 EMT: EMT associated with embryo formation and organ development

This type of EMT is well-defined and typically accomplished by a cell fate decision with neither causes fibrosis nor induces pathological invasiveness [1]. In regards embryonic development, EMT maintains critical significant role in the implantation of the embryo and the formation of the placenta throughout the initial stages of embryogenesis. Throughout development, EMT induces

cells with a mesenchymal phenotype with the aim of developing new tissues with various functions and activities **see figure 4**. EMT gastrulation is recognised as one form of Type 1, [68] which is seen to be an embryonic event that facilitates primitive germ layers: the ectoderm, mesoderm and endoderm. During gastrulation, the basement membrane underlying the epiblast breaks down leading to induction of EMT in the cells of the primitive streak by the action of FGF (fibroblast growth factor) resulting in up-regulation of SNAIL and repression of E-cadherin [69]. With complete EMT induction, the cells within the primitive streak undergo ingression and subsequently some either undergo MET and give rise to the endoderm or remain mesenchymal and give rise to the mesoderm [70]. In addition of gastrulation, EMT is also critical in neural crest formation [71] and heart valve formation [72], which are all types 1 EMT.

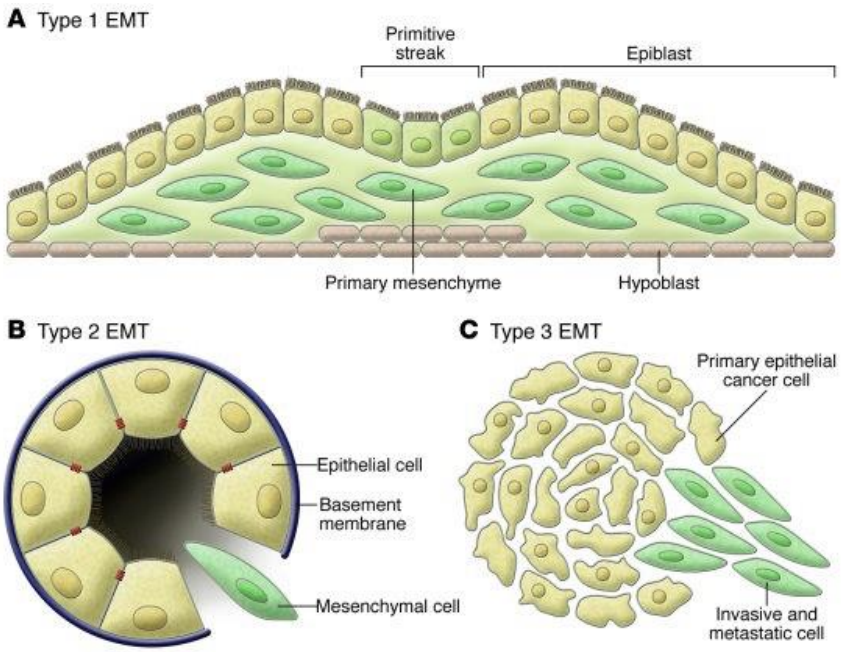


Figure 4. EMT subtypes. Taken from [1]

A. Type 1 EMT occurs during implantation, embryogenesis and organ development; this type of EMT is not associated with organ fibrosis or malignant metastasis, though type 1 EMT has the potential to be associated with MET to form secondary epithelia. **B.** Type 2 EMT is implicated in wound healing, organ fibrosis and tissue regeneration. It occurs in response to injury and contributes to repair processes by generating fibroblasts and other cells to help repair the tissue. **C.** Type 3 which are mainly involved with cancer progression (metastasis).

1.2.3.2 Type 2 EMT: EMT associated with wound healing, tissue regeneration, and organ fibrosis

In type 2 EMT, the program is initiated as part of repair process which leads to generation of fibroblasts and other cells to rebuild tissues following trauma and injury. In contrast to Type 1 EMT, Type 2, is associated with inflammation, and terminates once tissues recovered from the injury or once inflammation ends. Of note, Type 2 EMT could result in organ fibrosis and deconstruction in case of ongoing inflammation, eventually leading to organ destruction. In fact, tissue fibrosis is considered as an undiminished form of wound healing due to persistent inflammation [1]; **figure 4, type 2 EMT**. Organ fibrosis is mediated by inflammatory cells such as macrophages and myofibroblasts that can trigger EMT through the release of growth factors, such as TGF-β, EGF, and FGF [73], [74]. Activation of EMT via SNAIL leads to the acquisition of renal fibrosis and renal failure in transgenic mice [62]. High SNAIL expression and features of EMT have been also found in the kidneys of patients with renal fibrosis [62], [75]. Similarly, in patients with Crohn's disease, EMT was demonstrated in areas of fibrosis in the colon [76]. For that, developing novel therapeutics with a potential of reversing organ fibrosis via reversal of EMT is vital.

1.2.3.3 Type 3 EMT: EMT associated with cancer progression and metastasis

The EMT program has been widely demonstrated to enhance cancer cell migration and invasion in many *in vitro* models. EMT in regards tumour development and malignant transformation has been evidenced by a number of different studies and is recognised as Type 3 EMT. Nonetheless, EMT importance specifically in *in vitro* throughout cancer progression has been viewed with some degree of dispute throughout the last decade, predominantly owing to the lack of supporting evidence within clinical samples [9]. However, evidence has come from cancer cells at secondary sites which were found to resemble, at the histopathological level, the primary tumour indicating that these metastases colonise through activation of the reverse EMT process, MET [1]. Further evidence is the observation of small aggregates of tumour cells at invasive fronts detaching from the tumour mass into the adjacent stroma producing single migratory cells that lose E-cadherin expression [77]; **figure 4 type 3 EMT.** Without question, EMT has been the focus of much work and gathering of evidence in regards its capacity to enhance cancer cell invasion and migration across a number of different *in vitro* models. In consideration to such findings, EMT/MET significance in cancer has received much acknowledgment and further research efforts [78].

Importantly, EMT is not only involved in cancer metastasis, but also in other events highly relevant to tumour progression, including senescence, de-differentiation and immunosuppression, resistance to cell death and therapeutic resistance [41], [49], [79]. However, the link between EMT and drug resistance is not yet fully understood. A burgeoning body of literature now suggests that a better understanding of mechanisms underlying EMT and therapeutic resistance is urgently needed and will facilitate developing novel therapeutic strategies. The main focus in this thesis will be the association between EMT and therapeutic resistance as it the second aim of this project.

1.2.4 Signalling pathways in EMT

The regulation of EMT in cancer is yet to be completely clear. Indeed, the mechanisms underlying the acquisition of the invasive phenotype and the ensuring of systematic spread of the cancer cells have been the object of intensive research. As mentioned before, during EMT, epithelial cells lose expression of typical epithelial markers such as E-cadherin and keratins, and gain expression of mesenchymal markers, including vimentin and N-cadherin, which allow cells to migrate through extracellular matrix (ECM), thus increase motility and invasiveness [49], [80]. The acquired mesenchymal phenotype is associated with enhanced resistance to apoptotic signals and thus help circulating cells to survive in bloodstream escaping the immune defence and chemotherapeutic agents [1]. Down-regulation of E-cadherin is frequently found during tumour progression in various types of cancers [81], hence, loss of E-cadherin is an indicator for poor prognosis and metastasis [82], [25]. Despite a host of other events known to be involved in EMT, the repression of E-cadherin is often considered to be the critical event during EMT. This major event is supported by the observation that genetic deletion of E-cadherin in mice results in the formation of lobular carcinoma [83], [81]. Furthermore, breast ductal carcinomas with low abundance of claudin (a critical component of tight junctions) are shown to have decreased expression of E-cadherin, thus linking the expression of E-cadherin with the classical EMT factors in a clinically relevant fashion [84]. In this regard, mutations in the gene encoding E-cadherin have been detected in cancer cells, making them more vulnerable to EMT and metastasis. Although these mentioned above studies seem to provide convincing evidence for a definitive link between E-cadherin and EMT, the loss of E-cadherin alone is not necessarily indicative of a migratory phenotype, nevertheless, beside its crucial function in maintaining epithelial phenotype of the cells, it is the most notably and easily identifier marker of EMT [85].

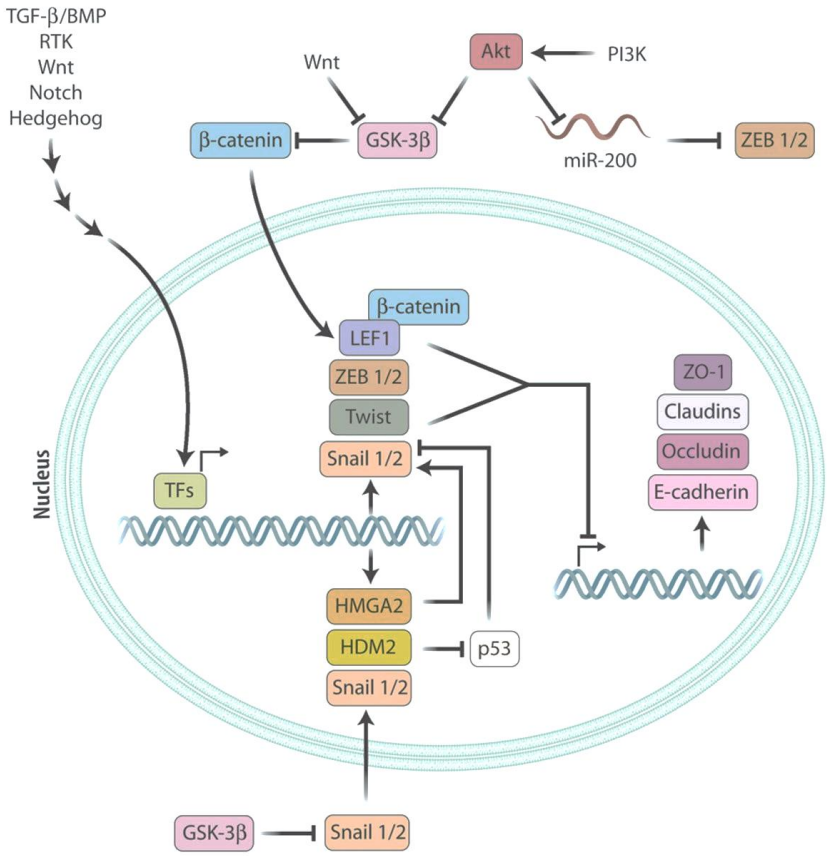


Figure 5. Signaling pathways mediating EMT.

Wnt, Notch, hedgehog, TGFβ and other growth factors of cytokines transduce signal cascades, modulate the expression EMT regulators and allow them to translocate to nucleus. They act as epithelial repressors (EpR) and/or mesenchymal activators (MeA) and bind with E box of promoter regions of epithelial genes and mesenchymal genes respectively. These complexes have an effect on EMT program by repressing epithelial genes and activating mesenchymal genes. [86]

The signalling pathways that contribute to EMT in cancer are not completely clear yet. EMT can be triggered by various intrinsic signals as well as extrinsic signals [280]. It has been suggested that the tumour-associated stroma is the main origin of extrinsic signals. In many carcinomas, HGF, EGF, PDGF and TGF-β are the main factors secreted by stromal cells which activate intracellular signalling proteins such as ERK, MAPK, PI3K, AKT, SMADs, β-catenin and RAS**.** Upon activation of these signalling networks, SNAIL1/2 and ZEB1/2 orchestrate EMT [41],[87],[88],[89]; **figure 5**. Activation of EMT programs is also facilitated by the disruption of cell-cell junctions and the cell -ECM adhesions [90]. The main biomarkers implicated in inducing EMT in cancer have been discussed in **section1.2.1**

As we previously mentioned, a group of transcription factors/repressors including Snail, Slug, ZEB1, ZEB2 (SIP1) and Twist, have been shown to suppress E-cadherin, thus induce EMT [79], [91], [92], [59]. These transcriptional factors are considered as a key initiating step in EMT, and their role has been discussed in detail in **section 1.2.2**. Expression and activation of EMT-inducing transcription factors occurs in response to several signalling pathways including transformer TGF-β [93], FGF [94], Wnt/β-catenin [95], Hh and Notch [96], all of which are involved in EMT regulation [9], [97], [11]. These pathways signal through intracellular kinase cascades to induce transcription factors that activate the expression of EMT-associated genes e.g. *ZEB1*. [98]. These pathways were reviewed in detail elsewhere [86], although a brief summary of TGF-β pathway which mostly contributed to our study is detailed below in order to have a better understanding of their contribution throughout the thesis. Understanding these mechanisms will help the therapeutic control of EMT to promote tissue regeneration and prevent cancer metastasis. Other signalling pathways as mentioned above were also shown to induce EMT however they are mostly context/cell type dependent and therefore cannot be listed as mainstream inducers of EMT.

TGF-β is the most physiological inducer of EMT which is utilised by cells during wound healing and inflammation [99]. It has been reported that adding TGF-β to epithelial cells in culture is an effective/efficient way to induce EMT in various epithelial cells [100], [101]. Upon TGF-β treatment, epithelial cells showed reduced expression of epithelial markers e.g., E-cadherin and increased mesenchymal marker such as vimentin and ZEB1. Morphologically, epithelial cells changed from cubelike to elongated spindle-like shape.

1.2.4.1 TGF-β

Although these above mentioned factors most likely contribute to induce EMT, TGF-β stand out as a major EMT inducer in cancer, and it is one of the best-studied pathways in EMT [102], [103]. This signalling regulates most cancers in which EMT is observed and acts through different intracellular messengers. TGF-β-induced activation of Smad complexes has crucial roles during induction of EMT [104].

The response to TGF-β depends on a signaling pathway that is initiated by the ligand-activated TGF-β receptor complex on the cell surface and is transduced into the nucleus by signaling mediators, the SMAD proteins [105]. Upon ligation, TGF-β binds to a specific pair of type I and type II receptor (TGF- β receptors) serine/threonine kinases, leading to the transphosphorylation and activation of the type I receptor (TβR-I) by the type II receptor (TβR-II). TGF-βRII phosphorylates TGF-βRI, resulting

in the activation of various intracellular signalling pathways including those mediated by SMAD2/3, RAS and PI3K Activated TβR-I then phosphorylates a specific subset of SMAD proteins, Smad2 and Smad3. Activated Smad2/3 complexes bind further to co-activator SMAD4, with the whole complex binds to DNA regulatory elements and subsequently activates a number of downstream effector proteins, most notably the Smad family of transcription factors **see figure 6**; [104]. Notably, Smads associate with Zeb proteins (Zeb1 and Zeb2/SIP1) to repress expression of E-cadherin during the initiation of EMT [106].

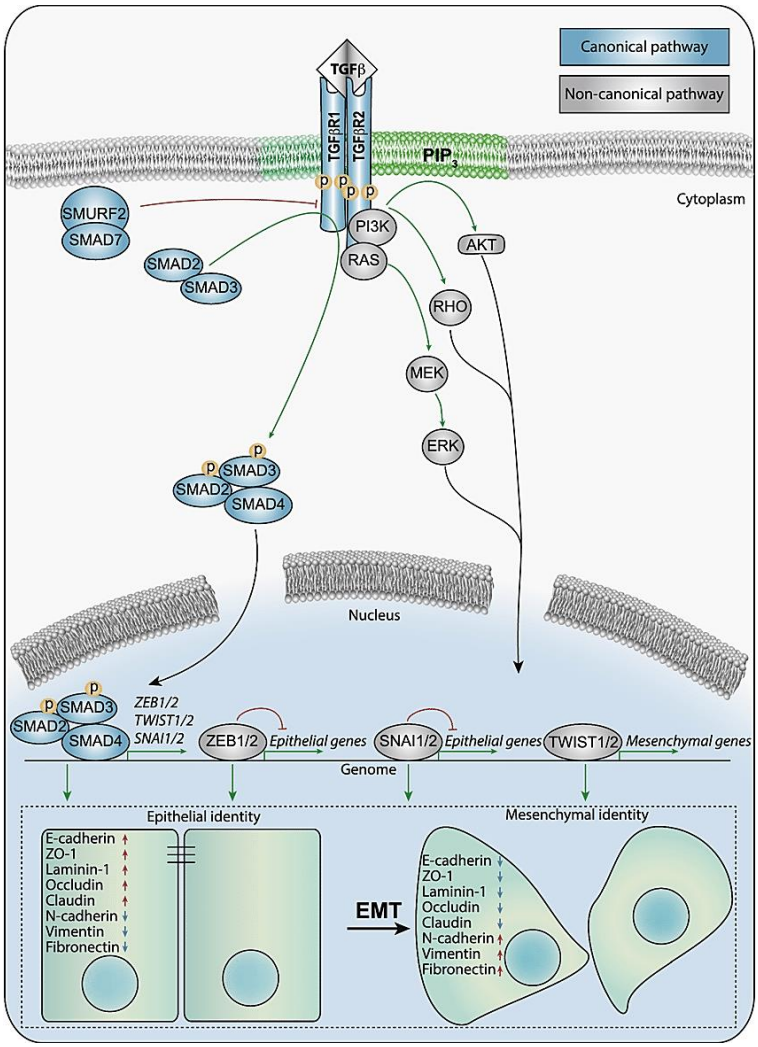


Figure 6. TGF-β- induced epithelial to mesenchymal transition.

TGF-β pathway can activate various signalling pathways that are either SMAD2/3-dependent or SMAD2/3-independent e.g., RAS and PI3K. The pathways activate transcription factors that induce the expression of various genes involved in enhancing cell proliferation and survival as well as invasion and metastasis. Taken from [107]

1.3 TNF- ligand family member: TRAIL

1.3.1 TRAIL

Tumour necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of TNF ligand family, and it was originally identified in 1990s as a cytokine secreted by most normal tissue cells, and rapidly induces apoptosis in different transformed cell lines [108]. TRAIL is a type II transmembrane protein composed of 281 amino acids, which binds with its cognate receptors (mentioned below in **section 1.3.2**). Since the discovery of TRAIL and its receptors most of the studies have focused on the apoptotic effect of TRAIL on carcinoma cells. TRAIL has been shown to kill a variety of tumor cells selectively, while sparing normal cells. This property has made TRAIL promising novel biotherapeutic agent for cancer therapy. Numerous studies using carcinoma cell lines expressing TRAIL receptors and treated with TRAIL have confirmed that TRAIL induces growth inhibition or apoptosis of carcinomas [109-113]. Nevertheless, resistance to TRAIL-mediated apoptosis in cancer cells remains a challenging issue for the successful application of TRAIL in gene therapy. Although many types of cancers are sensitive to TRAIL-induced apoptosis, considerable numbers of cancer cells are resistant to TRAIL [114], [115].

1.3.2 TRAIL receptors

TRAIL can bind to four membrane-bound receptors and one soluble receptor [116].These include TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and the fifth receptor reported for TRAIL is osteoprotegerin (OPG) [116]. TRAIL-R1 and R2 are both type I transmembrane proteins, containing an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The cytoplasmic domain contains the death domain DD, therefore TRAIL-R1 and R2 are called death receptors [117]. TRAIL-R1 and R2 can elicit an apoptotic death response upon binding of TRAIL in a variety of carcinomas and primary tumour cells [118]. Further to the ability TRAIL-R1 and R2 to induce cell death upon ligation with TRAIL, it has been found that both TRAIL-R1 and TRAIL-R2, might also be involved in non-apoptotic functions e.g. NF-kβ activation [119].

TRAIL also can bind to both TRAIL-R3 and TRAIL-R4 are also referred to as decoy receptor 1 (DcR1) and decoy receptor 2 (DcR2). However, due to the complete lack of intracellular DD that is essential for signalling apoptosis in these decoy receptors, TRAIL binding to each of these receptors fails to induce apoptosis [120].

Lastly, TRAIL can bind to a fifth receptor (OPG), a soluble member of the TNF receptor superfamily [121]. This receptor is involved in regulation of osteoclasts activation in the development and bone remodelling when bound by other members of the TNF family [122]. Given the low affinity of TRAIL binding to OPG [123], the physiologic role of TRAIL binding to OPG remains uncertain [124].

TRAIL receptor expression and distribution of has been studied as a mechanism by which normal tissues are resistant to TRAIL-mediated apoptosis, while cancer cells are not. In the beginning, it was proposed that the differential sensitivity to TRAIL-mediated apoptosis might be explained by the presence of TRAIL decoy receptors in normal tissues and TRAIL receptors on diseased cells or cancer cells [125], [126]. However, the expression of TRAIL decoy receptors did not correlate with the TRAIL susceptibility of several different cancer cell lines [127]. Studies have shown increased expression of TRAIL-R1 and TRAIL-R2 in many tumors, with decreased expression of both in surrounding normal tissues [128]. Nonetheless, normal colonic tissue expressing TRAIL receptors remained TRAIL-resistant [129]. Thus, simply having TRAIL receptors dose not confer sensitivity to TRAIL-mediated apoptosis.

1.3.3 TRAIL Signal transduction

TNF ligands such as TRAIL activates cell-survival, cell-death and necroptosis mechanisms simultaneously **see figure 7.** In the following sections, TRAIL-induced apoptosis, necroptosis and cell-survival will be described.

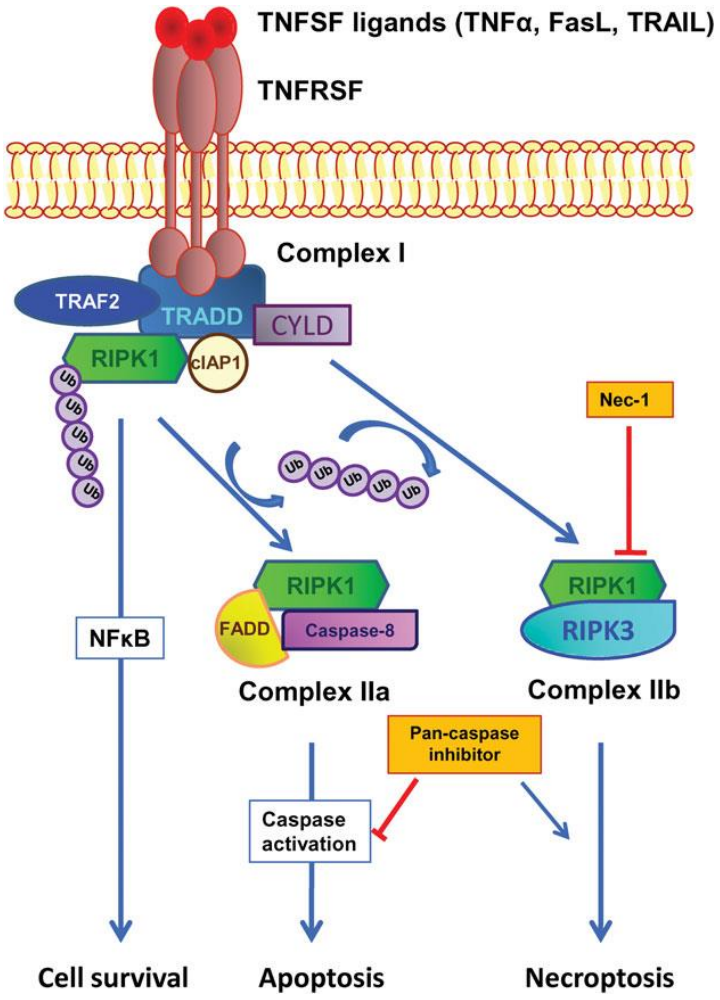


Figure 7. TNFSF ligand signalling of apoptosis, necroptosis, and cell survival.
Taken from: [130].

1.3.3.1 Induction of apoptosis

The interaction between TRAIL and its receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) is essential for TRAIL-induced apoptosis in carcinoma. Upon ligation of death receptors by their corresponding ligands, the extrinsic apoptotic pathway is initiated through oligomerization of the death receptors and recruitment of further adaptor proteins. When TRAIL binds to the pro-apoptotic receptors DR4 or DR5, TRADD promotes recruitment of Fas-associated protein with death domain (FADD) and deubiquitinated RIP1. Adaptor proteins, FADD, RIP1 and TRADD, enable cell death signalling to transduce from the exterior stimuli to the interior signalling cascade via homotypic proteins interactions [131]. Both FADD and RIP1 use their DD to interact with DDs within the initiator pre-caspase 8, leading to its cleavage and activation. FADD then recruits pro-caspase-8 or -10 and DISC (Death-inducing signaling complex) is formed which contains FADD, deubiquitinated RIP1 and the pro-caspase-8. At the DISC, pro-caspase-8 is then activated and promotes apoptosis [132]. Active caspase-8 released form the DISC complex is then activates downstream effector caspases such as caspase-3 and caspase-7 [116]. Subsequently, these active effector caspases cleave targeted cellular protein (such as PARP-1) and finally lead to cell death **see figure 8.** In addition, the inhibitory protein (c-FLIP) can compete with caspase-8 for binding to FADD, preventing the downstream activation of the apoptotic cascade [133]

In fact, cells can be classified into two different types (type I or type II), depending on how they activate death receptor-induced apoptosis. In type I cells, large amounts of caspase-8 is present in the DISC and thus facilitate activation of downstream effector caspases such as caspase-3, -6 and -7, directly. However, in type II cells, caspase-8 is less efficient, thus commitment of these cells to apoptosis pathway requires further signal amplification by intrinsic/mitochondria-dependent pathway [134]. Here, caspase-8 promotes cleavage of a protein called Bid (pro-apoptotic Bcl-2 family protein). When cleaved, truncated Bid (tBid) [135] translocate to the mitochondria and activates Bax and Bak, both pro-apoptotic Bcl-2 family member which cause depolarization of the mitochondrial membrane, and thus release of cytochrome c and other pro-apoptotic proteins. Bax, Bad has been shown to induce loss of mitochondrial membrane potential and lead to cytochrome c release [135]. Consequently, the apoptosome is formed leading to activation of caspase-9, which in-turn activates effector caspases -3, 6 and 7 leading to apoptosis vis the intrinsic pathway [134]. **see figure 8**.

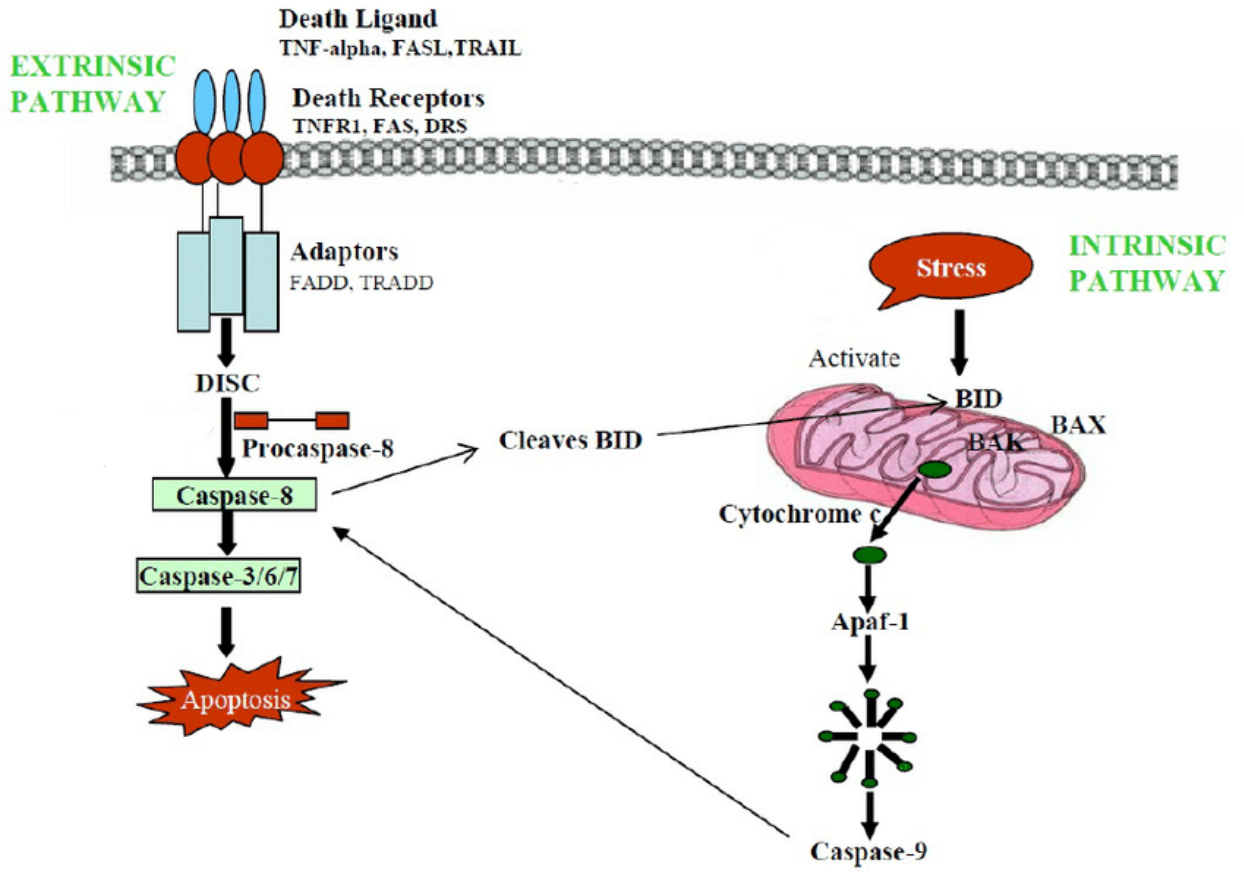


Figure 8. Extrinsic and intrinsic pathways of apoptosis.
Modified from: [136]

1.3.3.2 Initiation of necroptosis

Necrosis was firstly proposed in 1988 as an alternative form of programmed cell death. TNF is most frequently used stimuli for necroptotic cell death induction [137]. TRAIL also was found to induce necroptosis in a similar way to TNF, by ligation to its respective ligands. The apoptotic and necroptotic processes share some similar pathways and adaptor proteins. However, cell fate decision (apoptosis vs. necroptosis) is influenced primarily by the availability of caspase-8 and the cellular or inhibitors of apoptosis proteins (cIAP1, cIAP2, XIAP) [138]. For example, wherever caspase-8 is inhibited (by caspase-8 inhibiting protein such as CrmA or c-FLIP), apoptotic cell death is blocked and TNF is able to induce necroptosis [137].

Upon ligation, TNFR recruits several adaptor proteins to form complex 1, which contains TRADD-RIP1-TRAF2 and cIAP1/2. TRADD and RIP1 dissociate from plasma membrane into cytoplasm and recruit other proteins to form a secondary complex known as complex 2. By recruiting FADD and caspase-8, complex2 initiates apoptosis by activating the caspase pathway. However, in the absence of caspase-8 activity, RIP1 binds to RIP3 through a RHIM domain to form a complex with MLKL (mixed-lineage kinase-domain like) known as necrosome. In the necrosome, RIP1 utilize kinase activity to phosphorylate RIP3, subsequently phosphorylated RIP3 initiates the phosphorylation of MLKL at the threonine 357 and serine 358 residues for executing necroptosis [139]. MLKL is a key modulator of necrotic cell death signalling downstream of the kinase RIP3. Wang *et al.* (2012) found that RIP1, RIP3, and MLKL formed a necrosis complex in human cell lines [140].

Upon induction of necrosis by TRAIL, both isoforms of PGAM5, PGAM5L and PGAM5S, interacted with the RIP1-RIP3-MLKL necrosis complex and phosphorylated. Phosphorylated PGAM5S then recruited the mitochondrial fission factor DRP1 and activated DRP1 by de-phosphorylation, resulting in mitochondrial fragmentation and execution of necrotic death [140]. These observations establish a pathway consisting of RIP1, RIP3, MLKL and PGAM5 for mitochondria-mediated necroptosis in response to TNFR or any other stimuli.

In addition to trigger apoptotic/necroptotic pathways, other signalling pathways leading to NF-κB activation is induced by TRAIL receptors. TRAIL-R family has emerged as a key mediator of cell fate and survival [141]. A mechanism to protect cells from apoptosis can also be activated by TRAIL receptors. TRAIL-R1 activation result in the recruitment of RIP1 to TRADD, RIP1 can then activate NIK, which phosphorylates IKK leading to proteasome degradation of phosphorylated IκB as well as nuclear translocation and anti-apoptosis signaling of NF-κB [141]. In the following sections, we will describe NF-κB signaling pathway.

1.3.3.3 NF-κB classical pathway upon TNF/TRAIL stimulation

NF-κB is a key survival pathway that is activated by TNF family receptors. Upon activation, NF-κB family of transcription factors control the expression of a variety of genes involved in cell survival, differentiation and proliferation **see table 1.0** [142]. The contribution of NF-κB signalling in cell survival reveals its importance in cancer therapy. NF-κB components/ proteins are in a latent state (inactive) in the cytoplasm and requires extracellular stimuli for activation. Here I will briefly describe the classical NF-kB pathway that is triggered by TRAIL.

Numbers of studies show that NF-κB can be activated by TRAIL through ligation to TRAIL-R1 and TRAIL-R2 [143], [144]. Activated NF-κB by TRAIL involves the recruitment of receptor-interacting protein (RIP1) to the cytoplasmic domain of TRAIL-R1 and 2 by TRADD which results in further recruitment of other signalling molecules including TNF receptor associated factor2 (TRAF2) that can activate a protein complex [145]. Following this, TRADD-RIP1-TRAF2 is released.

In turn RIP1 and TRAF2 recruits key molecules that are responsible for further intra-cellular signalling. For instance, RIP1 is ubiquitinated and is essential for IKK complex activation. Studies showed RIP1 knockout cells fail to activate IKK in response to TNF-α [146]. Also, TRAF2 recruits the IKK complex by interacting with LZ motifs of IKKβ and IKKα. Taking together, RIP1 and TRAF2 are critical to TNFR-induced NF-κB activation as they activate IKK as well as the NF-κB-inducing kinase (NIK) [147], [148]. Once activated, the IKK complex then phosphorylates IκBα on Ser32 and Ser36, and subsequently, IκBα is ubiquitinated and degraded by the 26S proteasome and thus activates NF-κB, which then translocate to the nucleus and regulate the expression of variety of target genes such as c-IAP1, c-IAP2, XIAP, and c-FLIP, all of which found to have anti-apoptotic properties [149] **see table 1.**

Table 1. NF-κB target genes related to the enhancement of tumour progression.

Gene	Important for	Function	Reference	Human gene name
BCL-XL	Cell survival	Pro-survival Bcl-2 homologue	(Chen et al., 1999)	BCL2L1
cIAPs		Cellular Inhibitors of apoptosis	(You et al., 1997)	
XIAP		X- linked inhibitor of apoptosis	(Turner et al., 2007)	XIAP
c-FLIP		Pro-survival factor	(Kreuz et al., 2001)	CFLAR
TNF	Proliferation	Tumour necrosis factor	(Shakhov et al., 1990)	TNF
IL6		Interleukin-6, inflammatory cytokine	(Son et al., 2008)	IL6
Cyclin D1		Cell-cycle regulation	(Hinz et al., 1999)	CCND1
c-MYC		Proto-oncogene	(Duyao et al., 1990)	MYC
iNOS	Tumour promotion	Neuronal nitric oxide synthase	(Nakata et al., 2006)	NOS1
COX2		Cyclooxygenase, prostaglandin endoperoxide synthase	(Ackerman et al., 2008)	PTGS2
MMP-9		Matrix metalloproteinaase-9 (secreted collagenase involved in metastasis)	(Bond et al., 1998)	MMP9
ICAM-1	Metastasis	Intercellular adhesion molecule 1	(Bunting et al., 2007)	CD54
ELAM-1		Endothelial cell leukocyte adhesion molecule	(Whelan et al., 1991)	SELE
VCAM-1		Vascular cell adhesion molecule	(Iadermarco et al., 1992)	VCAM1

Metastatic/mesenchymal carcinoma cells are known to be highly resistant to apoptosis [55], [150]. A major focus in tumour biology has been identifying NF-κB targets involved in the regulation of survival and suppression of apoptosis which are activated as a result of EMT [151]. Inhibition of NF-κB activity has been reported to inhibit cell survival and tumour growth [152]

As we mentioned before, tumours with constitutive NF-κB activation usually show increased resistance to chemotherapy [153]. NF-κB has been suggested to be responsible for blocking the efficiency of chemotherapy and radiation in some types of tumour cells [149] **see table 1**. NF-κB activity inversely correlated with cellular sensitivity to chemotherapy in carcinoma cell lines [154]. Taken together, we have chosen to evaluate a key component in NF-κB pathway, apoptosis and necroptosis with a focus on EMT and resistance to apoptosis.

1.4 Hypothesis and the aims of the project

1.5..1 Hypothesis

EMT-induced RIP1 expression sensitizes metastatic cancer cells to TRAIL-induced apoptosis.

1.5..2 Aims of the project

1. Characterise the EMT status of our CRC and BC cell lines. The biological characteristics of 11 CRC and BC cell lines will be reviewed from the literature and comparisons of their morphological appearance will be carried out using a light microscope. In addition, various epithelial and mesenchymal markers will be used to assess the EMT status of the panel. Western blot (WB) will be the main technique used to obtain this goal.

2. To investigate TRAIL response in our panel. Apoptosis will be quantitatively and qualitatively analysed by western blotting and flow cytometry in both BC and CRC cell lines. **A.** To associate death as a response with EMT status and expression of proteins involved in TRAIL pathway to identify key molecules that control the outcome of TRAIL treatment. We will investigate vital molecules in the death receptor pathway components for apoptosis initiation in 11 cell lines with defined EMT status. From there, one molecule will be chosen to focus on further and most importantly to correlate its expression with the EMT status of some mesenchymal and epithelial BC and CRC cell lines. **B.** To associate whether TRAIL receptors DR4 and DR5 are increased during EMT and play a role in TRAIL sensitivity, we will investigate DR4, DR5 cell surface expression of 11 cell lines.

3. Finally, to check whether the morphology or EMT programming is critical for TRAIL sensitivity. To achieve this, we will knock *CTNND1* gene, encoding for the key epithelial junction protein p120-catenin, to generate a single-cell morphology and induce apoptosis by TRAIL. Also, we will knock down a master EMT transcription factor, ZEB1, in a mesenchymal cell line and examine TRAIL sensitivity in this knockout model. Additionally, we will induce EMT by TGFβ treatment and assess whether TRAIL sensitivity changes. In all these conducted experiments we will be questioning whether the chosen candidate molecule from death receptor mediated apoptosis pathway is altered in line with the response to TRAIL and EMT status.

Chapter 2: Material and Methods

2.1 Cell lines

2.1.1 Cell lines and morphology

The origin and morphology of six human colorectal cancer cells (CRC): RKO, HCT116, HT29, Coco-2, SW480, and SW620; and five breast cancer cells (BC): MDA-MB-231, HS-578T, MDA-436, T47D, and ZR-75-1 were reviewed from the literature and are summarized in the table below **(Table 2.0)**.

Table 2. The biological characteristics and suppliers of the CRC and BC cell lines.

CRC cell lines	***Source***	***Morphology***	***Phenotype***	***Supplier***
RKO	Human primary colon carcinoma	Mesenchymal	Adherent	Prof. Graham Packham
HCT116	Human colorectal carcinoma	Epithelial with few mesenchymal cells (Borderline)	Adherent	ATCC
HT29	Human colorectal adenocarcinoma	Epithelial	Adherent	ATCC
CaCo-2	Human colorectal adenocarcinoma	Epithelial	Adherent	ATCC
SW480	Human colorectal adenocarcinoma	Mesenchymal	Adherent	Not Known
SW620	Human colorectal adenocarcinoma	Epithelial with few mesenchymal cells (Borderline)	Adherent	ATCC
BCC cell lines	***Source***	***Morphology***	***Phenotype***	***Supplier***
MDA-MB-231	Human breast adenocarcinoma	Mesenchymal	Adherent	ATCC
HS-578T	Human breast carcinoma	Mesenchymal	Adherent	ATCC
MDA 436	Human breast adenocarcinoma	Mesenchymal	Adherent	ATCC
T47D	Human breast ductal carcinoma	Epithelial	Adherent	ATCC
ZR-75-1	Human	Epithelial	Adherent	ATCC

2.2Tissue culture

2.2.1 General principle

All tissue work was done in a laminar flow hood. Cells were grown at 37 C° in a humidified 5% CO2 incubator (Heraeus Hera Cell Incubator, Thermo scientific). All tissue culture reagents were stored in a dedicated 4 C° refrigerator.

2.2.2 Cell culture

Cells were grown in Dulbecco's Modified Eagle's Medium DMEM (Sigma) complemented with 10% Fetal Bovine Serum FBS (Sigma), 1% of 100 X Penicillin/Streptomycin (Sigma), and 2 mM L-Glutamine (Sigma). Of note, cell lines were regularly checked for Mycoplasma contamination (MycoAlert, Lonza). Only Mycoplasma-free cell lines were cultured and used.

2.2.3 Cell counting

A manual cell count was performed using a hemocytometer. A hemocytometer glass and coverslip were cleaned with alcohol. Afterwards, the coverslip was moistened with water and placed over the hemocytometer grid. After trypsinsation, the cell suspensions were diluted in 10ml complete medium DMEM. The cells were then placed in a falcon tube for counting. 60 µl of cells were taken from the falcon tube and inserted in the counting chamber from each side. Cells were counted under the microscope light. Only cells within the central square were counted, including the ones on the left and top line of the intersection grid. Afterwards, the average number was obtained. This number (per 0.1 µl) was multiplied by 10 (to find the number of cells in 1 µl) and then multiplied again by 10^3 to find cells per ml. A correct number of cells were taken and processed for further experiment.

2.2.4 Cell propagation and collection

The cells were propagated 2-3 times a week. The growth rate was monitored by using an inverted microscope and assessing confluency. The cells were trypsinised using 1X 0.05% Trypsin-EDTA (Sigma), resuspended in DMEM to inactivate trypsin. The correct number of cells (1/3-1/10, depending on the growth properties of cells) were taken to a new flask in DMEM. Where necessary, the remaining cells were then centrifuged at 300 x g for 5 min. Afterwards, the cells were washed in 1 x phosphate buffer saline (PBS) pH 7.0, pelleted, and frozen at -20C° to be used in further studies.

2.2.5 Freezing cells

Freezing media (73% complete DMEM, 20 % FCS and 7 % dimethyl sulphoxide (DMSO; Sigma) was prepared in advance and kept on ice. The confluency of cells was checked and accordingly trypsinised at 70-90% confluency in the same way that was explained in the previous section. Then, cells were collected in 15 ml Sterile Falcon tube (BD), counted, and centrifuged at 300 x g for 5 min. Pelleted cells were re-suspended in the prepared freezing medium. Next, 1-2 million cells /ml were aliquoted in cryo-vials (Grenier Bio-one Ltd), then transferred into a liquid nitrogen tank.

2.2.6 Defrosting cells

Cells were taken from the liquid nitrogen (-180°C) and placed immediately in dry ice to avoid sudden changes in the temperature. Rapid thawing was carried out at 37°C for 2 min in a water bath. Defrosted cell lines were mixed immediately with 10 ml warm complete DMEM and centrifuged at 300 x g for 5 min. After that, the supernatant containing DMSO was discarded, and the pellets were re-suspendered in 1ml complete DMEM and transferred into a T75 flask (Corning® Incorporated) with 10 ml complete DMEM. A small aliquot is taken at this stage and the cells were counted manually by a hemocytometer to calculate the percentage of viability, and then placed in an incubator. Defrosted cells were allowed to recover from the effects of cryo-preservation for at least 1 passage before being used in experiments.

2.3 Flow cytometry

2.3.1 General principle of flow cytometry

Flow cytometry (FC) is a widely used analytical technique in cancer science. The fundamental aim of FC is to count and profile cells in a heterogeneous liquid mixture as it passes through a laser. As the laser passes through the cell, it emits light - at different wavelengths - scatter in different directions. Light scattered in the forward direction of a laser beam is focused by a confocal lens and detected by a light detector that converts it into an electrical signal and then a digital signal to generate a parameter known as forward scatter (FSC). The size and shape of the cells can be determined by the FSC. The other parameter is the side scatter (SSC) where the morphology of the cell e.g., granularity can be determined at 90 degrees. The combination of the two parameters can help identify different types of cells. Different fluorochromes will emit light at different wavelengths and these are split into specific colours by optical filters and sent to optical detectors called photomultiplier tubes (PMTs). PMTs, convert light into electrical impulses, which are translated into a digital signal. These signals are readable by a computer system as events, which are then used to generate a histogram and dot plot **see figure 9.**

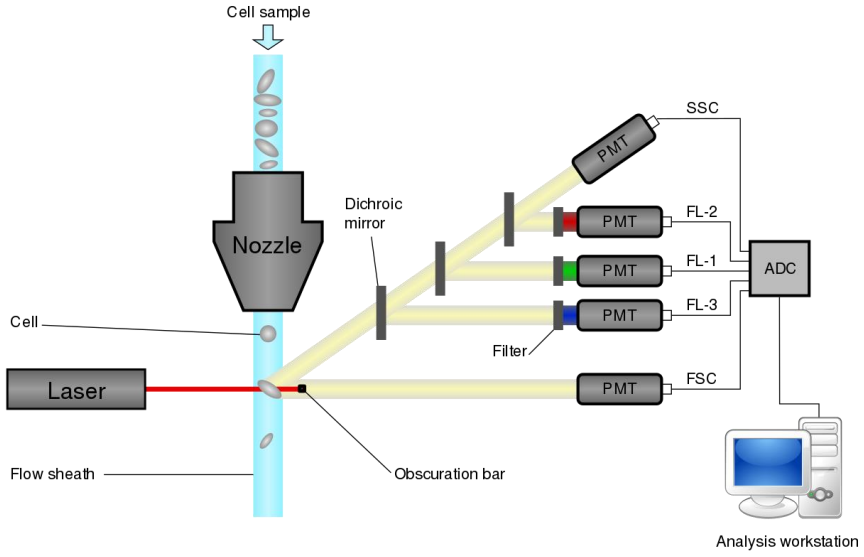


Figure 9. General principle of flow cytometry

Flowcytometry is a laboratory technique that can detect the physical (size, granularity) and biochemical (antibodies, dyes) properties of a single cell in suspension. The cell sample is passed through a pressurised fluidics system to assess each cell individually. During the passage, cells interact with inbuilt lasers set to specific wavelengths, resulting in disruption of the beam. This results in light scattering and fluorescence signals, which will ultimately manifest as a stream of electrons, generated by a photomultiplier tube (PMT). The electrical signal generated by the passage of a cell past the laser beam is studied for physical and biochemical characteristics through specialized computer software.

2.3.2 General preparation for flow cytometry for apoptosis detection following TRAIL treatment

To conduct my experiments, around $2X10^5$ cells of 11 cell lines MDA-MB-231, HS-578T, MDA436, T47D and ZR-75-1 breast cancer cells and RKO, HTC116, SW480, SW620, Caco-2 and HT-29 Colorectal cancer cells, were equally seeded in a 6 wells plate. The next day, the cells were treated with different concentrations of TRAIL 25ng/ml (low) and 250 ng/ml (high). Then, cells were collected after 6 hours of treatment using the following procedure. The supernatant containing apoptotic cells of each sample was transferred in labelled falcon tubes. PBS was used to wash the samples and again it was added to the supernatant. Then, the attached cells were trypsinised and added to the previously collected cells. Cells were, then, centrifuged at 1500 rpm for 5 minutes. After centrifugation, the supernatant was carefully discarded. Finally, the cells were gently resuspended by using 1ml of PBS, and then 900µl from that was placed in Eppendorf tubes for western blotting, while 100µl was kept for flow cytometry **see below section 2.3.3**.

2.3.3 Apoptosis detection and MMP quantification using flow cytometry

In order to investigate mitochondrial depolarisation during apoptosis, Tetramethylrhoamine, ethyl ester (TMRE) (Molecular Probes™ #T669) was used. 100µl of previously suspended cells, as described above in **section 2.3.2**, were stained with TMRE (100ng/ml in 400µl DMEM) and incubated for 20 minutes. After incubation, all samples were analyzed by a Becton Dickinson FACSCalibur flow-cytometry machine. Gate A was drawn around the visible cell population using the FSC versus SSC as a dot plot. A number of 5000 events were collected for each sample. The fluorescent colours were detected in the FL-3 (red) channel and the results were displayed on a logarithmic scale as a dot plot or as a histogram. After this, the percentages of cell death, the cells having lower mitochondria potential, were determined from the gate (M1 or % gated) for each sample. In addition, TMRE was also used to quantify the resting Mitochondrial Membrane Potential (MMP) in live cells by flow cytometry. Only untreated samples were investigated in the same way for MMP.

2.3.4 Assessment of cell surface TRAIL receptor expression

Cell surface expression of TRAIL-R1 (DR4) and TRAIL-R2 (DR5) was analysed using FACS as previously described. Cell surface TRAIL receptors (DR4 and DR5) were analysed after 3 passages once the cells were defrosted. Around 2 million cells of CRC and BCC cell lines were harvested using Trypsin, washed once with FACS buffer (1x PBS + 2.5% BSA), then centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and then cells were re-suspended in 300 µl FACS Buffer. Then, cells of each cell line were distributed equally in 3 tubes (100µl). Following that, each tube then treated with primary (conjugated) antibodies against DR4, DR5 or negative control (IgG) as follows:

1- 2 µl of Isotype IgG negative control (5 µl).
2- 2 µl of CD261 (DR4) monoclonal antibody, PE, eBioscience (5 µl).
3- 2 µl of CD262 (DR5) monoclonal antibody, PE, eBioscience (5 µl).

All tubes were incubated for 30 min at 4 °C in the dark. Then, the cells were washed with 1 ml FACS Buffer, centrifuged at 1500 rpm for 5 minutes, the supernatants were carefully discarded, and the pellets were re-suspended in 150 µl FACS buffer and analysed immediately. The data were collected using Becton Dickinson FACSCalibur and emission spectra of PE fluorochrome were checked. The mean fluorescent intensity (MFI) was measured at an excitation of 488 nm and an emission of 564 nm. The experiment was repeated independently three times. Obtained data were then analysed using GraphPad Prism.

2.4 Western Blotting

2.4.1 Main principle

Western blotting is an important technique used in cell and molecular biology. Western blot allows us to separate and identify specific proteins from a complex mixture of proteins in the cells. In this technique, a mixture of proteins is separated based on molecular weight through gel electrophoresis. Then it transferred to special solid support (nitrocellulose membrane). This membrane is then incubated with specific antibodies to be detected. The unbound antibodies are then washed off to leave only the protein of interest. Appropriate secondary antibodies are used to target primary antibodies followed by a chemo-luminescent substrate-based detection system. The light emitted from the reaction can be detected by developing a film. **See figure 10**. In order to do western blotting, several steps must be done in order. These steps are sample preparation, protein quantification, gel preparation, protein loading and gel electrophoresis, transfer of proteins to a solid support, induction of primary and secondary antibodies and film development.

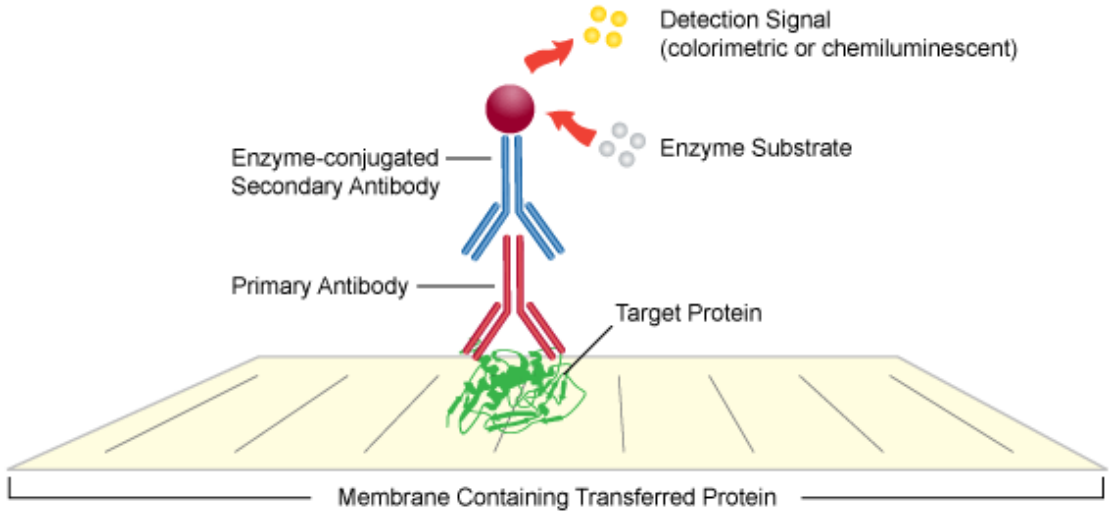


Figure 10. Illustration of a general principle of the western blotting detection method. Adapted from http://www.leinco.com/general_wb.

The main steps of Western blotting (WB) are:

- Preparation of Cell Lysate
- Protein Quantification
- Gel Preparation
- Protein Loading and Gel Electrophoresis
- Transfer of proteins to a solid support
- Protein Identification

Of note, the list of buffers used for this assay is summarised in the **supplementary table 2**.

2.4.2 Cell lysate and protein extraction

The frozen cell pellets were thawed on ice and then the proteins were extracted. The protein extraction was done in two steps. First, the cells were lysed with an appropriate volume of 2x SDS-PAGE loading (Laemmli) buffer to release the proteins. 2X Laemmli buffer can be prepared from 5X buffer solution by mixing 2ml of 5X buffer with 3ml dH_2O. Secondly, in order to ensure proper cell lysis, the cells were sonicated at 1.5 m Watts for 15 seconds (BioLogics Company). A Sonication machine is used to ensure that the cell membrane has dissolved enough by disrupting the cell membrane and releasing cellular contents. The sonication has started with untreated samples toward treated samples to avoid crossover. The extracted proteins were then quantified using a protein assay reagent (BCA; Thermo Scientific) as described in the next section.

2.4.3 Protein quantification

BCA mix was prepared (50:1, 50 parts of reagent A with 1 part of regent B). Using 96-well culture dish, 150 µl of the mixture was distributed in each well (depends on how many samples we have). The first well was left as blank and the next 3 wells were used for the standard curve. Bovine Serum Albumin (BSA; Fisher, main stock 2mg/ml) was used as standard and added at 1µl, 5µl and 12.5µl to wells. Also, 5 µl from each cell lysate (in triplicate) was added to individual wells and mixed well by pipetting. Following that, the plate was incubated for 30 min at 37°C to allow colour development. Finally, the standard curve was drawn using excel and the protein concentration of samples were calculated using the equation obtained by the BSA curve.

2.4.4 Gel preparation

Sodium Dodecyl sulfate-polyacrylamide (SDS-PAGE) gels were made according to Dr Emre Sayan's laboratory protocol- University of Southampton. Each SDS-PAGE gel was composed of two layers: Separating/running gel (lower part, 1.5M Tris: pH 8.8 is used); the density (percentage of acrylamide) of this part depends on the size of the protein of interest. Stacking gel (upper layer, 1M Tris: pH 6.8 is used): always 5%. In addition to 1.5M Tris, dH_2O is a part of the gel component. 30% acrylamide gel, 10% SDS and 10% APS are used. Finally, TEMED was added at the end for polymerization. Pure ethanol was used to straighten the gel line. The wells of gel were washed by pipetting running buffer or water inside of the wells.

Each SDS-PAGE gel was composed of two layers:

1- Separating/running gel (lower part, 1.5M Tris: pH 8.8 is used); the density (percentage of acrylamide) of this part depends on the size of protein of interest.
2- Stacking gel (upper layer, 1M Tris: pH 6.8 is used): always 5%.

Sodium Dodecyl sulphate-polyacrylamide (SDS-PAGE) gels were made according to Dr Emre Sayan's Laboratory protocol that was adopted from the Molecular Cloning book (Maniatis). Please refer to **supplementary table2;** for all buffers that were used in this project to prepare gels.

2.4.5 Protein loading and gel electrophoresis

After protein quantification and gel preparation, the lysates were supplemented with an appropriate volume of 5 x complete loading blue buffer. The samples, along with protein marker (Precision Plus ProteinT^M All Blue (10–250 KDa); Bio-Rad) were heated up at 95°C for 2 min. After that, 5 µl of the protein ladder was loaded in the first well of the polyacrylamide gel. Along with that, 20 µg of protein of each cell lysate was loaded in the subsequent wells. The loading was done as quickly as possible to minimize the diffusion of proteins. The gels were run at 180 V for 65 min using 1 x Tris/glycine/SDS buffer (TGS; Geneflow; Main stock 10 x).

2.4.6 Protein transfer

This step was done immediately and quickly after gel running is finished. The separated proteins were then transferred onto nitrocellulose membranes (Whatman Protran, GE healthcare). This was achieved by sandwiching the gel and the membrane in a transfer cassette (Bio-Rad) between 2 pieces of filter paper and 2 sponges and placing the cassette in a transfer tank containing 1 x Tris/Glycine buffer with 20% methanol (TGM; Geneflow; main TG stock 10 x) at 4°C, 20 V overnight for (16-20 hours). Ponceau red staining was used to check equal loading and efficient transfer of proteins. The Ponceau red was removed using Tris-buffered saline (pH 7.4) containing 0.01% Tween-20 (TBS-T).

The primary antibodies used in this work and their corresponding secondary antibodies summarised in **Table 3.**

Table 3. The primary antibodies used in this work and their corresponding secondary antibodies

Primary antibody	*Species*	*Molecular Weight* **(KDa)**	*Dilution*	*Catalog no.*	*Supplier*
E-cadherin	Mouse	120	1:1000	610181	BD (Becton, Dickinson and company)
Vimentin	Rabbit	57	1:1000	C5741	Cell signalling
PARP	Rabbit	89-116	1:1000	9542	Cell signalling
β-actin	Mouse	42	1:1000	612656	BD (Becton, Dickinson and company)
RIP1	Rabbit	78	1:1000	4926	Cell signalling
RIP3	Rabbit	46-62	1:1000	95702	Cell signalling
ZEB1	Rabbit	210	1:1000	3396	Cell signalling
Pan-Keratin	Mouse	65	1:1000	8068	Abcam
FADD	Rabbit	23	1:1000	2782	Cell signalling
TRADD	Rabbit	34	1:1000	36945	Cell signalling
TRAF-2	Mouse	53	1:1000	126758	Abcam
Caspase-8	Rabbit	55	1:1000	108333	Abcam
TG2	Mouse	75	1:1000	2386	Abcam
PKC-α	Rabbit	75	1:1000	32376	Abcam
P120-Catenin	Mouse	90-120	1:1000	1499	Thermofisher
IAP	Rabbit	72	1:1000	25939	Abcam

2.4.7 Protein identification

After blotting, membranes were incubated in blocking solution (4% semi-skimmed milk in TBS-T) for 30 min at RT to block non-specific epitopes. Subsequently, the membranes were incubated for 30 min-1 hour with appropriate primary antibodies (diluted in TBS-T with 2.5% BSA). After being washed three times with 5 min interval incubation in TBS-T, the membranes were incubated with the appropriate type of secondary antibodies (diluted in 4% semi-skimmed milk in TBS-T) for 30 min- 1 hour at RT. After a further 3 washing steps, the antigen-antibody interactions were visualized by using 1:1 (v/v) supersignal®west Dura luminal/enhancer solution (Thermo Scientific), which was incubated with the blots for 5 min at RT. The enzymatic reaction and the band intensity were then detected by X-ray film and films developed using an autoradiography machine.

2.4.8 Validation of EMT status of CRC and BCC cell lines

Cells can drift to epithelial or mesenchymal phenotype due to cell culture condition. So, maintaining a defined EMT status of every cell line is critical for this project. During the course of this thesis, we validated the EMT status of our cell lines regularly either in every experiment or at two-week intervals when propagating cells. We did this as follows:

Cell lysates were prepared, and western blotting was performed, as previously described in this section. The expression of several proteins implicated in EMT (EMT markers) was studied. These proteins were detected with ZEB1 (Santa Cruz Biotechnology; 1:500), E-cadherin (clone 36; BD Biosciences; 1:1000), Pan-keratin (C11; Cell signaling Technology; 1:1000), and Vimentin (3B4; Dako;1:1000) primary antibodies. β -actin (BD; Biosciences; 1:1000) was used as an equal loading control.

2.5 TGF-β induction

2.5.1 Induction of EMT by TGF-β

To determine the optimal duration for TGF-β exposure, ZR75-30 breast cancer cells were seeded into 6-well plates at a density of approximately $4x10^5$ cells per well. Following cell seeding, cells were treated with 2 ng/ml TGF-β. Untreated (control) cells were collected at 24h and remaining cells were collected at 24, 48, 72, 96 and 120h after addition of TGF-β, and stored at -20C until further use. Cell lysates were prepared, and western blotting was performed as described in **section 2.4.** Proteins were detected with TG2 (Abcam; ab2386; 1:1000) primary antibody. Beta-actin (BD; Biosciences; 1:1000) was used as an equal loading.

Once the optimum time point was determined (day 5), functional studies were performed on that day.

2.6 Transfection

Transfection is the process by which nucleic acids are introduced into eukaryotic host cells, with the intention to be integrated into the genome (stable transfection), or to get transcribed and translated temporarily (transient transfection), There are different methods of transfection:

1- Calcium Phosphate transfection:

   It is a chemical transfection method that depends on DNA condensation by Calcium phosphate co-precipitation. A calcium phosphate co-precipitation agent is generated via mixing DNA with calcium chloride in a buffered saline/phosphate. The reaction mixture is then dispensed into cultured cells, and DNA enters the cells via endocytosis. This method is labour, time and cost-effective, and suitable for many difficult types of cultured cells. However, it is cytotoxic to a lot of cells, and it is a pH, temperature, and buffer salt concentrations dependent procedure.

2- Electroporation:

   It is a physical transfection method which aims to create pores in the cell membrane by electronic pulses that facilitate nucleic acids to pass into cells. Although this method is easy, rapid, and effective, it has a major drawback which is a high percentage of cell death due to high voltage pulses.

3- Lipid mediated transfection:

   The principle of this transfection technique relies on the positive surface charge of liposomes interacting with negatively charged nucleic acids (phosphate backbone of DNA, RNA or siRNA). The liposome/ nuclei acid complex fuses into cells through direct interaction with the negatively charged cell membrane and endocytosis. Once the transfected nucleic acids get inside the cells, they diffuse through the cytoplasm and are either expressed temporarily or integrate into the genome for stable gene expression.

The lipid-mediated transfection is a fast, simple, non-toxic, and reproducible transfection method. It is a very suitable technique for many types of cells including adherent, suspension, and insect cells, as well as primary culture.

The lipid-based transfection reagent (Lipofectamine 2000) was used in this project as the reagent of choice for transfection**.**

2.6.1 RNA interference with siRNA

Successful gene silencing in siRNA-mediated RNA interference (RNAi) experiments requires efficient uptake of siRNA into the cells of interest. For *in vitro* experiments, transfection is an easy and rapid method of siRNA delivery. two variations are commonly being used: standard/forward transfection and reverse transfection. They differ in the order of timing of the addition of the three necessary components of transfection: siRNA, lipid-based transfection reagent, and cell. In this project, we used both of the siRNA transfection methods: the standard/ forward siRNA and the reverse transfection.

2.6.2 Forward/standard siRNA transfection

To start with, cells were seeded into a 6-well plate, with each well containing 2 ml DMEM. This occurred 24h prior to transfection, allowing the culture to reach 70-80% confluency. On the transfection, 70% of confluent MDA-MB-231 cells were transfected with ZEB1-si (cat no. 1027417; QIAGEN). For each siRNA being transfected, 100 pmol was added to 250 μl OptiMEM (cat no. 31985070; ThermoFisher) in the Eppendorf tube. In a separate tube, 5 μl Lipofectamine 2000 transfection reagent (cat no. 11668027; Thermofisher) was added to 250 μl OptiMEM. After exactly 5 minutes, the Lipofectamine/ OptiMEM mixture was combined with siRNA/OptiMEM

mixture and left for 20 minutes. This combination total volume of 500 µl was then added to one 6-well of MDA-MB-231 cells and left for 6 hours in the incubator. Six hours later, the medium was changed. After 48h, transfected cells were split into two for further experiment. Transfection efficiency was determined 48h post-transfection by western blotting for ZEB1 expression. The western blotting protocol is described above **(section 2.4).**

2.6.3 Reverse transfection of siRNA

The reverse transfection method was used for siRNAs, meaning that the cells were seeded in the wells after the addition of the transfection mix. This method of transfection is suitable for cells which its exposed surface area is small such as epithelial cells. For this reason and to increase transfection efficiency trypsinised cells are seeded on the transection mix.

The number of cells seeded varied among the cell lines and was calculated such that the cells would be 50 - 70% confluent one day after the transfection. The transfection mix was prepared according to the manufacturer's protocol using a ratio of 2µl of Lipofectamine 2000 for every 50 pmol of siRNA transfected. In our case, 70% of confluent HT29 and T47D cells were transfected with *CTNND1*-si (P120-catenin-si). To start with, first, the transfection mix was prepared as previously described. cells were seeded in 6-well plates on the transfection mix with each well containing 2 ml DMEM.

2.7 RNA sequencing analysis

Raw-read counts from the CCLE-CRC and CCLE-BC cohorts of ExpressionAtlas database were loaded into (edgeR) software and heat maps were created using *pheatmap* add-on.

2.8 Statistical analysis

All data were analysed using Student's t-test and one-way ANOVA to compare two groups of results with an equal variance that were obtained from our result. Data were considered statistically significant when the *p*-value was equal to or less than 0.05. Results are expressed as mean ± values of Standard Error of Mean (SEM). Each experiment was repeated at least three times. Prism 8 (GraphPad software version 7) was used as post-hoc analysis.

Chapter 3: EMT characterisation of CRC and BC cell lines

3.1Introduction

One of the central hallmarks of cancer is the ability of tumour cells to metastasize by acquiring invasive and migratory properties. Cancer metastasis remains the leading cause of cancer-related mortality worldwide. In cancer, the metastatic mechanism necessitates the detachment and propagation of cancer cells from a primary tumour site to a secondary distant site, and this is accomplished through EMT. EMT is a conserved and fundamental biological programme that contributes to development, tissue regeneration, and cancer. EMT is described as a multistep program in which epithelial cells lose their close association and acquire phenotypic characteristics similar to mesenchymal cells [9]. During EMT, epithelial cells down-regulate genes which are involved in tight junctions, adherent junctions, desmosomes, and epithelial intermediate filaments such as cadherins, ZO1, epithelial integrins, and cytokeratin. Destruction of these junctions leads to redistribution of other molecules at the cell surface reorganisation of cell cytoskeleton proteins and up-regulation of Extracellular Matrix (ECM) components [155], [90], [156]. For example, downregulation of E-cadherin, which is considered a hallmark of EMT in cancer metastasis [26], [41], [51], [157], causes the translocation of B-catenin from the cytoplasm to the nucleus which can then activate several EMT-inducing transcription factors [95]. The main transcription factors involved in regulating EMT are ZEB1/2. SNAIL1/2 and TWIST1/2. They are all implicated in facilitating the suppression of epithelial gene expression and activating mesenchymal genes [158], [28], [159], [61], [160], [161], [162]. Mesenchymal cells are defined as single cells with a spindle shape having a front-back-end polarity and expressing mesenchymal proteins such as vimentin and FSP-1. They are highly motile and invasive [163], [35]. In addition, these mesenchymal tumour cells may subsequently intravasate into lymphatic system or blood circulation, extravasate at a distant site, re-epithelize via mesenchymal-epithelial transition (MET), proliferate and form a secondary tumour in a distant region [164].

Interestingly, not all cells undergo complete EMT. Instead, some cells undergo partial EMT with an intermediate or metastable phenotype. The intermediate phenotype is characterised by cells retaining some epithelial junctions and showing some of the mesenchymal features at the same time [1], [21], [22], [165], [166], [167]. In this thesis, we will refer to such cells as “borderline” or “metastable”.

Overall, this is how the EMT program is believed to play a major role in cancer progression and metastasis. Loss of epithelial markers, e.g., E-cadherin and gain of mesenchymal markers, e.g., Vimentin and EMT-related transcription factors, most importantly ZEB1 can predict patient outcome.

3.2 Aims of the chapter

One of the key aims of this project is to investigate whether the EMT program and TRAIL response are related.

As we hypothesized that TRAIL response and EMT are linked, and in order to test our hypothesis, the first thing we need to do is correctly identify the EMT status of a panel that we will be using in this project, which includes eleven well-known and often studied CRC and BC cell lines. Therefore, the aims of this chapter are:

1- Review the biological characteristics of six CRC (RKO, SW480. SW620, HCT116, HT29, and Caco-2), and five BC cell lines (MDA-MB-231, HS-578T, MDA-MB-436, T47D, and ZR-75-1), and compare the morphological appearances as observed with a light microscope.

2- Identify the EMT status of the CRC and BC panel using epithelial markers (E-cadherin and Pan-keratin) and mesenchymal biomarkers such as Vimentin and an EMT-inducing transcription factor (ZEB1). This will be performed using Western blotting.

3.3 Results

3.3.1 EMT status of CRC and BC panel cell lines

3.3.1.1 Morphological appearance

Pictures of eleven cell lines were taken with a light microscope at 100 or 200X magnification, and descriptions of the phenotype of each noted.

3.3.1.2 Phenotype of the CRC and BC panel cell lines

The origin morphology, oncogene expression, and supplier of the 11 CRC and BC cell lines (RKO, SW480. SW620, HCT116, HT29, Caco-2, MDA-MB-231, HS-578T, MDA-MB-436, T47D, and ZR-75-1) were reviewed from ATCC literature and are summarized previously in **Table 2** (Material and Methods chapter).

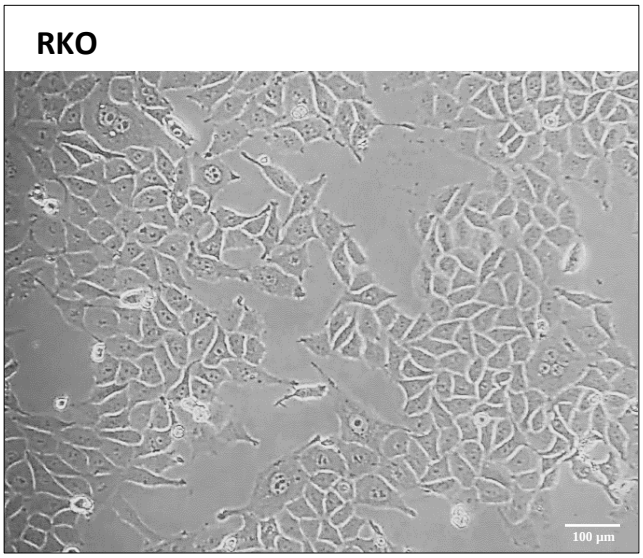


Figure 11. Morphology of RKO cells using a light microscope.

The RKO cell line originates from a poorly differentiated primary colon carcinoma cell line. The cells grow as uniform bipolar fibroblast-like cells. At 100% confluence, the cells covered the entire growth surface **see figure 11**.

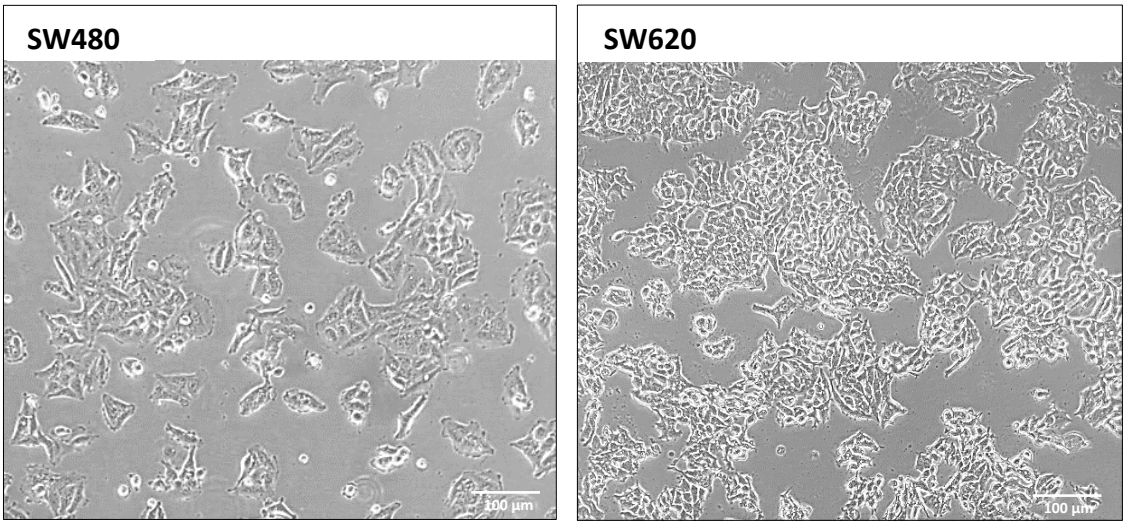


Figure 12. Morphology of SW480 and SW620 cells using a light microscope.

SW480 and SW620 cells were isolated from the same patient. SW480 was isolated from primary colon adenocarcinoma, whereas SW620 was isolated from a metastatic region in a lymph node. SW480 cells grow as bipolar elongated fibroblast-like cells. On the other hand, SW620 cells grow as a mixture of small individual sphere-shaped cells, which are clustered in unpacked epithelial islands, and small bipolar individual fibroblast-like cells; **see figure 12**.

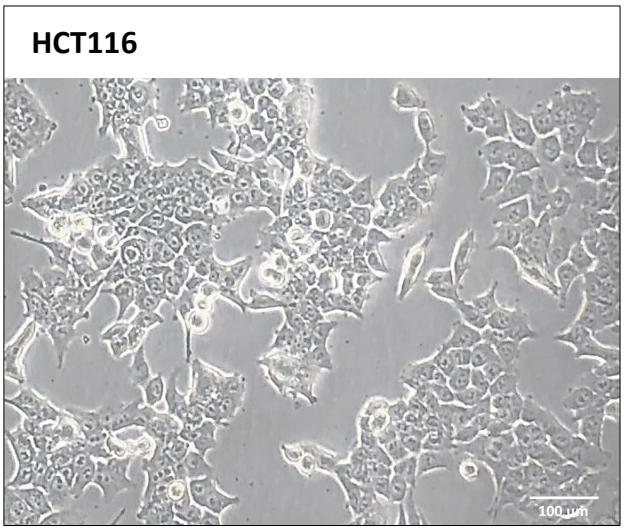


Figure 13. Morphology of HCT116 cells using a light microscope.

The HCT116 cell line was obtained from primary colon carcinoma. The cells are polygonal in shape and form tightly packed islands. In addition to the formed islands, a few small fibroblast-like cells grew at the borders of the islands, which indicate the presence of two cell populations **see figure 13**.

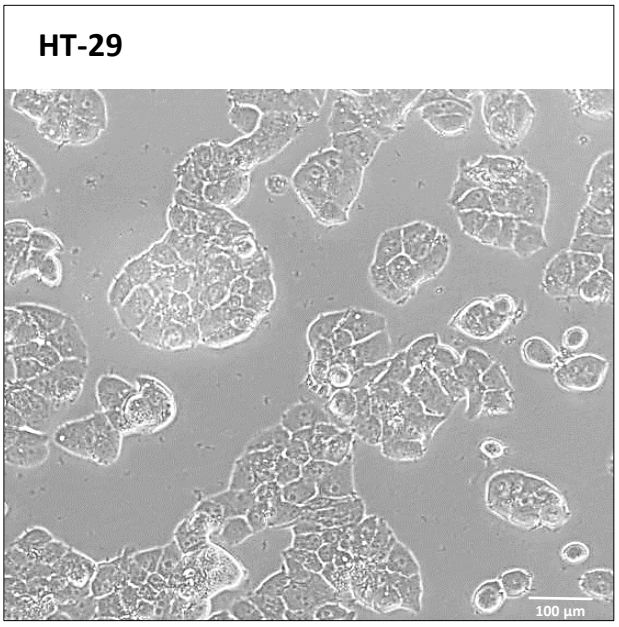


Figure 14. Morphology of HT-29 cells using a light microscope.
HT-29 is a well-differentiated primary colon cancer cell line. The cells are polygonal in shape and form tightly packed islands with visible sharp edges. The epithelial islands are intermediate in size and this cell line barely forms a monolayer; **see figure 14**.

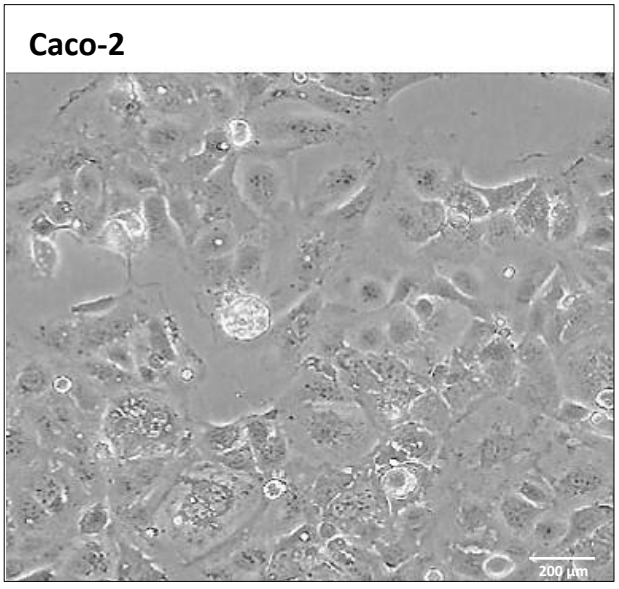


Figure 15. Morphology of Caco2 cells using a light microscope.
Caco-2 is a human colorectal adenocarcinoma cell line. The cells are adherent, and they grow as polarized tightly packed epithelial islands, and it forms a monolayer when it reaches 90% confluency; **see figure 15**

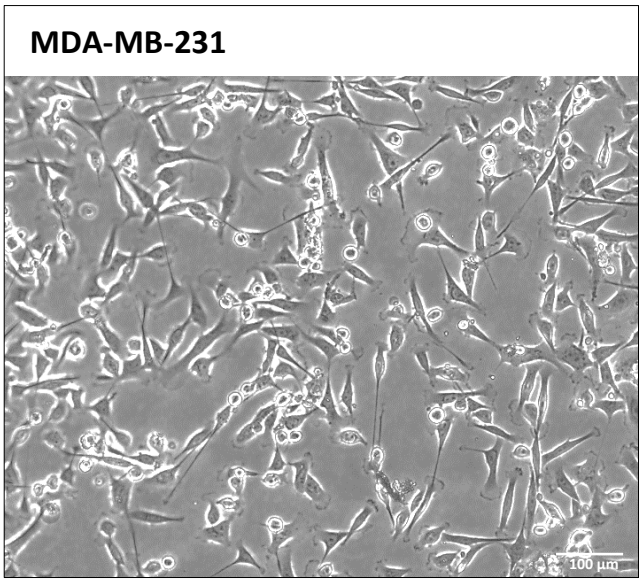


Figure 16. Morphology of MDA-MB-231 cells using a light microscope.

The MDA-MB-231breast cancer cell line was obtained from a patient in 1973 at M. D. Anderson Cancer Centre. It was isolated from a pleural effusion of a patient with invasive ductal carcinoma is commonly used to model late-stage breast cancer. The MDA-MB-231 appear phenotypically as spindle-shaped cells. These cells are adherent and very elongated in shape resembling that of fibroblasts; **see figure 16.**

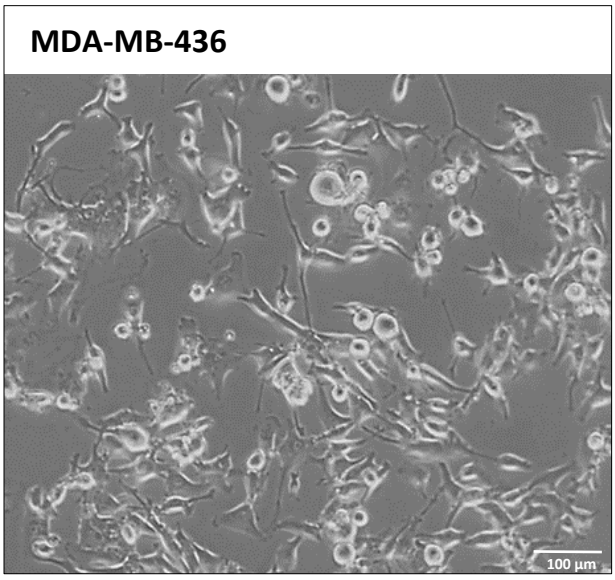


Figure 17. Morphology of MDA-MB-436 cells using a light microscope.

MDA-MB-436 cell line was first isolated from the pleural effusion of a metastatic breast adenocarcinoma from a 43-year-old female. MDA-MB-436 morphologically is displayed a fibroblastic phenotype which characterized by neuron-like cells; **see figure 17.**

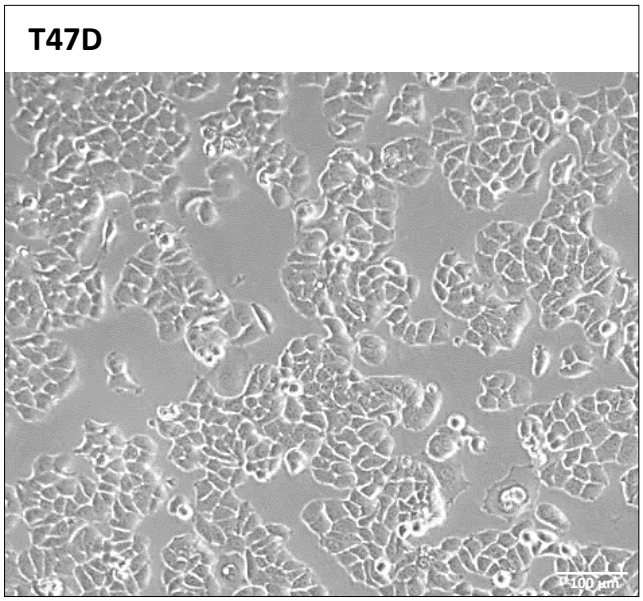


Figure 18. Morphology of T47D cells using a light microscope.

The T47D cell line is human breast cancer cell line. The cells were derived from a ductal carcinoma found in the mammary gland of an elderly human patient, and it has been widely used in cancer research. The cells demonstrate a tightly cohesive cobblestone appearance; **see figure 18**.

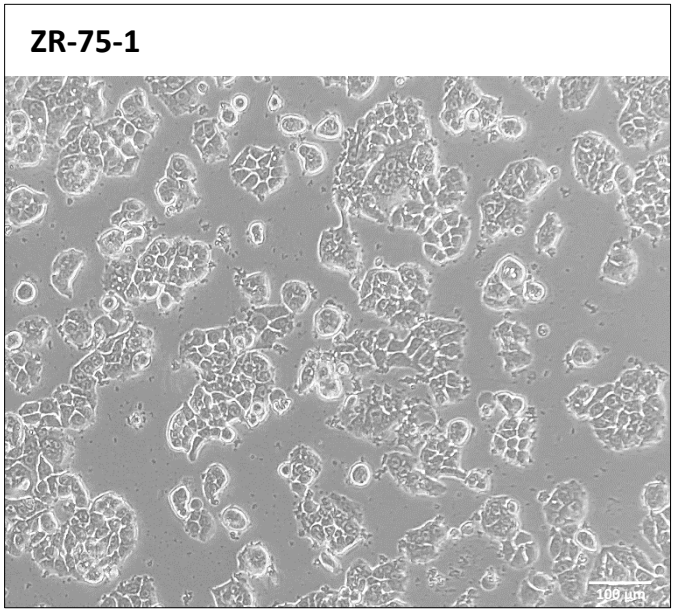


Figure 19. Morphology of ZR-75-1 cells using a light microscope.

The ZR-75-1 cell line is human breast cancer that was derived from a ductal carcinoma. The cells are grown with monolayer epithelial-like morphology; **see figure 19**.

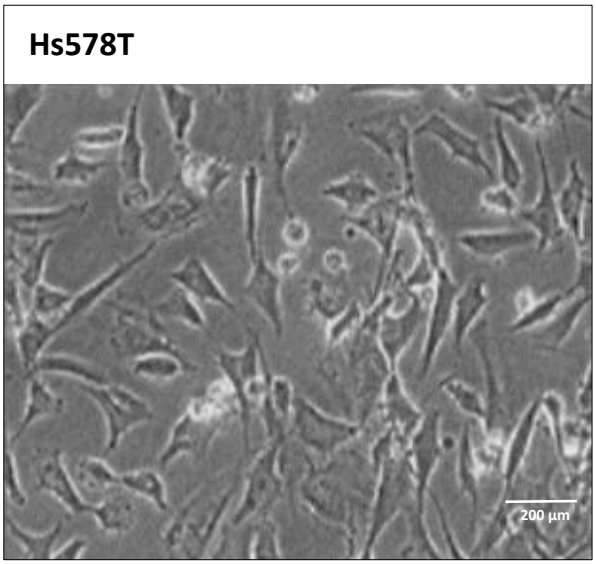


Figure 20. Morphology of Hs578T cells using a light microscope.

The Hs578T cell line was derived from a carcinoma of the breast. Morphologically, the Hs-578T had a mixed polygonal morphology initially, but a stellate cell type was selected during passage; **see figure 20**.

3.3.2 Qualitative assessment of EMT markers

3.3.2.1 Western blotting results of the 11 CRC and BC cell lines

The EMT status of 11 cell lines was identified using EMT biochemical markers (E-cadherin, Pan-keratin expression highlights epithelial status whereas vimentin and the EMT-inducing transcription factor (ZEB1) were used as mesenchymal markers). All markers were detected at the protein level using the western blotting technique; **see figure 21**.

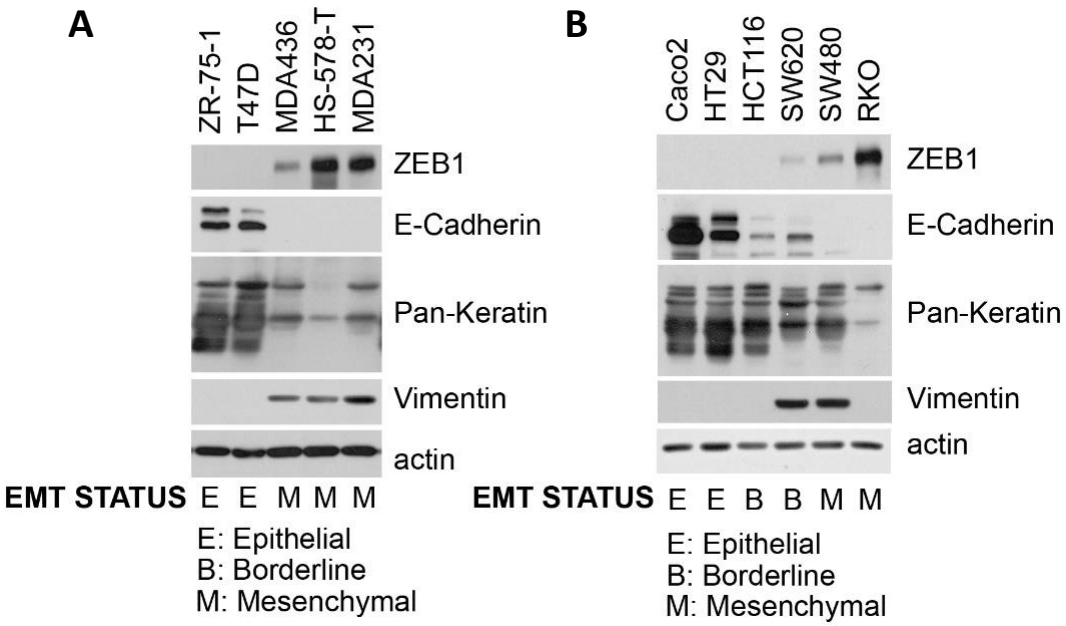


Figure 21. The protein expression of canonical EMT markers and EMT-inducing transcription factor in a panel of five BC (A) and CRC (B) cell lines.

E-cadherin and pan-keratin are epithelial markers whereas vimentin and/or ZEB1 are indicative of mesenchymal status. β-actin was used as an equal loading marker. 20 µg of total protein was loaded. EMT status abbreviations: E (epithelial), M (mesenchymal) and B (borderline/metastable). The figure is a representative demonstration of at least 3 independent results.

In BC cell lines, at the protein level, the epithelial marker (E-cadherin) protein was found abundantly expressed in ZR-75-1 and T47D cells. Whilst no E-cadherin was observed in MDA-MB- 231, Hs578T, and MDA-MB-436 cells. On the other hand, the mesenchymal marker (vimentin) was abundantly found in MDA-MB-231 and less abundantly expressed in MDA-MB-436 and Hs578T as shown in **figure 21 (A)**. The intermediate epithelial filament protein (Pan-keratin) was detected abundantly and at similar levels in T47D and ZR-75-1 cells, although Pan-keratin was seen much less and at varying amounts in the

other cell lines. Significantly, Hs578T cells showed no or barely detectable level of Pan-keratin. Crucially, the EMT- inducing transcription factor (ZEB1) was significantly detected in mesenchymal cells only, specifically in MDA-MB-231 and Hs578T; **see figure 21 (A)**.

In the CRC cell lines, surprisingly, RKO showed no vimentin at all. Although RKO expressed no vimentin, ZEB1 was heavily expressed in these cells. Whilst ZEB1 was moderately expressed in SW480 and SW620 cells. No ZEB1 expression was detected in any other CRC cell lines; **see figure 21 (B)**. SW480 and SW620 cells were found to have an equal amount of vimentin at the protein level, whilst HCT116, HT29, and Caco-2 cells showed no mesenchymal, but instead, epithelial markers and E-cadherin especially was found strongly in Caco-2 and HT29 cells. In contrast, a low level of E-cadherin was observed in SW620 and HCT116 cells, whilst no E-cadherin was seen in RKO and SW480 cells. Significantly, RKO cells showed no or barely detectable level of Pan-keratin, whilst HCT116 exhibited more filaments compared to SW480 and SW620 which expressed a lower level of Pan-keratin **as shown in figure 21 (B)**. The results of β-actin indicated that there was an equal loading of the proteins in all the 11 CRC and BC cell lines for the western blotting detection technique; **see figure 21**.

3.4 Discussion

3.4.1 Colorectal and breast cancer cell lines used

Cell lines are powerful experimental tools and in many instances in the past, the information derived from these cell lines has translated into numerous clinical improvements. Despite their crucial role in improving clinical outcomes, their ability to accurately reflect phenotypes of tumours remains controversial. However, many studies have shown that these cell lines actually mirror many of the characteristics of the colorectal or breast cancer subtypes from which they have been derived.

During the study period, finding cell lines that most accurately resemble EMT was an important step to start the project. Thus, eleven colorectal and breast cancer cell lines in the case of epithelial and mesenchymal morphology were chosen that represents each status of EMT after careful consideration. We selected these cells based on our previous experience and on published papers. Importantly, these cell lines were most frequently used within preclinical settings, and they have gene expression data in public databases.

A detailed phenotypic characterisation of each cell line used is demonstrated previously in material and methods chapter **section 2.1.1** and a brief overview of each cell line will be described in detail in this chapter.

3.4.2 EMT status of the 11 CRC and BC cell lines in relation to their morphological appearance

This chapter aimed to analyze the EMT status in a panel of CRC and BC cell lines and relate this to their morphological appearance and biological characteristics such as response to death receptor mediated apoptosis upon TRAIL treatment. To attain this goal, 6 CRC cell lines (RKO, SW480. SW620, HCT116, HT29, Caco-2) and 5 BC cell lines (MDA231, HS-578T, MDA436, T47D, and ZR-75-1) were chosen. All the cell lines are human cell lines. EMT status was identified by western blotting using various EMT markers such as E-cadherin and Pan-keratin as epithelial markers and vimentin as a mesenchymal marker. Moreover, the EMT transcription factor (ZEB1) was also included in the analysis as a mesenchymal marker. β -actin was used as a loading marker for western blotting. To the best of our knowledge, this is the first study to investigate EMT using such a wide variety of cancer cell lines from CRC and BC and EMT markers; **see figure 21.** The use of 11 cell lines from two different cancer types will let us generalise our findings and maybe to other cancers.

Based on our findings from the western blotting and the light microscopy, it has been decided to categorise the CRC and BC cell line panel into three distinct groups. The groups are:

A. An epithelial phenotype category, which includes cell lines having epithelial cell shapes and moderate to high levels of E-cadherin, with no expression of mesenchymal markers or EMT regulator (ZEB1).
B. A mesenchymal phenotype category, which includes cell lines expressing no E-cadherin, but with mesenchymal morphological appearances.
C. An intermediate phenotype category, which includes cell lines with low levels of E-cadherin and one or more mesenchymal markers or EMT regulator markers. In addition, they have a mixed population of both epithelial and mesenchymal cells.

Cancer therapeutic resistance, including radio resistance and drug resistance, is a major challenge in cancer research and treatment. Cancer stem cells, a small subpopulation of cancer cells with tumour-seeding and self-renewing ability, have been found to contribute to therapy resistance in all cancer types [168], [169]. Since tumour cells that have undergone EMT can acquire cancer stem cell properties [49], the EMT program has been implicated in therapy resistance in cancer, including radio-resistance, chemoresistance and other targeted therapies [11], [170], [171]. The activation of EMT has been shown to play a critical role in tumour initiation and metastatic spread [85]. EMT is described as multiple programs in which epithelial cells lose their epithelial junctions and acquire mesenchymal phenotype characterized by loss of E-cadherin expression [9]. The downregulation of the epithelial marker E-cadherin is considered the hallmark of EMT in cancer metastasis [27] . Loss of E-cadherin leads to tumour cell dissociation and enhanced ability to migrate, invade and metastasize, which is associated with poor prognosis in human cancer patients [172]. E-cadherin is repressed at the transcription level by multiple EMT inducers including ZEB1 which has the most consistent inverse correlation with E-cadherin expression across carcinomas.

Due to the pivotal role of ZEB1 in the downregulation of E-cadherin, ZEB1 acts as an inducer of EMT and cancer progression [173]. Aberrant expression of ZEB1 has been observed in many human cancers, such as pancreatic cancer [174], osteosarcoma [175], lung cancer [176], colon cancer [173] and breast cancer [51], [177]. In these tumors, ZEB1 expression correlates with loss of E-cadherin and is associated with advanced diseases or metastases, which indicates the relevance of ZEB1 induction of EMT and tumour progression in a clinical setting. Overexpression of ZEB1 in cancer has frequently been reported and associated with invasiveness, metastases, poor prognosis and presence of EMT [178], [179]. ZEB1 is overexpressed in various colorectal and breast cancer cell lines [51],[59]. A study reported that breast cancer cells overexpressing ZEB1 showed a 10-fold higher resistance to doxorubicin treatment compared with the control, non-transfected cells [180]. On the other hand, another study revealed that knockdown of ZEB1 in SW480 cells inhibits lung metastases after intrasplenic or intravenous injection in nude mice [59]. Further research carried out by Spaderna *et al.* found that E-cadherin expression was enhanced following ZEB1 knockdown, whereas vimentin expression was reduced, hence leading to reduced tumour cell migration in SW480 colorectal cancer cells as well as in MDA231 breast cancer cells [59]. ZEB1 was also found to play a critical role in regulating tumour radiosensitivity. Knockdown of ZEB1 in different cancer cells was observed to increase radiosensitivity, while overexpression of ZEB1 in radiosensitive mammary epithelial cell lines, MCF7, led to elevated radio-resistance [98]. These studies confirm that as a driver of EMT, ZEB1 plays an important role in tumor progression and metastasis and correlates with poor clinical outcomes in cancer patients.

Mesenchymal cell lines are defined as single cells with a spindle shape having a front-back-end polarity and expressing mesenchymal protein such as vimentin. They are highly motile and invasive [35]. Interestingly, not all cells undergo complete EMT. Instead, some cells undergo partial EMT with an intermediate or metastable phenotype. The intermediate phenotype is characterized by cells retaining some epithelial junctions and showing some mesenchymal features at the same time [21]. EMT and its reverse process, MET, are involved in different stages of metastasis. The induction of EMT in epithelial cancer cells promotes migration, invasion and dissemination, whereas MET facilitates metastatic colonization of distant sites by disseminated tumour cells [164]. Mesenchymal-like tumour cells generated by EMT have been shown to exhibit characteristics of cancer stem cells, including self-renewal, radio-resistance and drug resistance [49], [181]. Thus, research on EMT and MET will not only advance our understanding of tumour progression but also shed light on improving cancer treatment.

For our project, we started by correctly identifying the EMT status of our cell lines using mesenchymal and epithelial markers (vimentin and E-cadherin, respectively) as well as ZEB1. We hypothesized that mesenchymal carcinoma cells have altered pro-survival pathways making them resistant to chemotherapeutic agents that induced apoptosis. However, we believe that the altered pro-survival pathways make them vulnerable to death receptor-induced apoptosis. To test our hypothesis, the expression levels of epithelial and mesenchymal markers for a panel of 11 colorectal and breast cancer cell lines were determined by immune blotting through rigorous testing.

The colorectal cancer cell lines RKO, SW480, SW620, HCT116, HT29 and Caco2, and breast cancer cell lines MDA-MB-231, Hs578T, MDA-MB-436, T47D and ZR-75-1 were cultured and assessed. Confirming our morphological observations, in breast cancer cells, MDA-MB-231, Hs578T, MDA-MB-436 are classified as mesenchymal cells, whereas T47D and ZR-75-1 are found to be epithelial. For colorectal cancer cells, RKO and SW480 are considered as mesenchymal cells, whereas SW620 and HCT116 could be classified as intermediate (borderline), while HT29 and CaCo2 are epithelial.
During the initiation of EMT, ZEB1 expression is an early event. Thus, we first investigated the abundance of ZEB1 protein in our cell lines. Based on our findings from western blotting, we clearly found that all mesenchymal cells expressed ZEB1. It has been previously found that ZEB1 is overexpressed in various mesenchymal cells of colorectal and breast cancer origins [51], [59]. On the other hand, no ZEB1 expression was detected in any of the epithelial cells. We further investigated the association between ZEB1 and the expression of the mesenchymal marker, vimentin. We found that the majority of cells that expressed ZEB1, also expressed the mesenchymal marker vimentin **see figure 21**.

Looking into the details of the EMT status of each cell line, we found:

Caco2 cells showed high expression of E-cadherin at the protein level with no expression of any mesenchymal markers. In addition, The EMT-transcription factor (ZEB1) was not detected in these cells. On top of the abundant level of E-cadherin, other epithelial markers such as occludin, claudin1 and desmosomes have been reported in these cells as well [182]. Morphologically, the cell line was described as a polarised epithelial cell monolayer that provides a good model to study the physical and the biochemical properties of epithelial barriers for the passage of small molecules [183]. The reported formation of the monolayer matches our observations by light microscopy. It is also known that these cells are non-invasive or non-metastatic [184], [185]. For all of the above-mentioned results, Caco2 can be considered as a highly epithelial cell line.

HCT116 displays a low level of E-cadherin and few keratins compared to Caco2 cells. Although no expression of vimentin was detected, these cells are expressing a reasonable level of alpha-SMA which is considered as a mesenchymal marker [186]. The same observation was seen by other authors [187]. In fact, expressing both epithelial and mesenchymal markers at the same time may explain the phenotype of this cell line. In culture, two populations of un-tightly packed epithelial islands and fibroblast-like cells are seen; **see figure 13**. The same morphology had been described by the scientist who deposited this cell line to the ATCC. It is also reported that this cell line has a degree of metastatic potential in mouse experiments [185], [188]. The metastatic potential could be due to the presence of fibroblast-like cells. These findings can categories this cell line as an intermediate "metastable" phenotype representing a partial EMT.

HT29 is a mucus-secreting human colonic cancer cell line that grows in culture as a tightly packed cluster of epithelial islands. Other researchers reported similar growth descriptions [189], [190]. The epithelial morphology of these cells was confirmed by assaying some EMT markers. This cell line showed a moderate level of E-cadherin expression with a similar amount of keratins in comparison to Caco2 epithelial cells. No mesenchymal markers or EMT regulators were detected. A controversial result was reported which found that HT29 cells have low levels of vimentin and ZEB1 proteins [191]. Nevertheless, I confirmed our results by repeating them multiple times, whilst Alsaihti *et al.* also confirmed similar results by different techniques (protein and RNA expression) [192]. In addition to the EMT markers, the morphological appearance of the cells displayed an epithelial morphology. Celesti *et al.* also found similar findings as ours [193]. Moreover, desmosomes, cytokeratin, zonula adherent and tight junctions were reported for HT29 cells [194],[195]. Therefore, HT29 is classified as epithelial.

RKO cells express no E-cadherin or cytokeratin at all. This representative finding of E-cadherin is supported by a study by Breen *et al*., (1995) which found that RKO completely lacks E-cadherin, explaining why it is so aggressive, poorly differentiated and highly invasive compared with the transfected with E-cadherin [196]. Importantly, this cell line has a very mesenchymal phenotype in terms of morphology [196]. Surprisingly, RKO cells, which expressed a high level of ZEB1 have shown no vimentin expression among all mesenchymal cells. The same result had been found previously: the highest level of ZEB1 was observed for RKO cells, however, it had not acquired the expression of vimentin or other filamentous proteins associated with a mesenchymal state, including fibronectin[10]. The absence of both vimentin and E-cadherin was also reported by Hur *et al*. (2013) who also found that among CRC cell lines, the highest level of ZEB1 was observed for RKO cells, a cell line that lacks E-cadherin expression although it has not acquired of vimentin, the intermediate filamentous protein associated with a mesenchymal state [197]. We believe that this might be due to a specific mutation or polyploidy associated with loss of chromosomal segment harboring the *VIM* gene. Previous work from Buck *et al*., group has suggested that owing to methylation-induced transcriptional silencing of the promoter region, RKO cells did not express transcripts for either E-cadherin or vimentin [198], [10]. Morphologically, the cells grow as uniform individual cells and look like small fibroblasts. Taking together, RKO is classified as a mesenchymal cell line.

SW480 and SW620 colon cancer cell lines originated from the same patient; thus, they have the same genetic background. SW480 was isolated from primary colon cancer and SW620 was isolated from a metastatic site in a lymph node [199]. In culture, we observed that SW480 has a different morphological appearance than SW620. The SW620 cell line tends to be more epithelial morphologically and they grow as a mixture of small epithelial islands, whereas SW480 cells grow as small spherical cells and elongated bipolar cells. Our data shows that SW620 cells express low levels of E-cadherin proteins, whereas SW480 do not show any expression of E-cadherin protein. In addition, SW480 cells have a lower level of Pan-keratin in comparison to SW620 cells. On the other side, the mesenchymal marker vimentin was seen in an equal amount in SW480 and SW620 cells. However, contrary findings describing the EMT status of each cell line and metastatic potential have been reported. SW620 cells were seen to express mesenchymal markers including nuclear β-Catenin and vimentin as well as EMT-transcription factors (EMT-TFs) such as ZEB1 [200], [201]. Detecting the expression of mesenchymal markers in morphologically epithelial cells such as SW620 is curious, hinting that this may be due to a mixed population of mesenchymal and epithelial cells. It is well known that each carcinoma cell line contains a small stem cell population. This population usually accounts for only 1%–5% of all tumor cells and is not detectable by common molecular biology techniques [202]. It is quite likely that SW620 cells have high percentage of stem cells, which allowed us to detect ZEB1 and vimentin. In accordance

with our data, SW620 expressed vimentin was reported by Prof. Elizabeth Hay *et al.* [10]. In fact, there are two possible explanations for this: first, as previously mentioned, this might be due to a mixed population of cells; and second, the expression of the mesenchymal marker in SW620 could indicate that this cell line had undergone an EMT-like transition [10]. The same author also reported that SW620 had undergone EMT, expressing vimentin and gaining a fibroblastic morphology.

Although these cells are derived from the same patient, our data shows that SW480 expresses a slightly higher level of ZEB1 compared to SW620 cells. The result of the very low or no expression of E-cadherin, and the high expression of ZEB1 in SW480 cells was found previously [160], [55]. However, contrary findings reported that the expression levels for ZEB1 were 2-fold higher for the cell line SW620 compared with SW480 [10]. The controversial result of this cell line's status led to a literature search for their depositors [189], [194], and we found that the differentiation stats of the cell lines is in agreement with our reproducible findings (n=3). Importantly, the cell is sold by ATCC, and they classified SW480 as mesenchymal, therefore, we believe that clearly there was a mix-up of cells in the above-mentioned study.

Many studies reveal that cancer cells at the distant metastatic region re-gain the expression of E-cadherin [27], [28], [165], [203]. and this aligns with our E-cadherin findings with SW620. In line with our findings, SW620 cells were reported to have less metastatic and migration potential compared to SW480 [204], [205]. Indeed, many studies have shown that SW480 cells are more invasive, migratory and metastatic compared to SW620 cells [205]. From all of these biological data, SW480 is a mesenchymal cell line whereas SW620 could be classified as intermediate as cells have undergone a re-differentiation process or MET although not entirely. In fact, SW480 and SW620 cells can be considered as suitable models to study colon cancer progression with respect to EMT and MET.

MDA-MB-231 cells is a highly metastatic breast cancer cell line that was originated from a pleural effusion of a patient with invasive ductal carcinoma [206]. In culture, MDA-MB-231 cells display a spindle-like morphology, they are very elongated in shape resembling that of a fibroblast. This pattern was also previously reported [207],[208]. Biochemical characterisation through western blotting also confirmed the morphological appearance as mesenchymal. At the protein level, the cell line showed heavy expression of all mesenchymal markers and transcription factors that we studied in this project; Vimentin and ZEB1. Among the five breast cancer cell lines, MB-MB-231 cells expressed ZEB1 most abundantly. Invasiveness and migration studies conducted by Hughes *et al.* (2008) showed that the MDA-MB-231 cells were the most migratory among a variety of breast cancer cells [209]. Similar results have been also obtained by other investigators [208], [210]. This cell line did not express detectable E-Cadherin. From the above description, the MDA-MB-231 cell line is grouped under the mesenchymal category.

As these cells are considered to be an ideal EMT representative, MDA-MB-231 was chosen to be extensively used for further genetic manipulation as an ideal experimental model to study EMT in this project.

MDA-MB-436 cell line was first isolated from the pleural effusion of a metastatic breast adenocarcinoma from a 43-year-old female. These cells express mesenchymal markers such as ZEB1 and vimentin less abundantly compared to MDA-MB-231cells. They also showed no E-cadherin at all, with some expression of Pan-keratin. These results also accord with the researchers who showed similar findings [211]. Notably, MDA-MB-436 cells have been previously characterized as having low metastatic activity in comparison to MDA-MB-231[212]. Yi-Fang *et al*. (2011) also concluded that MDA-MB-231 is a highly metastatic breast cancer cell line, while MDA-MB-436 is less metastatic [213]. Both these cells were derived from pleural effusion and display no E-Cadherin expression so the lower metastatic ability of MDA-MB-436 was explained by having a significantly lower mitochondrial function [214]. Nevertheless, MDA-MB-436 has been shown to be invasive by *in vitro* assays [215]. Morphologically, this cell line displayed a neuronal phenotype characterized by neuron-like cells. Hence, MDA-MB-436 cells are classified as mesenchymal.

Hs578T the cells displayed a strong mesenchymal features and reduced level of the epithelial marker Pan-keratin. As a matter of fact, this cell line showed the lowest level of Pan-Keratin expression (almost none) and high levels of ZEB1. We found that Hs578T displays a slightly lower levels of vimentin compared to MDA-MB-231 cells. Despite the lower level of vimentin, this cell line has been characterised as a highly invasive and metastatic cell line [216]. According to Hughes *et al*. (2008), Hs578T was the most invasive cell line *in vitro* among different metastatic breast cancer cells. Hs578T demonstrated substantial invasion capacity with cells exhibiting primarily stellate morphology and growing in cultures as fibroblast-like cells [209]. Given this, Hs578T is characterised as a mesenchymal cell line.

T47D cell line was isolated from a 54-year-old female patient's pleural effusion having invasive ductal carcinoma. The epithelial morphology of these cells was confirmed by assaying some EMT markers. This cell line showed an abundant level of E-cadherin and Pan-keratin. On top of the abundant level of E-cadherin and Pan-keratin, other epithelial markers such as claudin, along with occluding, have been reported as well [217]. In addition, none of the mesenchymal markers was detected in this cell line. Further, T47D is also categorised as a poorly invasive cell line with a classical luminal epithelial

phenotype [216]. Morphologically, the cell line was exhibited a tightly cohesive cobblestone appearance (tight cell-cell junctions) and typical epithelial phenotype. The same morphology had been described by the scientist who deposited this cell line to the ATCC [218], [219]. The reported formation of tightly cohesive structures displaying robust cell-cell adhesions matches our observation by light microscopy. For all of the aforementioned results, T47D can be placed with the epithelial category, and it can be considered as a highly epithelial BC cell line.

ZR-75-1 cell line is derived from malignant ascites fluid from a 63-year-old female with infiltrating ductal carcinoma of the breast. Similar to the T47D cell line, ZR-75-1 cells showed robust expression of E-cadherin, which is consistent with an epithelial phenotype. On the other hand, no mesenchymal markers or any EMT regulators were detected. Morphologically, ZR-75-1 cells present a monolayer rounded cell morphology, and this can be linked to the high expression of E-cadherin and other epithelial markers. Other authors reported similar growth descriptions [220], [221]. It is also reported that this cell line has a low level of CEA Carcinoembryonic antigen which could classify the cell line as a non-metastatic cell line [222]. These observations provide evidence that the ZR-75-1 cell line is epithelial and should therefore be placed in the epithelial category.

From this discussion, it can be concluded that the 11 CRC and BC cell line panels can be categorised into three groups based on their EMT status and morphological appearance:

- Epithelial cell category, which includes: HT29 and Caco-2 from colorectal cancer cells, T47D and ZR-75-1 from breast cancer cells.
- Mesenchymal cell category, which includes RKO and SW480 from colorectal cancer cells, MDA231, HS-578-T and MDA436 from breast cancer cells.
- Intermediate cell category (borderline), which includes: SW620 and HCT116.

This study reporting the EMT characterization and gene expression patterns of this panel of cell lines represent an important initial step to realizing the potential involvement of EMT in cancer therapy. Hence, further studies may help to identify novel targeted therapies for the clinically highly relevant cancer cell type.

In summary, we investigated eleven cell lines of two cancer types in total and we found them to be reasonably stratified in terms of EMT status. Without question, EMT has been the focus of much work and gathering of evidence in regard to its capacity to enhance cancer cell invasion and migration across a number of different *in vitro* models. As mentioned before, EMT is linked to chemoresistance in different cancers including breast and colorectal cancers [150] yet the mechanisms are not well defined. Empirical evidence suggests that cells that have undergone EMT have a stem cell-like property, thus sharing key signaling pathways and drug resistance phenotypes with cancer stem cells (CSCs) [49], [150]. We believe targeting these cells in cancer is of utmost importance for true cancer therapy.

As TRAIL can induce apoptosis selectively in tumor cells, leaving other cells unharmed [112], we will therefore attempt to highlight the basis for this tumor cell selectivity which remains an active area of investigation and may have important implications for cancer therapy. To our knowledge, this study is the first to link the response of carcinoma cells to TRAIL with respect to their phenotype (EMT status). In the following chapter, therefore, we will investigate whether TRAIL response could be related to EMT status in our panel of eleven cell lines.

Chapter 4: TRAIL sensitivity and Death Receptor Components (DRC) in epithelial and mesenchymal CRC and BCC cell lines

4.1Introduction

Resistance to chemotherapeutic drugs is the major obstacle to successful cancer treatment. Some mechanisms were resolved but currently, there is no targeted therapy for eliminating metastatic cancer cells and CSCs [150], [223]. One proposed anti-cancer treatment is TRAIL. TRAIL has been shown to kill a variety of cancer cells, but it is non-functional in others. The selectivity of TRAIL towards some cancer cells combined with its relatively low toxicity has made it a very attractive potential therapeutic agent in different cancers [112], [224]. Although how TRAIL induces apoptosis is fairly well understood, no protein associated with TRAIL-induced apoptosis has been linked with its selective killing activity. TRAIL protein has been found naturally in many different human tissues. While most of the normal tissues are resistant to TRAIL-mediated apoptosis [133], TRAIL has been shown to kill cells in natural physiology or during development. These cells are neural cells and hepatocytes [225],[226]. Some established fibroblast lines also respond to TRAIL [227],[112]. The broad TRAIL expression and the inability of TRAIL to induce apoptosis in normal cells and some cancer cells suggest that these cells contain mechanisms that protect them from TRAIL-induced apoptosis. For example, a recent analysis of breast cancer cells have indicated that half of them were resistant to TRAIL, and the remaining show response to TRAIL and these cells were mostly highly metastatic triple-negative/basal-like breast cancer [228]. These studies support that the metastatic potential of cancer cells could influence TRAIL response. As we previously mentioned, EMT plays an essential role in promoting metastasis and it is considered as the key process in tumor cell invasiveness and formation of CSCs [229], [150]. Thus, studying TRAIL response in corresponding with EMT may suggest a mechanism for TRAIL sensitisation. A variety of mechanisms have been proposed to underlie the possible reason behind TRAIL resistance/sensitivity, including possible defects in the TRAIL receptors expression on the cell surface, mainly DR4 and DR5 [230], [115], or inhibitory binding of TRAIL by decoy receptors DcR1 and DcR2 [231], [232]. Additionally, upregulation of pro-apoptotic proteins and suppression of anti-apoptotic proteins such as Bcl-2 and c-FLIP respectively, in addition, to the inhibitors of apoptosis (IAPs) family members; has been reviewed in many studies [233-236]. However, these studies did not demonstrate that the levels of these proteins are the primary reason for TRAIL response in resistant and sensitive cancer cells. Therefore, to enhance the TRAIL-medicated apoptosis, finding a novel TRAIL sensitizing agent or a biomarker of TRAIL response possibly represent the best clinical option.

In this chapter, our objective was to explore TRAIL response in a panel of eleven colorectal and breast cancer cell lines. Here we demonstrated TRAIL-response, for the first time to our knowledge, in such a wide variety of different cancers of different origins in relation to their EMT status. Furthermore, we sought to define the gene that most highly correlated with TRAIL-sensitivity to identify a common determinant of TRAIL-sensitivity in our selected cancer cell lines. As EMT induces major changes in gene expression we will also inquire whether selected proteins determining TRAIL sensitivity are also associated with EMT status of our cell lines.

4.2 Aims of the chapter

1- We hypothesized that EMT status could be a defining parameter for TRAIL response. To identify the association of EMT status and TRAIL sensitivity, a panel of eleven (mesenchymal and epithelial) colorectal and breast cancer cell lines will be subjected to TRAIL.

2- To assess DR4 and DR5 expression in our cell line panel and to see whether TRAIL response and death receptor expressions are correlated.

3- To investigate the expression of proteins involved in TRAIL mediated apoptosis pathway and identify key molecules that link EMT status and TRAIL response.

4.3Results

4.3.1 Assessment of mitochondrial membrane depolarisation induced by TRAIL in CRC cell lines using flow cytometry

Mitochondria membrane depolarization is an essential step in apoptosis and marks the point of no return. The CRC cell lines panel was subjected to TRAIL (25 and 250 ng/ml). 6 hours after treatment, samples were collected for flow cytometry, and some spared for western blotting to assess caspase activity using PARP cleavage. We performed flow cytometry to check the mitochondria depolarization in each sample. **Figure 22** reveals the effect of TRAIL on our representative CRC cells. RKO, classified as a mesenchymal cell line, recorded a strong apoptotic response with 55.6% and 65.0% with low and high concentration of TRAIL respectively. On the other hand, HCT116 cells, which were considered as borderline, the percentages of cell death were 14.9% treated with (25 ng/ml). Increased cell death was observed 27.0% for the sample treated with a higher concentration of TRAIL (250 ng/ml).

Epithelial cell lines such as HT29 and Caco-2 did not respond to TRAIL at any given concentration. From our experiment we concluded that mesenchymal and borderline cells responded to TRAIL, and epithelial ones did not. The response was linear and paralleling EMT status.

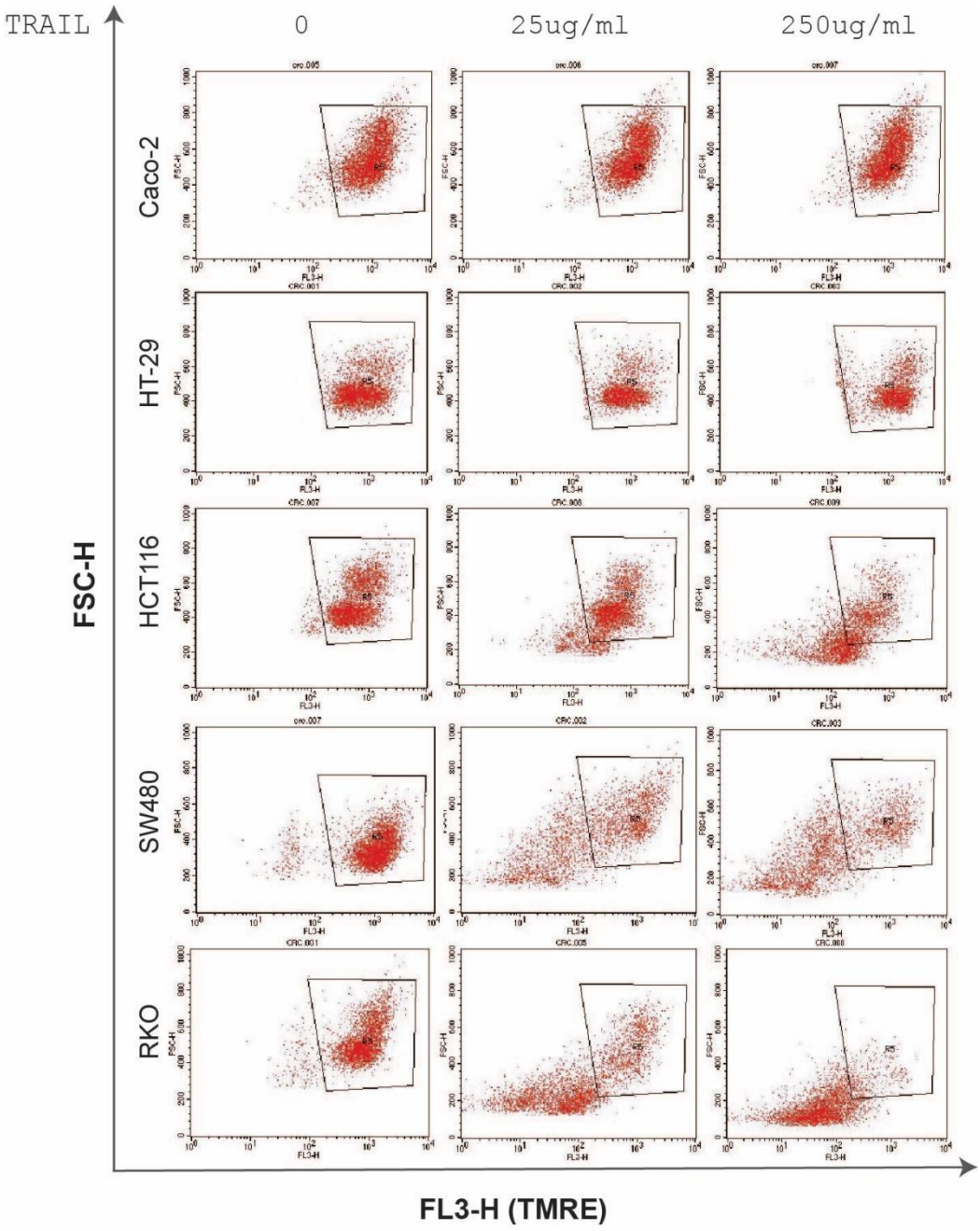


Figure 22. Flow cytometry analysis of the mitochondrial membrane depolarisation in CRC cells treated with different concentrations of TRAIL (25 and 250 ng/ml) for 6 hours.

Cells were stained with TMRE and examined using FACSCalibur in FL3 channel. RKO (mesenchymal cells) died more with low and high concentrations of TRAIL. HCT116, a borderline cell, hardly died with low concentration (25ng/ml) but showed significant death with high concentration (250ng/ml). Both epithelial cell lines tested (HT29 and Caco-2) did not show any mitochondria depolarisation with any of the doses of TRAIL.

4.3.2 Detection of cleaved PARP as an apoptotic marker in response to TRAIL in our panel of CRC cell lines

An important step in apoptosis is activation of effector caspases such as caspase 3, -6 and -7. These caspases cleave vital proteins such as PARP and Lamins to make sure cell death occurs. Therefore, and also to make sure cell death observed upon TRAIL treatment is apoptosis, we performed western blotting to check PARP cleavage as an indicator of effector caspase activation.

Cleaved PARP has been clearly observed in mesenchymal CRC cells (RKO and SW480) treated with lower concentrations of TRAIL (25ng/ml) and more significantly with a higher concentration (250ng/ml). PARP cleavage was also observed in high concentration TRAIL treated HCT116 and SW620 cells, which were considered borderline/metastable according to our EMT classification. However, the response to lower TRAIL concentration was much less pronounced in these cell lines than observed in mesenchymal cell lines such as RKO.

On the other hand, there was no PARP cleavage in HT29 or Caco2 cells, both of which are epithelial in nature, at any concentration of TRAIL. In conclusion, the most significant PARP cleavage was seen in RKO and SW480 cells (mesenchymal). Moderate cell death was observed in HCT116 and SW620 (borderline) and very little or no cell death was detected in HT29 and Coco-2 (epithelial cell lines). Overall, we can say that mesenchymal cells die more with TRAIL; **See figure 23.** These results are in line with quantitative data that we obtained from flow cytometry.

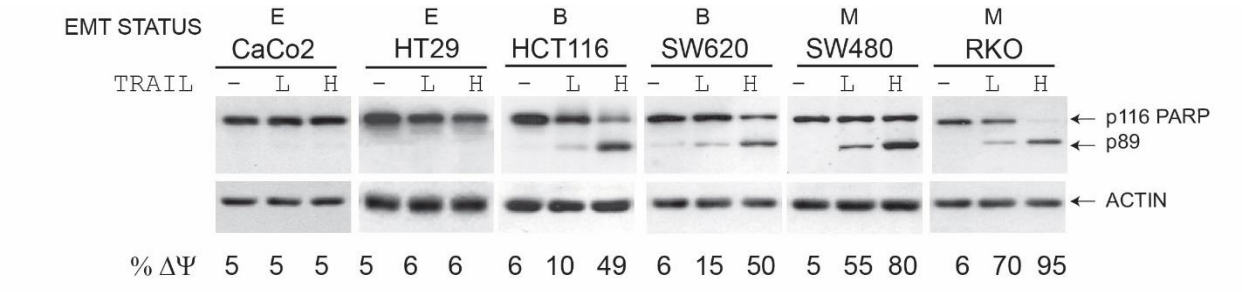


Figure 23. PARP expression and formation of cleaved PARP (p89) in CRC cell lines treated with TRAIL (25 ng/ml and 250 ng/ml) for 6 hours.

The mesenchymal cells (RKO, SW480) die with low and high concentrations of TRAIL. The borderline cells (*e.g.,* HCT116) die*d* only with high concentration of TRAIL. Epithelial cells (HT29 and Coco-2) do not die with TRAIL with any concentration. Actin was used for equal loading. % of apoptosis ($\Delta\psi$) was assessed using flow cytometry.

4.3.3 Assessment of mitochondrial membrane depolarisation induced by TRAIL in BC cell lines using flow cytometry

To extend our understanding related to TRAIL response and EMT status, we further checked mitochondria depolarisation in a panel of BC cell lines. We found that there was substantial apoptosis in mesenchymal cells, such as MDA-MB-231, treated with TRAIL (25, 250ug/ml). As observed in figure, healthy (live cells) are located in gate R1. Upon TRAIL treatment, a significant number of these cells (more than 80%) were found to have shifted left, indicating a lower or no mitochondria potential. When exposed to a higher TRAIL concentration, almost all cells shifted left indicating more cell death. Also, Hs578T showed apoptosis even with low concentrations (25ug/ml). A moderate shift was detected in MDA-MB-436 with different concentrations (slightly more apoptosis with high concentration, 250ug/ml). On the other hand, epithelial cells, T47D and ZR-75-1 showed no mitochondria depolarization (apoptosis) at all even with a high concentration of TRAIL at the end of a 6-hour treatment. From the figure, these cells remain in the gate even with the treatment suggesting they are still alive; **See figure 24**. Briefly, only mesenchymal BC cells responded to TRAIL and showed mitochondria depolarisation.

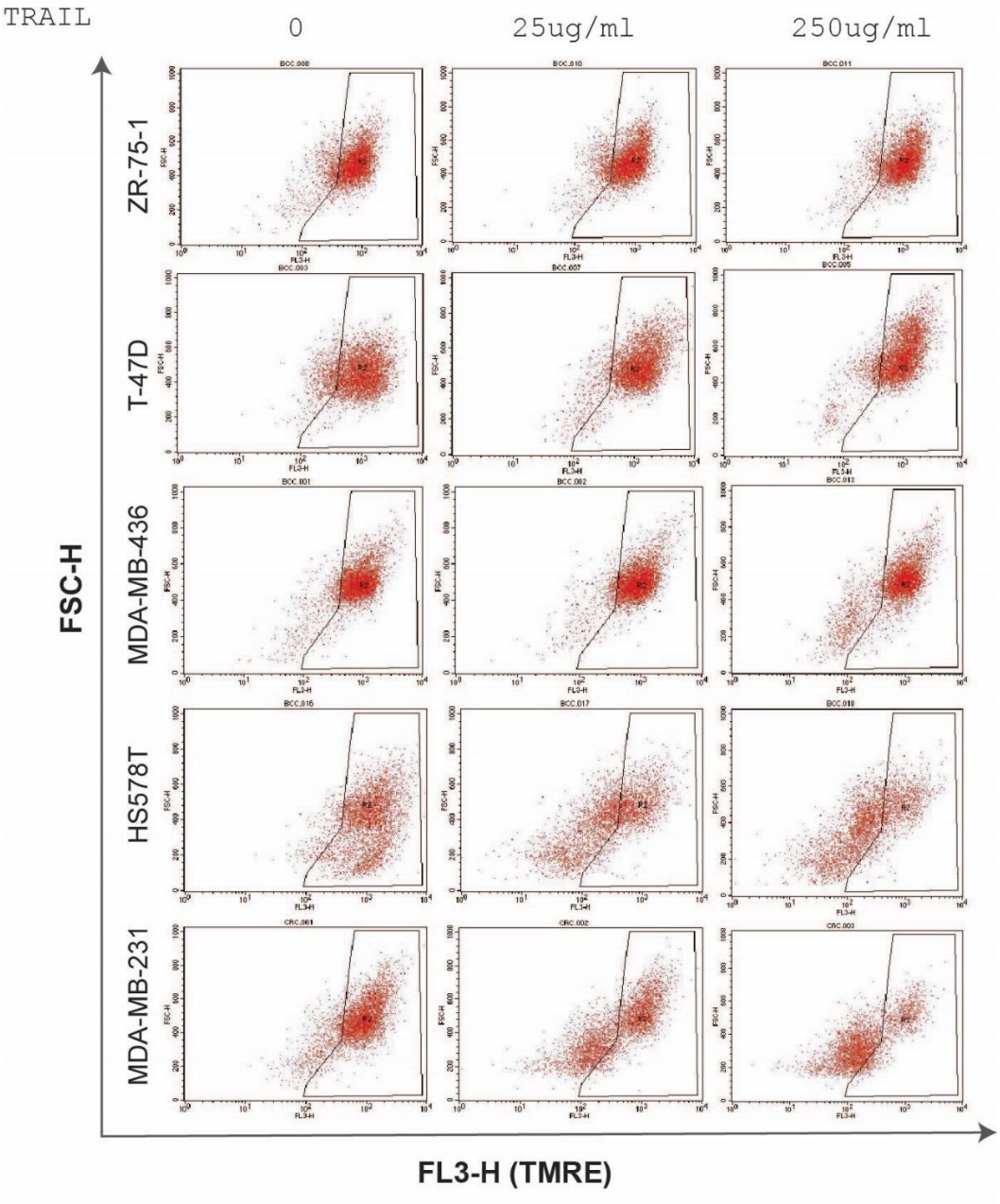


Figure 24. Flow cytometry analysis of the mitochondrial membrane depolarisation in BC cells treated with different concentrations of TRAIL (25 and 250 ng/ml) for 6 hours.

The cells were stained with TMRE and examined by flow cytometer in FL3 channel. MDA-MB-MB-231 and Hs578T (mesenchymal cells) died more with low and high concentrations of TRAIL. T47D and ZR-75-1, epithelial cells, do not die with any concentration of TRAIL.

4.3.4 Detection of cleaved PARP as an apoptotic marker in response to TRAIL in our panel of BC cell lines

Similar to the observations by flow cytometry, a considerable cell death (PARP cleavage) was observed in the mesenchymal cells MDA-MB-231 for the samples treated with different concentrations of TRAIL (25, 250 ng/ml) for 6 hours. It is worth noting that MDA-MB-231 cells showed complete cell death even with low concentration of TRAIL. On the other hand, MDA-MB-436 cells showed low (some) cleaved PARP, which increased with high concentration of TRAIL. Whilst T47D and ZR-75-1, which are classified as epithelial cells, were shown to have no cleaved PARP even with highest concentration tested. In conclusion, no PARP cleavage was detected in any of the epithelial cells **See figure 25.** This is in line with flow cytometry data,

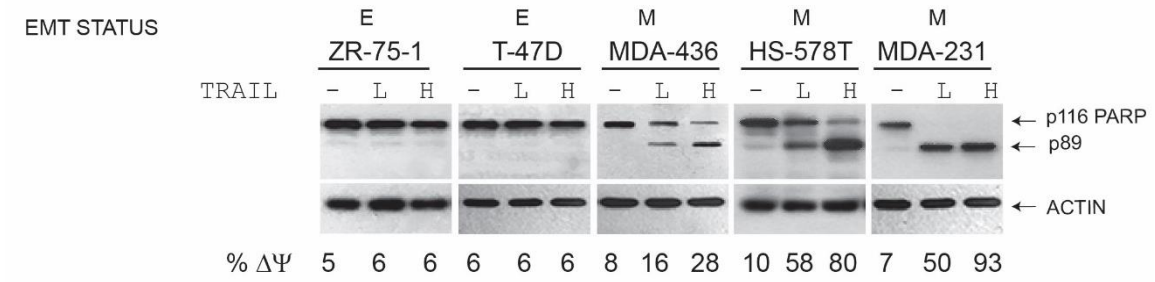


Figure 25. Protein expression for PARP in BC cell lines treated with TRAIL (25 ng/ml and 250 ng/ml) for 6 hours.

The mesenchymal cells (MDA-MB-231, MDA-MB-436 and HS-578T) die more with high concentrations of TRAIL. Epithelial cells (T47D and ZR-75-1) do not die with TRAIL with any concentration. Actin was used for equal loading. % of apoptosis (Δψ) was assessed using flow cytometry.

4.3.5 Quantitative analysis of mitochondrial depolarisation for colorectal and breast cancer cell lines

As we have performed quantitative experiments (mitochondria depolarisation) 3 times, we were in a position to analyse our results and to assign statistical significance to see whether TRAIL response and EMT status correlate. A chart was derived from flow cytometry analysis showing the relative TMRE fluorescence indicating mitochondrial depolarisation in CRC and BC cell lines treated with different concentrations of TRAIL; **See figure 26.**

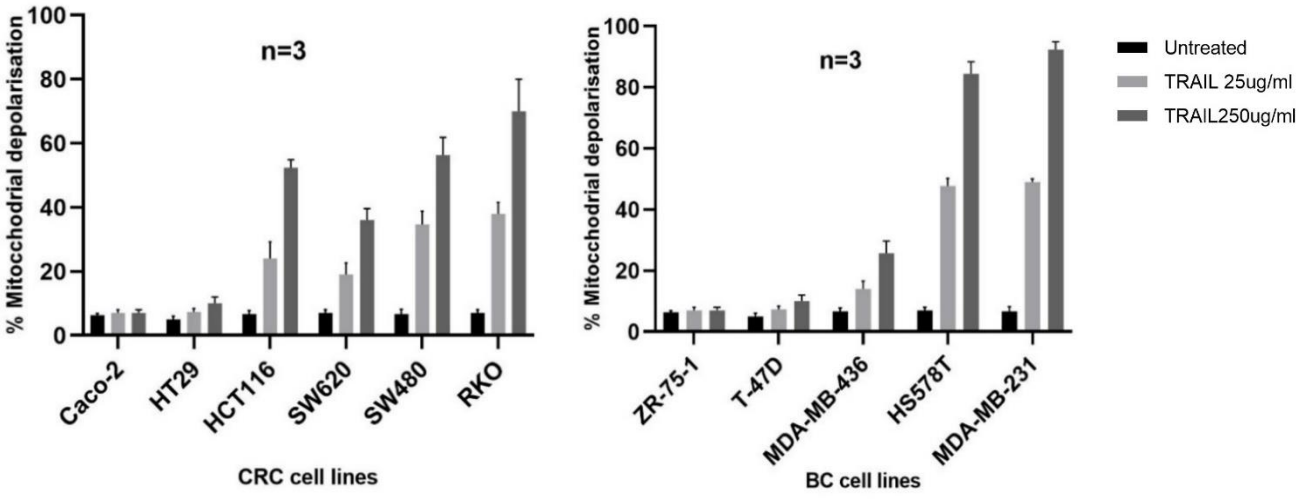


Figure 26. Relative TMRE Fluoresce for mitochondrial depolarisation during apoptosis for untreated and TRAIL treated samples.

A one-way ANOVA revealed that there was a statistically significant differences between the two groups (epithelial and mesenchymal cell lines) in both CRC and BC cell lines p value = 0.025 and 0.013 respectively.

4.3.6 Expression analysis of TRAIL receptors (DR4 and DR5)

The interaction between TRAIL and its receptors is the first step triggering apoptosis, and TRAIL sensitivity may be influenced by the level of expression of these receptors on the cell surface. It was initially speculated that some cells would express greater levels of the TRAIL receptors therefore they are more sensitive to TRAIL, and this will help to explain their differential sensitivity [237]. The question arose in our study whether TRAIL receptors are highly expressed in the responding cells compared to the non-responders. Cells surface TRAIL receptors were therefore measured using TRAIL receptor-specific antibodies and flow cytometry as previously described; **see section 2.3.4**. Our result showed that both sensitive (mesenchymal) and resistant (epithelial) cells were found to express DR4 and DR5; **see figure 27**. The presence of these receptors on the cell surface is indicated by a right shift of the peak after binding to a specific antibody relative to a control IgG-PE. Surprisingly, SW480 cells, which responded to TRAIL, did not appear to express DR4 but only DR5. In general, as illustrated in the table below, some cells were found to express similar levels of DR4 and DR5. On the other hand, higher levels of DR4 and DR5 were present in some cells more than the others. However, despite the variation in DR4 and DR5 expression levels, this difference is found to be statistically not significant in terms of TRAIL response and the calculated *p* values were equal to 0.18 and 0.13 for DR4 and DR5 respectively. Overall, this result showed that the TRAIL receptors DR4 and DR5 are present in both sensitive and resistant cancer cell lines. Partial lack of TRAIL receptors was also not a valid reason for TRAIL response.

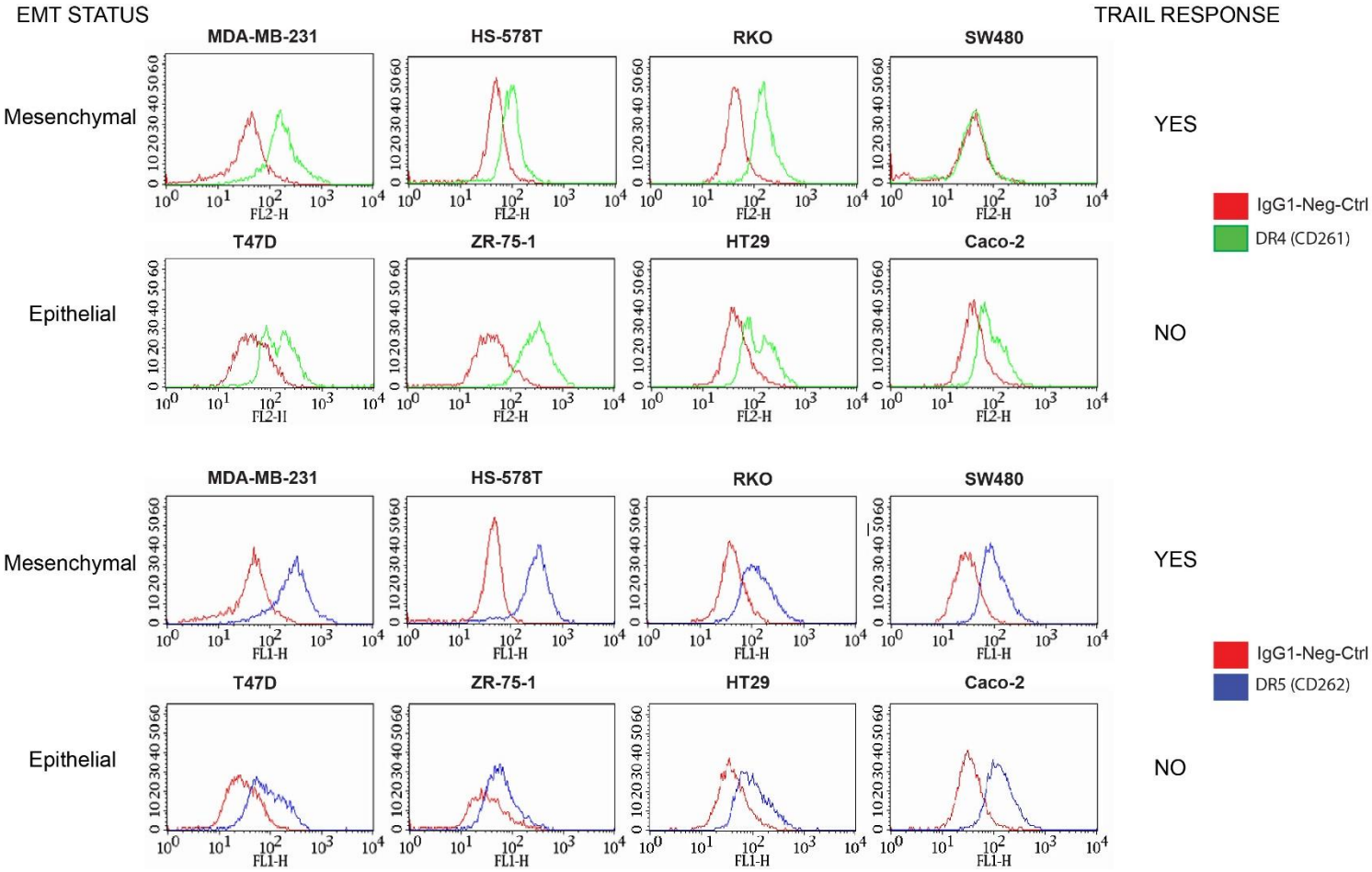


Figure 27. Expression analysis of TRAIL receptors DR4 and DR5 measured by flow cytometry.

Histograms represent the IgGI negative control (red) DR4 (green) and DR5 (blue) on the surface of the live cell population. The intensity of the fluorescent signal is reported on the X-axis. Plots are representative of three independent experiments (n=3). A shift to the right of fluorescence intensity corresponds to the increase in DR4 and DR4 expression at the cell surface. Cell were human colorectal and breast cancers.

Table 4. TRAIL cell surface receptors expression.

Cell line	***EMT status***	***TRAIL 250ng/ml* (sensitivity)**	***TRAIL-R1 (DR4) Expression***	***Mean Florescence Intensity* (MFI)**	***TRAIL-R2 (DR5) Expression***	***Mean Florescence Intensity* (MFI)**
MDA MB 231			High		High	
HS 578T			Low		High	
RKO	Mesenchymal		Medium	166 ± 60	Medium	160 ± 35
SW480		Sensitive,	Not expressed		Medium	
MDA MB 436		Responders (R)	Medium		High	
HCT116			Medium		Medium	
SW620	Borderline		Low		Low	
T47D			Medium		Medium	
ZR 75 30		Resistant,	High	90 ± 22	Low	85 ± 17
HT 29	Epithelial	Non-responders	Medium		Medium	
Caco2		(NR)	Low		High	

*MFI values of 1-50 are considered low, 50-250 are medium and 250+ are high.

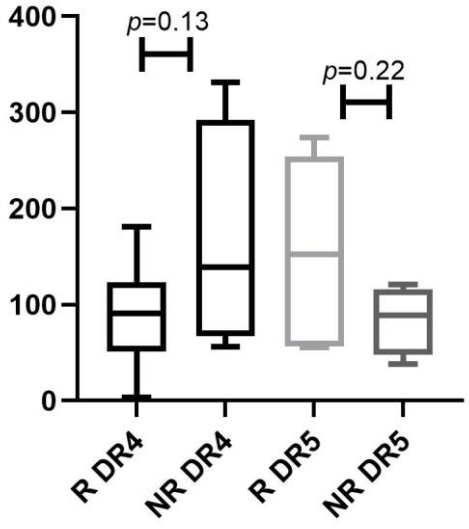


Figure 28. Relative Mean Fluoresce Intensity of the TRAIL receptors.

Cell surface expression of TRAIL-R1(DR4) and TRAIL-R2 (DR5) do not correlate with TRAIL-sensitivity. Mean cell surface expression of DR4 and DR5 in seven TRAIL-sensitive (R: responders) and four TRAIL-resistant (NR: non-responders) breast and colorectal cancer cells was measured by flow cytometry. Data are presented from three independent experiments (n=3), error bars represent the mean ± SEM. DR4 and DR5 calculated *p* value = 0.18 and 0.13 respectively.

4.3.7 Death receptor components in our panel of CRC and BC cell lines

Our previous results suggest mesenchymal carcinoma cells are more susceptible to TRAIL induced apoptosis and increased expression of DR4 and DR5 cannot be reason for this activity. A potential reason for enhanced TRAIL sensitivity can be altered expression of death receptor complex components. For example, increased expression of caspase 8, as the initiator caspase in TRAIL induced apoptosis pathway, can be the reason why mesenchymal carcinoma cells are more sensitive to TRAIL. Therefore, we investigated a number of TNF-receptors-associated molecules (Caspase-8, FADD, TRADD, RIP1, RIP3. c-IAP1 and TRAF2); **see figure 29**. Among all proteins assessed. RIP1 was detected more abundantly in mesenchymal cells compared to epithelial ones, as well as the borderline-metastable cells which also responded to TRAIL but a lesser extent. This observation valid in both breast (left panel) and CRC (right panel) cell lines.

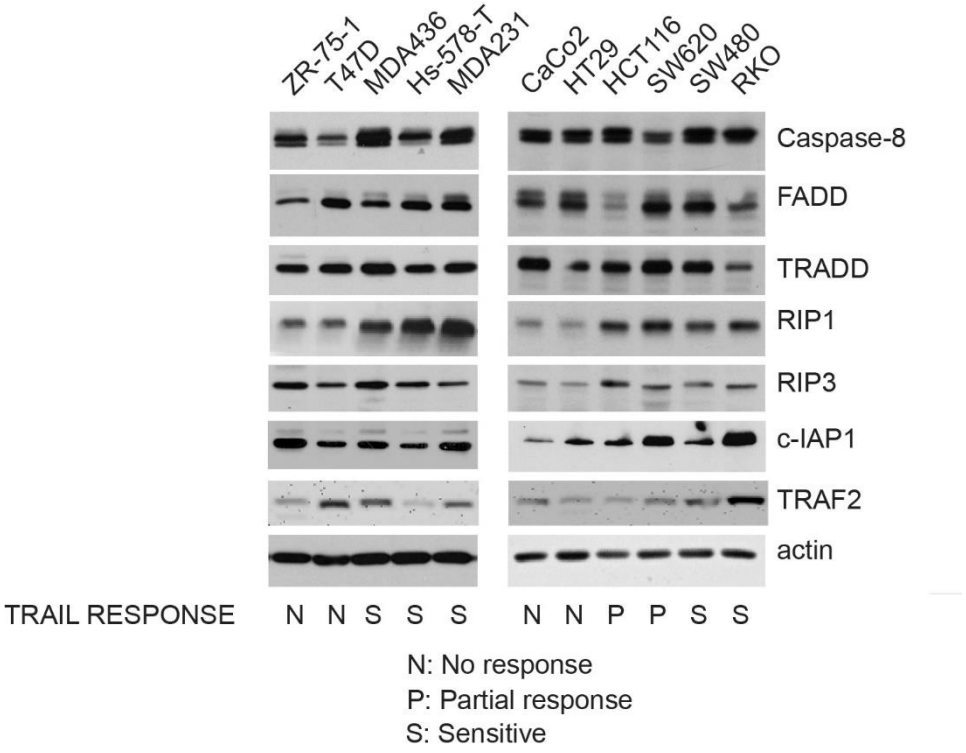


Figure 29. Protein expression of death receptor mediated apoptosis components.

Protein expression of Caspase-8, FADD, TRADD, RIP1, RIP3. c-IAP1 and TRAF2 in BCC (left panel) and CRC (right panel) cells treated with TRAIL. In both panels. RIP1 was found more abundant in all mesenchymal cells compared to epithelial cells. Actin was used to confirm equal loading.

The investigation focused on a number of TNF-receptors-associated molecules that were involved in up-stream signaling death receptor mediated apoptosis as well as NF-kβ activation. The results from this suggest that, despite minor variation, most proteins were equally expressed for example, FADD, which recruits Caspase 8 to DR complex, was observed in all cell lines but less in ZR-75-1, HTC116 and RKO cells. RIP1 was expressed more in mesenchymal cells and barely detected in epithelial cells i.e., HT29 and Coco-2 in CRC cell lines. Similarly, less expression of RIP1 was observed in ZR-75 and T47D as compared to MDA-MB-231, HS578T and MDA-MB-436 cells**; see figure 29**.

4.3.8 The winner/ final candidate

In principle among all proteins investigated, RIP1 was the only discriminator of EMT or borderline status in our cell line panel. Cells expressing more RIP1 should have more active NF-kβ pathway [238], [239]. This may partly explain their chemoresistance associated with mesenchymal phenotype.

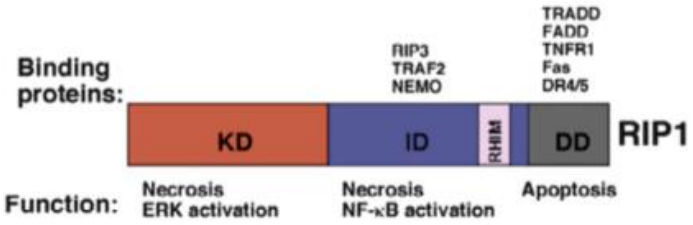


Figure 30. The domain structure of receptor-interacting protein 1 (RIP1).

The domain structure of RIP1 consists of an amino-terminal kinase domain, a receptor-interacting protein homotypic interaction motif (RHIM)-containing intermediate domain and a carboxy-terminal death domain. The interacting molecules and proteins are indicated above each domain.

RIP1 kinase, is a multifunctional protein that contains an N-terminal Ser/Thr kinase (KD) and a C-terminal death domain (DD), has emerged as a key regulatory molecule involved in regulating both cell death and cell survival. When the proinflammatory cytokine TNFα stimulates its receptor, TNFR1, RIP1 regulates whether the cell lives by activating NF-κB or dies by apoptosis or necroptosis, two distinct pathways of programmed cell death that are activated by different signalling and resulting in different outcomes.

4.4 Discussion

4.4.1 TRAIL sensitivity in CRC and BC cell lines

Although TRAIL is proposed to selectively kill cancer cells, many breast and colorectal cancer cell lines are known to be resistant to TRAIL [110],[240], [241]. To investigate the matter in detail, a panel of eleven human colorectal and breast cancer cell lines were screened for TRAIL sensitivity by determining 1- PARP cleavage using immunoblotting and 2- mitochondrial depolarization using flow cytometry following a 6h treatment.

TRAIL induces apoptosis through binding its death receptors, DR4 and DR5. These receptors are characterized by an intracellular death domain that facilitates assembly of the DISC and subsequent activation of caspase cascade [116]. Binding of TRAIL to a cell surface receptor either DR4 or DR5, leads to apoptosis through the extrinsic pathway **see section 1.3.3.1**. Briefly, the trimerization of the receptors on the cell surface results in recruitment of FADD, which in turn recruits procaspase 8. Next, caspase 8 is cleaved through proximity activation. The formation of the active caspase-8 triggers an intracellular signalling cascade either through 1- direct activation of effector caspases e.g., caspase-3 which can cleave PARP [132] or 2- indirectly by activating caspase-9 through cleaving Bid and inducing mitochondria depolarisation [242]. These two different mechanisms were classified as type I and type II extrinsic apoptosis, respectively and in the order I defined them above, dependent on the involvement of mitochondria. Mitochondria depolarisation is a key event in apoptosis as it is often regarded as the point of no return, therefore we used it to quantitatively analyse apoptotic cells. Interestingly, most cells in our panel represent type II mechanism, where mitochondria is depolarised at early stages of apoptosis. This can be visualised from our results as PARP cleavage and mitochondria depolarisation numbers align with the exception of MDA-MB-436 where PARP cleavage was very evident at low dose TRAIL, but mitochondria depolarisation was minor. As mentioned above, we also investigated PARP cleavage as readout of apoptosis. PARP is an important protein in the cellular response to DNA damage; and once cleaved by caspase-3, -6 or -7, it is no longer active, and DNA cannot be repaired. Therefore PARP cleavage and formation of p89 is a readout of effector caspase activation [243].

In our study, we examined TRAIL-sensitivity in breast and colorectal cancer cells. Our panel consisted multiple cell lines from each cancer, representing all the spectrum of EMT phenotypes such as epithelial, metastable/borderline, and mesenchymal. We found that TRAIL sensitivity aligned with the EMT status of cancer cell lines, e.g., the more mesenchymal the cell, the more it responds to TRAIL. Strikingly, seven of eleven colorectal and breast cancer cell lines showed high sensitivity to TRAIL-induced apoptosis with 25 and 250 ng/ml. By contrast four cell lines of the eleven cell lines tested were resistant

to TRAIL-induce apoptosis at any given dose (low and high). Two of the eleven cell lines showed a modest sensitivity to TRAIL, only displaying detectable apoptosis with high concentration of TRAIL (250ng/ml). Other studies, although not designed to look at TRAIL sensitivity based on EMT status, found similar results. For example, our results showed that MDA-MB-231 cells, apoptosis was detected in ≥ 90% of cells after a 6-hour treatment at doses (25 and 250ng/ml). In fact, microscopically, we observed an increase in cell death within 2 hours after treatment (data not shown). This result can be explained by the fact that caspase-8 and caspase-3 were cleaved and activated as early as 2 hours in these cells. Another mesenchymal BC cell line, MDA-MB-436, also showed strong response to TRAIL but slightly less than MDA-MB-231. These results mirror those of the previous studies that have examined the same cell lines [228], [244], [110], [245]. Another breast cancer cell line which showed high sensitivity to TRAIL even with low concentration was Hs578T cells with a mesenchymal phenotype. This finding was also reported by Rahman *et al.* who showed that Hs578T cells were highly sensitive to TRAIL-induced apoptosis [228]. Furthermore, and most importantly, we observed the same effect of TRAIL on in colorectal cancer cell lines stating mesenchymal phenotype. In consistent with previous study, our results showed that RKO cell line showing mesenchymal phenotype despite lack of vimentin expression, was also highly sensitive to TRAIL [246]. Visually, apoptosis was observed in RKO cells treated with 250ng/ml as early as 2h post-treatment. While HCT116, a borderline cell line, exhibited the same apoptotic response to the high concentration of TRAIL required 6h for a similar magnitude of apoptosis as compared to RKO. These results are in agreement with Saturno *et al*. findings which showed that both RKO and HCT116 cells were sensitive to TRAIL-induced apoptosis [247], but RKO is more sensitive.

Importantly, we observed a different TRAIL response for the colorectal cancer cell lines SW480 and SW620, although they were generated from the same patient [199]. In the previous chapter, we showed that SW480 cell line was classified as a mesenchymal cell line, whereas SW620 cells were shown to be a metastable/ borderline. Here, SW480 showed more PARP cleavage upon TRAIL treatment even with low concertation (25ng/ml) compared to SW620 cells. This result agrees with the findings of other studies, in which SW480 found to be TRAIL-sensitive cell line [248]. On the other hand, SW620 cells showed PARP cleavage only with high concentration of TRAIL (250ng/ml).

Our findings that SW620 cells respond to TRAIL is not in full agreement with others. White-Gilbertson *et al.* (2000) found that SW620 is TRAIL-resistant [240]. A possible explanation for this might be due to a lower concentration of TRAIL or duration of treatment as well as the commercial source of TRAIL, as in our case we only observed a response with high concertation and after 6h. Of note, all TRAIL treatment experiments were repeated more than 3 times for each cell line.

On the other hand, no caspase cleavage was detected up to 6 hrs in our colorectal and breast cancer cell lines with epithelial morphology. T47D and ZR-75-1 cells which are classified as epithelial show no apoptosis upon TRAIL treatment. In addition, TRAIL resistance was sustained in these cells even at the highest concentration (250 ng/ml). Similarly, Guseva *et al*. and others found that these cells were highly resistant to TRAIL-induced apoptosis [249], [250]. TRAIL resistance was similarly observed in the selected epithelial colorectal cancer cells (Caco2 and HT29) cell lines. These cells are also known to be resistant to TRAIL [251], [114], [252], [253]. From western blotting findings, it is therefore likely that such connections exist between EMT status and TRAIL sensitivity.

We also determined the mitochondrial depolarization using flow cytometry in our tested panel of breast and colorectal cell lines, and in line with our western blotting results, only mesenchymal cells responded to TRAIL and showed mitochondria depolarisation. The flow cytometry results were consistent with the data obtained by western blotting, especially at high concentration. Only MDA-436 was an outlier as it didn't significantly depolarise mitochondria with low dose TRAIL. An increase in the mitochondrial depolarization was observed in all TRAIL-sensitive cell lines (mesenchymal and metastable-responders) cells, which was statistically greater than that seen in the TRAIL-resistant (epithelial-nonresponses) group which showed no mitochondrial depolarization at all **see figure 22, 24**.

These results and literature support our proposed hypothesis that EMT status is associated with TRAIL responses seen in breast and colorectal cancer cells and indicate a potential for therapeutic selectivity for cancers that have propensity to metastasize. To this end, we characterized and labelled cell lines according to their TRAIL sensitivity and EMT classification **see figure 21,23**. Next, we investigated cell surface TRAIL receptors DR4 and DR5 in our panel

4.4.2 TRAIL Receptors

It has been speculated that the expression of TRAIL receptors at the cell surface or expression level of pro- and antiapoptotic proteins are the key determinists for sensitivity to TRAIL. To address these questions, we studied cell surface expression levels of TRAIL receptors in relation to TRAIL sensitivity in a panel of eleven breast and colorectal cancer cell lines. Furthermore, we analysed intracellular components of the apoptotic pathway related to TRAIL sensitivity.

TRAIL resistance has been associated with various mechanisms, including defects in TRAIL receptors such as loss of cell surface expression. There are two receptors for TRAIL that can induce apoptosis upon

ligand binding: TRAIL-R1 (DR4) and TRAIL-R2 (DR5) [230], [115]. Interaction between TRAIL and its receptors is the first step triggering apoptosis and TRAIL sensitivity may be influenced by the level of expression of these receptors on the cell surface. [117], [126], [254]. Upon ligand stimulation, death receptors initiate apoptotic signalling. Importantly, not all cells respond to death receptor ligation leading to the conclusion that there may be cell type-specific differences in TRAIL signalling. Moreover, cancer cells generally express TRAIL-binding death receptors, and for unknown reason, signal transduction of apoptosis induced by these receptors appears to be selective for tumour cells [237]. Not only in cancer cells, but studies have also shown increased expression of TRAIL receptors in many normal cells. Even though some cells are expressing TRAIL death receptors, they are still resistant to TRAIL. For example, normal colonic epithelium tissue expressing TRAIL receptors remained TRAIL-resistant [129], suggesting that the presence of these receptors is not sufficient to confer sensitivity to TRAIL-mediated apoptosis.

From our screen of eleven human CRC and BC cell lines, we found that seven of them responded to TRAIL, while the remaining four cell lines were highly resistant up to a concentration of 250 ng/ml. We have previously identified that the responders were morphologically mesenchymal, while the non-responders were epithelial.

Further, we analysed DR4 and DR5 cell surface expression by flow cytometry in our panel of TRAIL-resistant and TRAIL-sensitive tumour cell lines of various origin but defined EMT status. We found there was no correlation between expression of TRAIL receptors DR4 and DR5 on the cell surface and resistance to TRAIL; **see figure 27**. This result support previous research in this area which links TRAIL sensitivity and the expression of its cell surface receptors [255], [256].

In our findings, with one exception, all cancer cell lines analysed exhibited cell surface expression of DR4 and DR5, irrespective of TRAIL response. The exception was the SW480 cells that expressed DR5, but not, DR4; **see figure 27**. This finding is consistent with that of Jin *et al.* (2004) who showed that the level of death receptor 4 on the cell surface was significantly down-regulated in SW480 cells which were sensitive to TRAIL [230]. Moreover, in accordance with this result and despite having no DR4, several tumour types have been shown to respond to TRAIL only by one of its death receptors (DR4 or DR5) [257], [115]. Furthermore, similar to our results, Saturno and colleagues (2013) showed that SW620 cells also were sensitive to TRAIL, although it expresses low level of DR4 [257]. Moreover, HT-29 cells, which are very epithelial and unresponsive to a high concentration of TRAIL were expressing considerable amounts of DR4 and DR5. In consistent with our result, Galligan *et al.* (2005) have confirmed that HT29 which is TRAIL-resistant cell line expressing both TRAIL receptors [258]. Importantly, TRAIL-resistant ZR-75-1 cells express DR4 much greater that the hypersensitive cell line Hs578T. Additionally, the

levels/amounts of DR5 are similar in RKO (responders/sensitive) and Caco-2 (non-responders/resistant) cells. This result is in agreement with a study performed by Geelen *et al.* (2003) who showed that Caco-2 cells which were TRAIL-resistant expressed both receptors on the cell surface at similar level to SW948 cells which is TRAIL-sensitive [241].
In general, independent of TRAIL sensitivity, all cell lines tested (MDA-MB-231, HS-578T, MDA-MB-436, RKO, SW620, SW480 and HCT116/ sensitive), and (T47D, ZR75-1, HT-29 and Caco2/resistant) cancer cells were expressing comparable level of DR4 and/or DR5, indicating that these receptors can be present in cancer cells irrespective to their sensitivity to TRAIL.

In general, our results are in line with previous studies showing that surface expression of TRAIL receptors is not a determinant for TRAIL sensitivity [228], [256], [247], [255], [127], [256] .Furthermore, despite comparable levels of both DR4 and DR5 in our cell lines, some cells are failed to response to TRAIL treatment. This suggest that the expression levels of TRAIL receptors DR4 and DR5 was unlikely to be a cause of TRAIL resistance, at least for the cell lines tested here. Statistically, we observe no correlation between TRAIL sensitivity and TRAIL receptors DR4/5 expression on the cell surface. From the table we can see regardless of the sensitivity of the tumour cells to TRAIL, all these cells are expressing DR4 and DR5 (p=0.16) and (p=0.13) respectively.

In principle, the presence of receptors is necessary for TRAIL-induced apoptosis, however, cells expressing these receptors will not necessarily respond to TRAIL treatment. TRAIL also can bind to other death receptors such as DcR1 and DcR2, but they only have a decoy function. They do not transmit pro-apoptotic signals to downstream molecules, but they compete with DR4 and DR5 for TRAIL binding. Therefore, they do not have a positive function but can inhibit TRAIL-induced apoptosis [231]. Several reports have shown that no correlation between decoy and TRAIL sensitivity as mentioned in the literature review; **see section 1.3.2.** Decoy receptors have been shown to inhibit TRAIL- induced apoptosis when overexpressed, however, the endogenous expression of decoy receptors has not been found to correlate with TRAIL resistance in several different cancer cell lines [127], [255], [228], [259]. For example, the TRAIL-resistant cell line (Caco-2) express no DcR1 or DcR2 on their surface but DR4 and DR5 [241]. We investigated the expression of DcR1 and DcR2 using publicly available expression databases, such as the “gene expression atlas” [260]; **see supplementary figure 2**. The data shows no association between decoy receptors expression and TRAIL susceptibility. For example, in our data, TRAIL-resistant cell line (Caco-2) express very low or no DcR1 or DcR2, whereas the highly TRAIL-sensitive cell line (Hs578T) expresses both decoys.

Although the surface expression of death receptors does not necessarily predict TRAIL sensitivity, the presence of both or one receptor is essential to initiates apoptosis in the cell. Thus, TRAIL sensitivity could be due to potential defect in downstream signalling components. we hypothesised that the development of resistant to TRAIL-induced apoptosis in these tested colorectal and breast cancer cells may be due either to increased expression of survival proteins or decreased expression of pro-apoptotic factors. In the following section we examined several intracellular proteins in our panel with respect to EMT status and TRAIL sensitivity.

4.4.3 Components of death signalling pathways

Our previous findings suggest that mesenchymal carcinoma cells die upon TRAIL treatment and their selective death is unlikely to be associated with the expression of TRAIL receptors. This has been investigated thoroughly via cell surface profiling using flow cytometry.

We further characterised expression of intracellular regulatory proteins that are involved in the death receptor-mediated apoptosis signalling pathway. Other than TRAIL cell surface receptors (DR4 and DR5), a potential common mechanism associated with TRAIL response is the expression of DISC components such as TRADD, FADD, caspase-8 [261]; or several other accessory proteins such as c-FLIP, c-IAP1 or c-IAP-2 can be important in determining TRAIL response [262], [228], [255]. However, these studies do not demonstrate that the level of these proteins are the primary reason for TRAIL resistance. Our results suggest that TRADD, FADD and caspase-8 are either equally expressed or the expression pattern do not correlate with TRAIL response in our cell line panel of eleven cancer cell lines tested here **as shown in figure 29**.

Moreover, as we previously mentioned in the literature review, some studies suggest differential expression of Bcl2 may also play a role [263], [264], [249]. Whereas others confirmed no association found between Bcl2 level and protection against TRAIL in their study performed in different cancers includes neuroblastoma, glioblastoma, and breast carcinoma cell lines [235]. They believe there might be intrinsic differences between cell types. In other words, TRAIL signaling pathway may be regulated in a cell type-dependent manner at different levels, e.g., at the mitochondrial level by Bcl-2 and at a level downstream of mitochondria by XIAP. In addition, the differences between the studies may be due to different TRAIL preparation which may vary in their cytotoxic potential [265]. Furthermore, we looked at gene expression profile of c-FLIP in these cancer cell lines and found no correlation between

c-FLIP expression and TRAIL sensitivity; **see supplementary figure 1**. Also, there was no association between c-FLIP expression and EMT status.

In general, manipulating any gene in the DRC could have an impact on TRAIL sensitivity. Many previous studies proposed various mechanisms in which TRAIL sensitivity can be altered. For example, overexpression of genes that can positively contribute TRAIL induced apoptosis such as caspase 8 or Bid would increase TRIAL sensitivity. Similarly, reducing any negative contributor such as cIAP1/2 or c-FLIP can have a positive contribution in sensitising TRAIL sensitivity. These factors might contribute to the observed TRAIL sensitivity in individual cell lines, but a literature review demonstrated none showing a consistent correlation. Thus, in this study our main aim was to identify a common determinant of TRAIL-sensitivity, therefore we sought to define a common gene in our panel that most highly correlated with EMT related TRAIL-induced apoptosis.

As a result, we tested some of the abovementioned molecules in a panel of eleven breast and colorectal cancer cell lines representing different molecular phenotype (EMT) and TRAIL sensitivity. Among investigated proteins, we were able to identify only one protein, RIP1, which is in highly expressed in TRAIL sensitive cells. RIP1 is also interesting in the context of EMT for the reason that it is expressed at the lowest levels in epithelial carcinoma cells.

RIP1 known to be an important regulator of cell survival through activating NF-κβ pathway as much it is involved in apoptosis [239]. Therefore, overexpression of RIP1 in chemo-radio resistant carcinoma cells, namely mesenchymal-metastatic cancer cells, is not a surprise but a novel finding. To our knowledge our findings are the first to associate EMT status and RIP1 protein overexpression. We also found that, in mesenchymal cells and upon TRAIL ligation, RIP1 can switch from a pro-survival to pro-apoptotic protein as it complexes with caspase-8. In line with our hypothesis, upon TRAIL treatment, both PARP cleavage and mitochondria depolarization has been observed more in mesenchymal cancer cells that also express RIP1 abundantly. Basically, those cells that have high RIP1 showed response to TRAIL through RIP1-facilitated apoptosis. On the contrary, epithelial cells had low RIP1 expression and they did not show any response to TRAIL even with high concentration.

Notwithstanding, the non-definitive proof of mechanism, our findings suggest a potential use for this promising apoptosis/survival-related molecule as biomarker for monitoring the TRAIL sensitization process. Our result here suggests a mechanism by which EMT induced RIP1or RIP1 can increase sensitivity of cancer cells to TRAIL. In the following chapter, we will investigate and discuss the mechanism in more detail.

Chapter 5: Modulating TRAIL sensitivity through EMT and cell morphology

5.1Introduction

While the basic machinery that drives TRAIL-induced apoptosis is known, the molecular mechanism of the regulation of apoptosis induction by TRAIL, especially with respect to tumour-cell-specific sensitisation to TRAIL is still an unresolved issue. Therefore, cancer cells' sensitisation towards TRAIL response studies were carried out by many researchers with the aim to identify novel factors that are required for apoptosis induction by TRAIL. As in previous chapters we provided evidence that mesenchymal cells respond to TRAIL and of the panel of proteins we analysed, RIP1 was the only discriminator between responding and nonresponding cells, we will explore whether RIP1 is a part of EMT programme. In this chapter, experiments will be carried out to modulate EMT status of cells and TRAIL sensitivity will be investigated with a focus on RIP1 abundance.

Numerous studies focused on manipulating EMT-TFs in cell culture or mouse xenografts [59]. In particular, ZEB1 has emerged as a key inducer of EMT. Additionally, ZEB1 protein expression showed the most significant inverse correlation with E-cadherin. ZEB1 was shown to repress E-cadherin and to induce EMT in various cancer cells [266],[267],[51]. Furthermore, in normal physiology or during wound healing, EMT can be induced by extracellular stimuli e.g., TGF-β. TGF-β signalling has been shown to effectively induce EMT in various epithelial cells. Thus, we will manipulate EMT status using these above-mentioned mechanisms.

5.2Aims the chapter

The aim of this chapter is to manipulate the morphology or EMT status of cancer cells and examine parallels with RIP1 expression and TRAIL response.

1- To generate epithelial cells with single-cell morphology and investigate whether RIP1 expression and TRAIL response change. To achieve this aim, we will knock out *CTNND1* gene, a master regulator of epithelial cell-cell junction, in two epithelial cell lines (T47D and HT29). Here we will address whether the morphology of cells or EMT transcriptional programming is important for TRAIL response.

2- To investigate if converting mesenchymal cells to epithelial will alter RIP1 expression and TRAIL response. To study this, we will knock down *ZEB1* gene, a master EMT regulator, in a mesenchymal cell line (MDA-MB-231) and see whether mesenchymal to epithelial transition (MET) is sufficient to alter TRAIL response.

3- To investigate if converting epithelial cells to mesenchymal will alter RIP1 expression and TRAIL response. To test this hypothesis, we will be treating our epithelial cell line (ZR-75-1) with TGF-β, a well-known driver of cancer progression and a potent inducer of EMT.

5.3Results

5.3.1 Validation of P120 knockdown in HT-29 and T24D, cell morphology and EMT markers

A key feature of mesenchymal cells is lacking epithelial junctions therefore, being unable to make cellular clusters. However, being a single cell, for example in the case of seeding at low density, does not induce EMT. A key question in our study was whether TRAIL response observed in mesenchymal cancer cells was due to having single cell morphology. To test this, we knocked down a master regulator epithelial junction gene, *CTNND1*, by siRNA in two epithelial cell lines (HT-29 and T47D). To validate the knockdown, the reduction in P120 catenin protein, encoded by *CTNND1* gene, was measured by western blotting 72h after transfection; **see figure 31.** *CTNND1* siRNA transfected cells acquired single cell morphology, similar to mesenchymal cells. Along with p120 catenin protein, EMT markers were examined to ascertain the epithelial gene expression programming was intact. MDA-MB-231 cells were used as a positive control for mesenchymal status. Knocking down *CTNND1* did not alter EMT status of both cell lines tested. There was still detectable E-Cadherin and no ZEB1 or vimentin expression upon *CTNND1* knock down. Importantly, no change in RIP1 expression was detected in the knock down cells compared to the control.

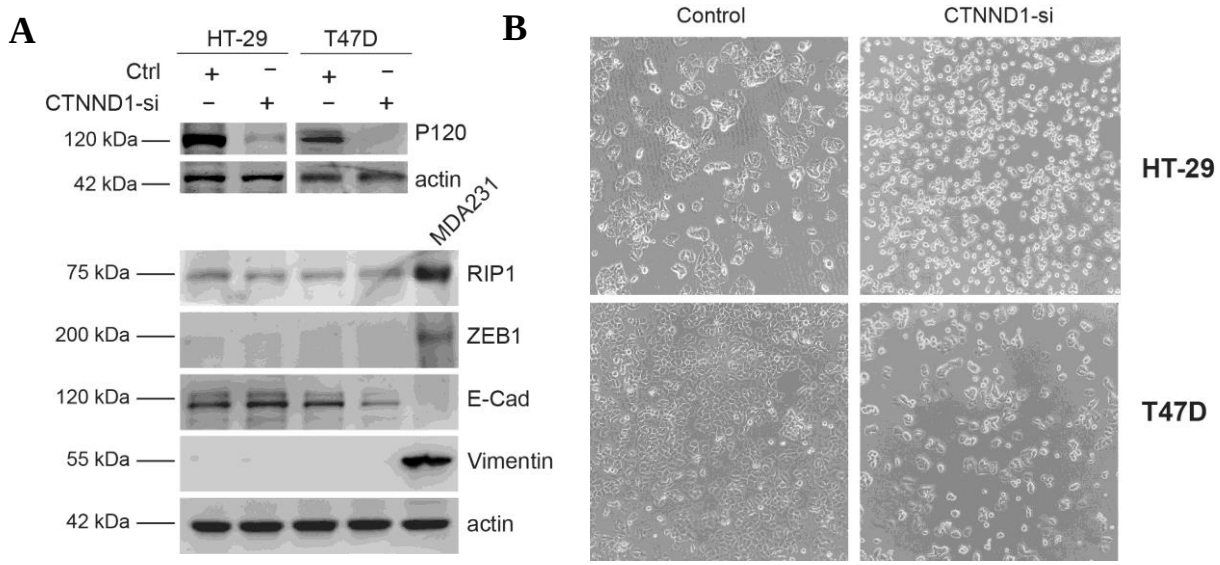


Figure 32. Biochemical and morphological validation of CTNND1-siRNA transfection in HT-29 and T47D cell lines.

A. Validation of *CTNND1* knock-down and the expression levels of EMT markers in T47D and HT29 both control and transfected cells 72h post-transfection. ZEB1, E-cad and vimentin showed no change. MDA-MB-231 was used as a positive control for the mesenchymal marker indicator. RIP1 expression showed no change in KD cells compared to the control. **B.** morphology of the control and *CTNND1*-si cells 72 h post-transfection. The KD cells show a single cell morphology.

5.3.2 TRAIL treatment of the P120 catenin knockdown (CTNND1-si) model in HT-29 and T47D cells

As only cells with mesenchymal morphology responded strongly to TRAIL, we, next, treated these cells with TRAIL (250ug/ml) for 6h. There was no response to TRAIL in both control and transfected cells. We didn't observe any differences in PARP cleavage. MDA231 was used as a positive control for TRAIL response which showed strong PARP cleavage.

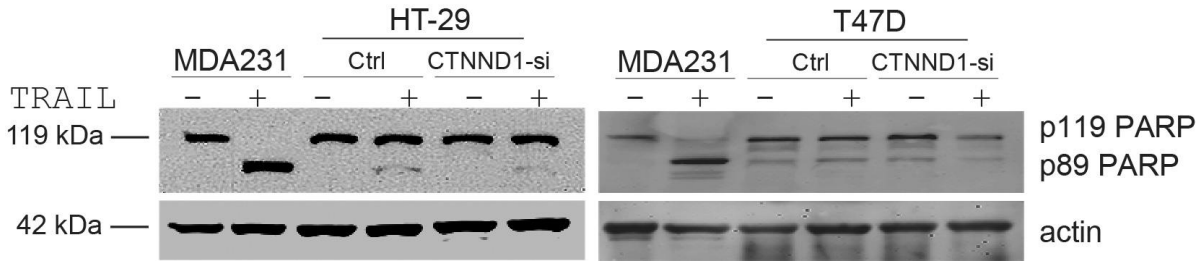


Figure 33. TRAIL response upon CTNND1 knock down in T47D and HT-29 cells.

Western blot analysis for PARP after TRAIL treatment (250 ng/ml) for 6 hours to assess the presence of cleaved PARP in T47D and HT-29 cells. PARP cleavage was not detected in both control and KD cells. MDA-MB-231 was used as a positive control for TRAIL response. Actin was used for equal loading.

5.3.3 Biochemical and morphological hallmarks of EMT for MDA-MB-231 ZEB1 knockdown model

After ascertaining the EMT programme, but not the morphology of cells as a discriminator of TRAIL response, we were obliged to investigate an isogenic model of EMT or MET for TRAIL sensitivity. Therefore, we knocked down the master EMT regulator ZEB1 using lentiviral shRNA in MDA-231 cells to have its epithelial version [177]. In order to validate the knockdown, we analysed the morphological (panel A) and biochemical (panel B) hallmarks of EMT. The results showed that ZEB1 knockdown was successful and produced the expected outcome as *ZEB1*-knock down cells showed epithelial morphology microscopically. ZEB1 protein was only detected in MDA-MB-231 WT cells. E-Cadherin and Pan-keratin expressions mark epithelial status and were detected in the knockout cells with no vimentin and ZEB1 expressions which identify mesenchymal features.

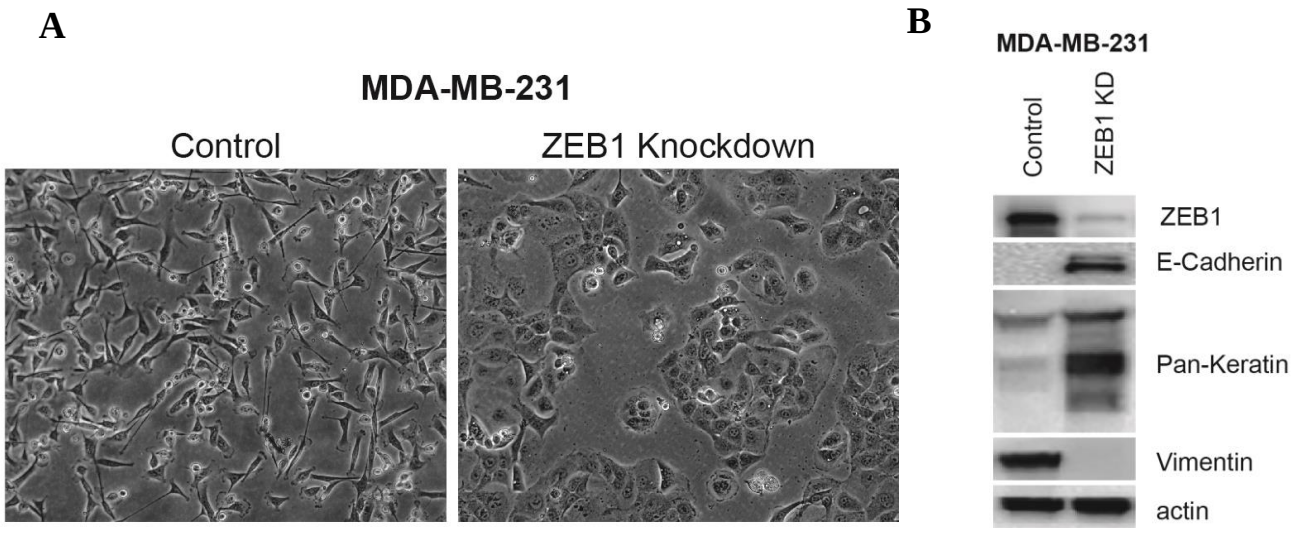


Figure 35. Validation of ZEB1 knockdown and EMT status in MDA-MB-231 cells.

A. Cell morphology of MDA-MB-231 WT (left) and ZEB1 knockdown model (right). **B.** ZEB1 and Vimentin (mesenchymal markers) and E-cadherin and Pan Keratin (Epithelial marker) were investigated by western blotting. β -actin was used as an equal loading control.

5.3.4 TRAIL response in MDA-MB-231 WT and MDA-MB-231 ZEB1 knockdown cells

When we treated these cells with TRAIL, ZEB1 knock-down (ZEB1 KD) cells did not respond to TRAIL (**panel A**). This result is in agreement with our hypothesis and previous findings. We also confirmed these results by flow cytometry as ZEB1-KD cells did not show any significant increase in mitochondria depolarisation (**panel B**). Importantly, RIP1 protein was decreased in ZEB1-KD cells along with mesenchymal markers (**panel A**). Next, we investigated the possibility of other death receptor-mediated apoptosis component proteins towards the lack of TRAIL response in ZEB1 KD cells.

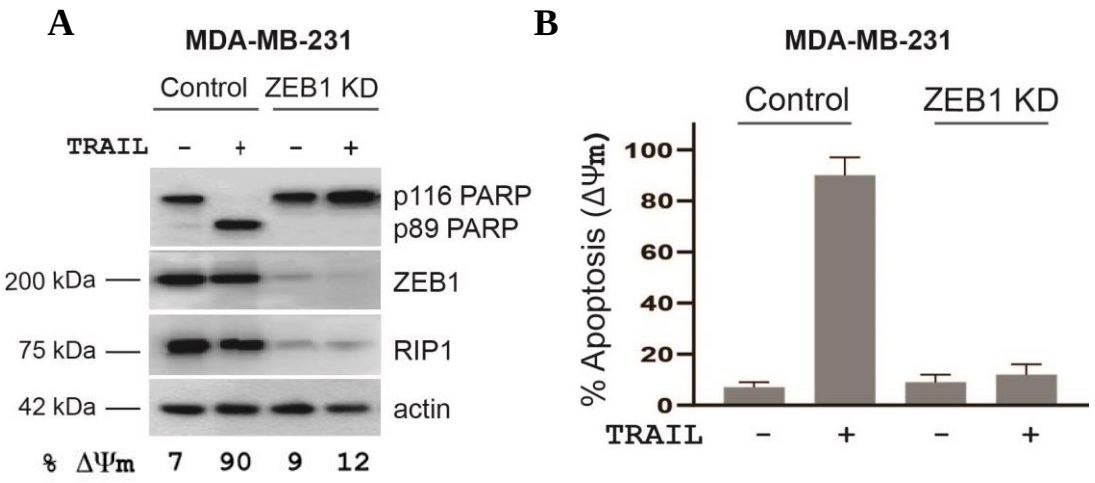


Figure 37. Assessment of apoptosis by PARP cleavage and mitochondria depolarisation in MDA-MB-231 and MDA-MB-231 ZEB1 KD cells.

A. Western blot analysis to assess the expression of PARP in control and KD cells upon TRAIL treatment (250 ng/ml) for 6 hours. RIP1 expression in both control and KD cells. Actin was used for equal loading. **B.** Quantitative assessment of apoptosis in the form of mitochondria depolarisation (Δψm) using flow cytometry in abovementioned cells.

5.3.5 Death receptor components of MDA-MB-231 and MDA-MB-231 ZEB1 knockdown model

To investigate the possibility of other death receptor-mediated apoptosis component proteins towards the lack of TRAIL response in ZEB1 KD cells we performed western blotting for FADD, TRADD, TRAF-2, c-Flip and caspase-8 along with RIP1. Crucially, our results suggest that none of the death receptor-mediated apoptosis components was altered significantly to contribute to reduced apoptosis in MDA-231-ZKD cells but RIP1.

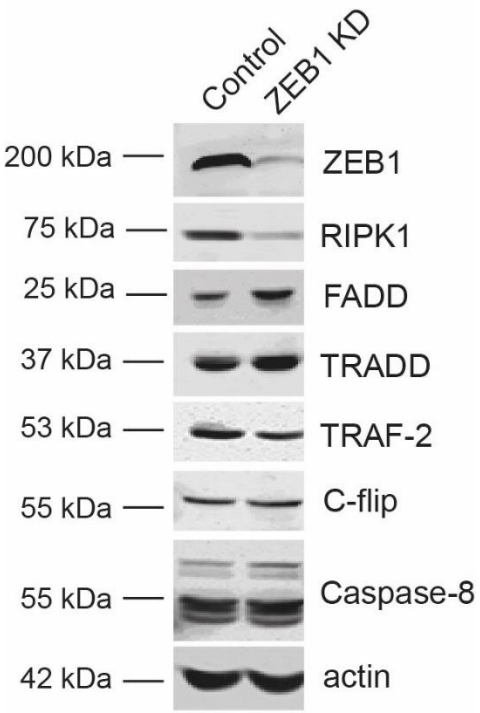


Figure 38. Death receptor components in MDA-MB-231 WT and MDA-MB-231 ZEB1 knockdown model.

Protein expression of RIP1, FADD, TRADD, TRAF-2 c-Flip and caspase-8. No observed Death receptor components in MDA-MB-231 WT and MDA-MB-231 ZEB1 knockdown model.

5.3.6 Kinetics of EMT activation in ZR-75-1 cells upon TGF-β treatment.

Our third aim in this chapter is to induce EMT using TGF-β and investigate TRAIL response. Most epithelial cells respond to TGF-β but the kinetics and magnitude of the response differs from cell to cell. To determine the optimal conditions for EMT induction, ZR-75-1 cells were exposed to a constant dose (2 ng/ml) of TGF-β for up to 120h (5 days) and cells were collected at daily intervals. We used a well-known marker for TGF-β pathway activation, Transglutaminase 2 (TG2), along with the morphological assessment of ZR-75-1 cells to assess the extent and magnitude of EMT phenotype. With the course of the treatment, TG2 protein gradually increased and reached very high levels at 120h (day 5) (Fig. 4). Therefore, we selected day 3, which showed a minimal but detectable, and day 5, for an evident, activation of TG2 (EMT) for future experiments. On day 5, also the morphology of ZR-75-1 cells has changed (Fig. 5). We observed cell flattening and individualisation, all hallmarks of EMT, which corresponded to TG2 upregulation observed by WB. In the following section, we sought to validate the EMT status and to investigate RIP1 expression levels on days 3 and 5.

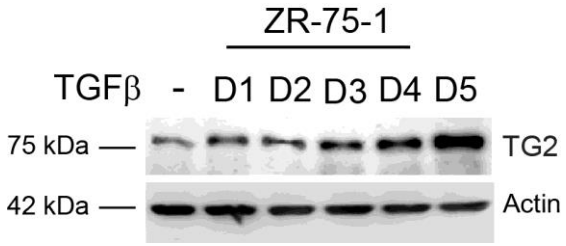


Figure 39. Kinetics of TGF-β response (time-course) in ZR-75-1 breast cancer cells.
TG2 expression in ZR-75-1 cells, treated with the TGF-β (2ng/ml) for up to 120h (5days). Control ZR-75-1 (-) were untreated. A steady increase in TG2 expression levels was observed over time. β -actin was used as an equal loading control.

5.3.7 EMT status and cell morphology of ZR-75-1 cell line treated with TGF-β

We next analysed EMT markers on day 3 and day 5 and RIP1 abundance as it is our focus. As expected, we detected significant upregulation of ZEB1 on day 5 on the protein level upon TGF-β induction. In another word, TGF-β induced overexpression of ZEB1 and it activated EMT, as ZR-75-1 cells became mesenchymal, morphologically and biochemically evidenced with downregulation of E-cadherin and increased vimentin. Importantly, we observed a remarkable increase in RIP1. Besides TG2, PKCα was selected to confirm TGF-β activation. It was demonstrated by Sayan's group that activation of TGF-β-induced EMT and increased PKCα abundance [177]. The next stage of this line of research was to investigate whether TGFβ induced EMT and accompanied RIP1 expression led to sensitization of TRAIL treatment.

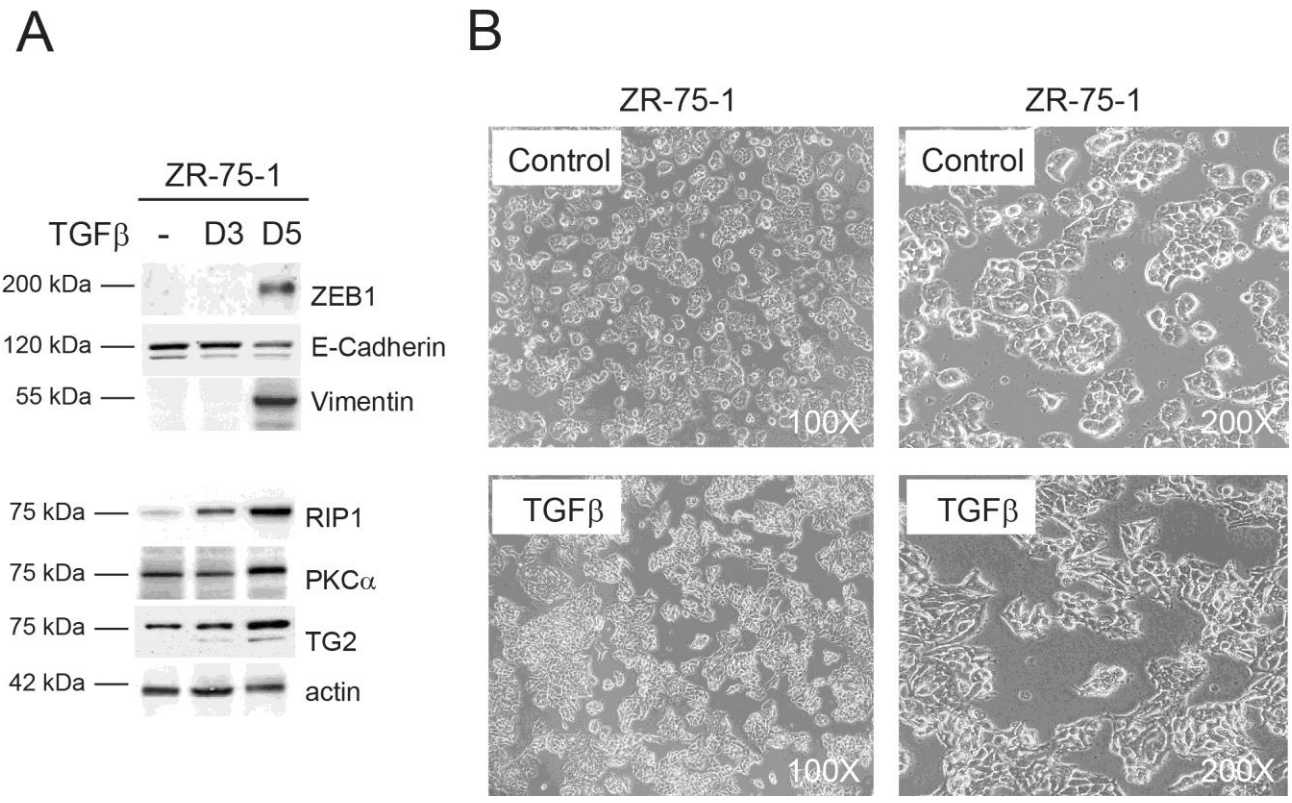


Figure 40. TGF-β induction in ZR-75-1 cell line.

A. EMT protein markers ZEB1, Vimentin (mesenchymal marker) and E-cadherin (epithelial marker). PKCα (mesenchymal marker) and TG2 (TGF-β marker). RIP1 expression (our gene of interest) in ZR-75-1 cells, tread or not with TGFβ. RIP1 expression increased on day 5 along with mesenchymal markers. β -actin was used as an equal loading control. **B.** ZR-75-1 cell underwent EMT and acquired a mesenchymal morphology on day 5 upon TGFB treatment.

5.3.8 TRAIL response upon TGF-β-induced EMT in T47D and ZR-75-1 cell lines

To further study the implication of TGF-β-induced EMT on TRAIL response, we performed western blotting to check the expression of PARP and its cleavage in each sample. Crucially, cleaved PARP was clearly observed only in cells treated with TGF-β and TRAIL (250ng/ml). On the other hand, there was no PARP cleavage in T47D or ZR-75-1 cells with no TGF-β, both of which are epithelial in nature. TGFβ did not induce apoptosis alone. In conclusion, PARP cleavage was only seen in cells with mesenchymal phenotype. Overall, we can say that cells that have undergone EMT respond to TRAIL; **see figure 38.** In agreement with our earlier studies, RIP1 expression was highest in cells that respond to TRAIL. Also, we concluded that TGFβ induces RIP1 as part of the EMT programme.

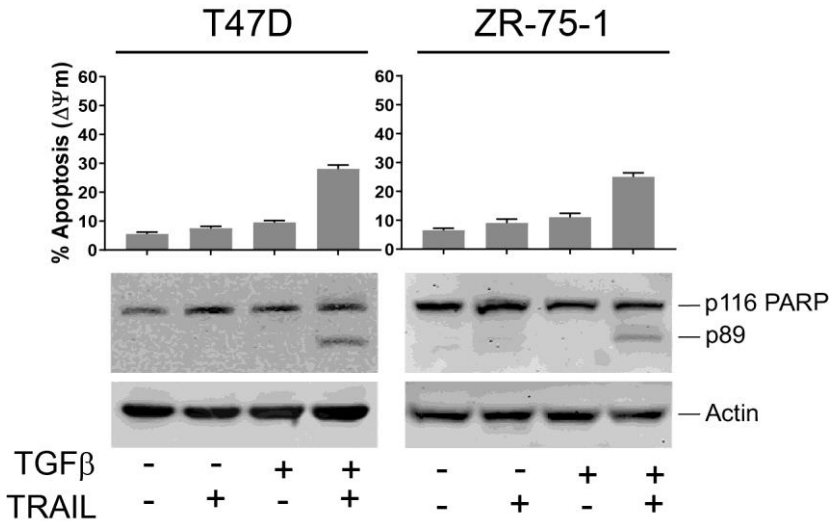


Figure 41. TRAIL response upon TGF-β-induced EMT in T47D and ZR-75-1 cell lines.

Western blot analysis of TRAIL treatment (250 ng/ml) for 6 hours to assess the expression PARP in T47D and ZR-75-1 cells with (+) and without (-) TGF-β. Apoptosis detected in the cells treated with TGF-β/TRAIL. Actin was used for equal loading. % of apoptosis (Δψm) was assessed using flow cytometry. Data are presented as the mean ± SD. All experiments were repeated at least three times. The data were analysed using GraphPad Prism software (GraphPad Prism 7, USA).

5.4Discussion

Metastasis is still the major cause of cancer-related mortality. This highlights the pressing need for the identification of new molecular targets for the treatment of metastases and markers of metastatic capability. Activation of EMT was considered a decisive driver of tumour progression from initiation step to metastasis [1]. Most studies involved manipulation of different EMT-inducing transcription factors (EMT-TFs), such as Snail, Slug, Twist, ZEB1 and ZEB2 in cell culture or xenograft mouse models. Several studies have since highlighted their roles in stemness, invasion and metastasis of cancer cells. Among these master EMT-inducing genes, ZEB1 expression was most significantly correlated with the mesenchymal phenotype (high Vimentin and low E-cadherin expression) [98]. A study carried out by T. Brabletz group showed that ZEB1 depletion strongly reduced progression towards highly malignant, metastatic pancreatic tumours. This is in stark contrast to the depletion of Snai1 or Twist1 in the same model, which did not affect malignant tumour progression [268]. ZEB1 was shown to repress E-cadherin and to induce EMT in various cancer cells such as uterine [269], pancreatic [267], [174], osteosarcoma [175], lung [176], liver [270], colon [173], [177] and breast [51]. In these tumours, ZEB1 expression correlates with loss of E-cadherin and is associated with advanced disease or metastasis, which indicates the relevance of ZEB1 induction of EMT and tumour progression in cancer progression. However, none of these studies have reported the association between ZEB1, the main activator of EMT, with RIP1-induced TRAIL sensitisation, which will be further discussed in this chapter.

Moreover, induction of EMT can be accomplished by many different extracellular factors both during development and in cancer. This include TGF-β signalling which has been shown to play an important role in EMT [271], [102], [93]. TGF-β is the most physiological inducer of EMT which is utilised by cells during wound healing and inflammation [99]. It has been reported that adding TGF-β to culture media is an effective/efficient way to induce EMT in various epithelial cells [100], [101]. Upon TGF-β treatment, epithelial cells showed reduced expression of epithelial markers e.g., E-cadherin and increased mesenchymal marker such as vimentin and ZEB1. Morphologically, epithelial cells changed from cubelike to elongated spindle-like shape.

However, mutations in genes e.g. *SMAD4* involved in TGF-β pathway is a common feature in some cancers including colorectal cancer [272], [273], [274], [102]. For that reason, we only performed this experiment in breast cancer cells as mutations in TGF-β pathway have not been found in breast carcinoma cell lines [275-278].

Basically, our reasoning behind all these two approaches in modulating EMT is to transform tumour cells from mesenchymal to epithelial-like (by silencing ZEB1) or take the opposite approach and induce EMT in epithelial cells by TGF-β stimulation. Hence, we will be able to examine the modulation of our target gene, RIP1, in these two models and, most importantly, to assess their TRAIL response.

Based on our earlier findings in chapters 3 and 4, it will be of further interest to evaluate if EMT-induced RIP1 is critical for TRAIL response. **In chapter 3,** we characterized 11 breast and colorectal cancer cell lines based on their EMT status: mesenchymal, borderline (metastable) or epithelial. We observed RIP1 is consistently overexpressed in mesenchymal/metastatic carcinoma cells. **In chapter 4**, we treated these cells with TRAIL and found that only mesenchymal cells are responding. We concluded that these cells, which responded to TRAIL, have a high abundance of RIP1 and this is our novel finding. These findings indicate that RIP1 could play a potential role in EMT-induced TRAIL response. In brief, RIP1 expression pattern matched with TRAIL-induced apoptosis response. I also have to mention that activation of a key survival pathway (NF-κB) is dependent on RIP1 abundance/activity, which is also observed in the naturally chemo-resistant metastatic cancer cells [279]. As RIP1 is also shown to be a decisive molecule in death receptor-mediated apoptosis, we propose treating mesenchymal carcinoma cells with TRAIL was able to direct the pro-survival activity of RIP1 to pro-apoptotic, as observed with extensive cell death.
Therefore, it would be reasonable to hypothesize that EMT-induced RIP1 mediates TRAIL sensitisation.

First and foremost, and before we started manipulating EMT in our selected cell lines, we attempted to investigate whether it is the "single-cell morphology" acquired during EMT or the "EMT transcriptional programme" itself that has a role in TRAIL sensitisation. Our reason to take this matter further is the differences in cell surface area and accessible cell surface between epithelial and mesenchymal cells. Epithelial cells cluster and they have apico-basal polarity. The only exposed surface of an epithelial cell is the apical side, which is quite small. On the other hand, mesenchymal cells do not cluster in groups or form epithelial junctions. They are elongated with a large surface area. Importantly, as they are not bound to other cells on the sides, they have a more accessible surface to ligands. Therefore, they may have more exposed death receptors compared to epithelial cells where the death receptors may be binding to TRAIL.

To investigate this, we reduced the expression of P120-catenin protein (encoded by *CTNND1* gene), a master regulator of epithelial junctions which links cell-cell junctions to cytoskeleton, by using siRNA [280]. There are many epithelial junctional proteins, for example, a-catenin, b-catenin, E- and P-Cadherins, which facilitate epithelial morphology. We chose to knock down *CTNND1* because it is

considered a master regulator of cell adherence. Other reasons include 1- There is a functional redundancy of E- and P-Cadherins. We needed to knock both these genes down to achieve a true functional inhibition, 2- Mutations in *CTNNB* gene (encoding for b-Catenin protein) are commonly observed in colon cancer with no morphological consequence. To extend our findings to multiple cancers, the transfection was carried out in two epithelial cell lines: HT-29 and T47D of colon and breast origin. We observed single-cell morphology in the transfected cells. Downregulation of P120 Catenin protein was also detected using western blotting **(Figure 7A)** indicating that P120 itself was enough to break the cell-cell junction acquiring single-cell morphology **(Figure 7B)**. Therefore, in HT-29 and T47D cells at least, P120 appears to be essential for cell-cell junction and stability. In contrast, some reports indicate that P120 is not essential, and its absence causes only a minor defect that is not fully apparent unless complemented by removing E-cadherin or α-catenin [281], [282]. Nevertheless, this matter could be dependent on the efficiency of knockdown or context (e.g., mutation profile of the cancer cell). Indeed, many other studies including ours, confirmed that when p120 is removed, single cell morphology is achieved effectively [283], [280]. Davis M. *et al.* showed that P120 acts at the cell surface to control cadherin turnover, and they knockdown P120 by siRNA which resulted in complete loss of cell-cell adhesion [283].

We also checked EMT markers and RIP1 expression which showed no change in the *CTNND1*-si samples compared to the control. These cells still express high levels of Keratins and E-Cadherin but no vimentin or ZEB1. This means *CTNND1* knockdown cells are still epithelial transcriptionally but present as single cells morphologically. This means that we achieved obtaining clustered and single epithelial cells with no change in RIP1 expression or EMT status **(see figure 31-A)**.

Next, we set out to investigate the consequences of this transfection. When we treated these cells with TRAIL (250ug/ml) for 6h, *CTNND1*-si (single cells) showed no response to TRAIL along with the control (clustered cells). In another word, these cells are exposed to TRAIL from all sides having no apical- polarity, similar to mesenchymal cells but did not respond to it. In short, we provide evidence that cell morphology alone is not a determinant parameter for TRAIL response. Here, we disclose that single-cell morphology has no role in TRAIL sensitisation without EMT programming. RIP1 expression level remains unchanged as the control supporting our previous observations.

In the following part, we hypothesized that EMT-induced RIP1 is critical for TRAIL response. As we previously showed, ZEB1 expression and the mesenchymal phenotype are correlated in breast cancer. To test our hypothesis, we knocked down *ZEB1* with shRNA in MDA-MB-231 human breast cancer cells as shown in **figure 33 A**. Western blot analysis showed that ZEB1 downregulation was successful. Initially, we analyzed EMT markers as a validation of the knockdown. Indeed, under ZEB1 knockdown we observed a remarkable increase in E-Cadherin and Pan-Keratin protein levels accompanied by a loss in spindle-like cell morphology with a shift into more cobblestone-like morphology which marks epithelial status **figure 33 B**. No vimentin expression was observed which identifies mesenchymal status. In many previous and ongoing research, knockdown of ZEB1 was achieved in MDA-MB-231 cells and resulted in upregulation of different genes most of which are determinants of epithelial differentiation and cell-cell adhesion [284], [285], [286]. Our results were in parallel with them.

Next, I sought to investigate whether EMT status was a determinant of RIP1 expression and hence TRAIL response. Initially, we analyzed RIP1 expression in both control and knockdown cells. ZEB1 knockdown facilitated a significant change in RIP1 expression level as we observed that RIP1 is going down as a result of this depletion as **shown in figure 34A**. Although we don't have a mechanistic explanation for these findings, the present study could represent a first step in elucidating a causal relationship between ZEB1 and RIP1 expression. Indeed, understanding the reasons of the concomitant changes in ZEB1 and RIP1 expression, for example by direct transcriptional control, will not only expand our knowledge of molecular mechanisms but also will aid our ability to develop therapeutic approaches.

Our following aim was to examine TRAIL response in MDA-MB-231 ZEB1 KD cells. Both control and KD cells were treated with TRAIL (250ng/ml) for 6 hours. As expected, and in accordance with our previous findings, unlike control cells (mesenchymal), there was no response to TRAIL in the KD cells (epithelial) concomitant with having decreased level of RIP1 protein **(see figure 34A)**. This reveals that RIP1 is a part of EMT mediated survival signalling in which metastatic cells become more chemo-resistant by activating a key survival pathway (NF-κB) through a higher abundance of RIP1, but at the same time, these cells are vulnerable to TRAIL due to high abundance of RIP1. In parallel with western blot analysis, we performed a flow cytometry assessment of the mitochondrial depolarisation to quantitatively assess apoptosis. These produced similar results to WB, as such KD cells did not die upon TRAIL treatment (**see figure 34B)**. The results of this study are also consistent with our main findings previously presented in **section 4.3.2, 4.3.4**. Of course, the influence of ZEB1 knock down to

chemoresistance and its consequences through NF-kβ pathway has to be investigated but this represents a different study.

Lastly, to exclude other death receptor-mediated apoptosis component proteins, contributing towards the lack of TRAIL response in ZEB1 KD cells, we performed western blot analysis for FADD, TRADD, TRAF-2, c-Flip and caspase-8. Crucially our results suggest that none of these was altered to convincingly reduced apoptosis but RIP1 **as shown in figure 35**. This result provides evidence that RIP1 is crucially involved in metastatic cells and TRAIL-induced apoptosis. Abhari *et al*. explored the role of RIP1 in apoptosis in a model and they showed that RIP1 silencing abolished the formation of the RIP1/FADD/caspase-8 complex and thus prevented caspase activation [287]. In fact, emerging evidence suggests that RIP1 kinase may modulate tumorigenesis

In the last section, we attempted to reach out for our third aim in this chapter. This aim is to study whether the induction of EMT would affect epithelial cells towards TRAIL response. TGF-β is well known to induce EMT in various epithelial cells. Many EMT transcription factors, including ZEB1, Twist, Snail and Slug, are induced by TGF-β signalling in a Smad-dependent manner and play critical roles in EMT induction [102], [93]. It has been shown, *in vitro* and *in vivo*, that TGF-β signalling act as a powerful stimulator of tumour progression [288]. Generally, TGF-β-induced EMT in breast carcinoma directly affected by TGF-β/SMAD signalling pathway [275] **See section 1.2.3** Herein, we attempt to induce EMT in epithelial cells. Specifically, we induced EMT in epithelial breast cancer cell lines (ZR-75-1 and T47D).

To study this, we set up a model using ZR-75-1 epithelial breast cancer cells. First, we wanted to determine the optimal conditions for EMT induction in ZR-75-1 cells. The cells were exposed to a constant dose (2ng/ml) of TGF-β for up to 120h and were collected at daily intervals. We used a well-known marker for TGF-β activation, TG2, along with a morphological assessment of ZR-75-1 cells. Previously published studies showed that TG2 is overexpressed in many malignancies and its expression is associated with a metastatic phenotype [289], [290]. Moreover, many other studies have concluded that TGF-β induced TG2 is not only an indicator for EMT, but also TG2 may have a function in promotion and sustaining EMT [290]**.** Herein, with the course of treatment, TG2 protein gradually increased and reached very high levels at 120h (day5) **(figure 36)**. Accordingly, for future experiments, we selected 72h (day 3), which showed a minimal but detectable, and day 5, very strong activation of EMT. On day 5, also the morphology of ZR-75-1 cells has changed as we observed cell flattening and individualisation, as morphological hallmarks of EMT as shown in **(figure 37B)**, which corresponded to TG2 upregulation observed by western blotting. Besides TG2, PKCα was selected as a marker to

confirm TGF-β induced EMT. It was demonstrated by Sayan's group that activation of TGF-β-induced EMT increased PKCα abundance [177]. In agreement with their study, we observed an increase in PKCα.

After effectively inducing EMT in the epithelial cells (ZR-75-1), we wanted to verify EMT markers on Day3 and Day5, as well as RIP1 abundance, as it is our focus. Long-term exposure to TGF-β (2mg/ml) resulted in more profound EMT as evidenced by cellular elongation, repression of E-cadherin and induction of vimentin and most importantly ZEB1 expression as shown in **figure 37A**. In addition to this successful attempt, we also study RIP1 expression in these cells. Interestingly, RIP1 was gradually increased in parallel with TG2 and showed a remarkable increase on day 5; **see figure 37B**. We provide here evidence, for the first time, that treatment of ZR-75-1 cells with TGF-β upregulates the expression of RIP1. Our results suggest for the first time that RIP1 is a part of the EMT programme induced by TGF-β similar to vimentin and ZEB1.

The next stage of our research was to investigate whether TGF-β induced EMT leads to sensitisation to TRAIL. Interestingly, we have shown that when ZR-75-1 cells underwent EMT and acquired mesenchymal phenotype, they respond to TRAIL (*n=3*) **see figure 38**. For validation, we repeated the same experiment with another epithelial cell line (T47D) which showed the same result following TGF-β treatment (*n=3*). Overall, we can say that cells that have undergone EMT are expressing more RIP1 and consequently respond to TRAIL.

Based on all these findings we are proposing a novel biomarker/therapy axis. We suggest metastatic propensity of a cancer cell can be assessed by RIP1 expression and such cells can be eliminated using a TRAIL based therapy.

Chapter 6: Conclusion and future perspectives

Overall, we had several key observations that lead to the progression of our study. Briefly, and knowing mesenchymal carcinoma cell lines are resistant to apoptosis, we found that the expression of RIP1 is increased in mesenchymal cells with acquired TRAIL sensitivity. These results suggest that RIP1 play a key role in acquired TRAIL sensitivity (response to TRAIL), making it a promising molecular target for sensitizing TRAIL-induced cancer cell death. In this thesis we made an attempt to cover the topics of TRAIL sensitivity and EMT, to elucidate molecular mechanisms behind TGF-β driven breast cancer progression and RIP1 expression for TRAIL sensitisation. We managed to propose a mechanism by which RIP1 upregulation or downregulation can be achieved through modulating TGF-β/Smad pathway or in more generic terms EMT. TGF-β pathway activation led to RIP1 upregulation TRAIL sensitisation and ZEB1 knock-down reduced RIP1 levels and caused TRAIL resistance. Briefly, we found EMT-induced RIP1 sensitise cells to TRAIL.

6.1Summary of the project findings

The results of this project were summarised in 3 chapters:

Chapter 3: EMT status in breast and colorectal cancer cell lines.

Chapter 4: TRAIL sensitivity, cell surface receptors expression and death receptor components in BC and CRC cell lines.

Chapter 5: Modulating TRAIL sensitivity through EMT and cell morphology in selected cell lines.

In chapter 3, EMT status was assessed in 11 cell lines from BC and CRC. I believe this is the first time such a large-scale study has been conducted using various cancer types. Our microscopy and western blotting data concluded to classify the 11 cell lines into three groups:

A- Epithelial cell category, which includes T47D, ZR-75-1, HT29 and Caco-2.

B- Mesenchymal cell category, which includes MDA-MB-231, MDA-MB-436, Hs578T, RKO and SW480.

C- Intermediate/borderline cell category, which includes SW620 and HCT116.

Our panel was classified based on the most prominent EMT markers, E-Cadherin and Pan-keratin as epithelial markers and vimentin as a mesenchymal marker. Also, ZEB1, the major regulator of EMT as a mesenchymal marker and was found only in mesenchymal cell lines.

In chapter 4, We examined TRAIL sensitivity in breast and colorectal cancer cells using a panel that included multiple cell lines of each phenotype. The response was confirmed by determining PARP cleavage in these cell lines using western blotting, also we checked the mitochondria depolarisation using flow cytometry. Our results found that TRAIL sensitivity varied with the EMT status of each cancer cell line, as the responders (TRAIL-sensitive) cell lines were morphologically mesenchymal, while non-responders (TRAIL-resistant) cell lines were epithelial. Intermediate/ metastable cell lines showed partial response to TRAIL.

Moreover, to associate whether TRAIL death receptors DR4 and DR5 are increased during EMT and play a role in TRAIL sensitivity, we examined TRAIL cell surface receptors (DR4 and DR5) in both sensitive and resistant cell lines. The results demonstrated that these death receptors can be present in these cancer cells independently of their TRAIL response. A similar conclusion applies to decoy receptors.

Lastly, our third aim in this chapter was to identify a key molecule in the apoptosis pathway. This objective is achieved, and we identified RIP1 as a potentially important determinant of TRAIL response in epithelial and mesenchymal carcinoma cells. We found that the expression of RIP1 is increased in TRAIL-sensitive mesenchymal cells compared to TRAIL-resistant epithelial cells. This result suggests that RIP1 play a key role in TRAIL sensitivity, making it a promising molecular target for sensitising TRAIL-induced cancer cell death.

Here, the result of this study indicated for the first time the possible implication of EMT-induced RIP1To our knowledge, this study is the first to link the response of mesenchymal carcinoma cells to TRAIL with their phenotype and this is via RIP1-mediated apoptosis.

In chapter 5, As we suggest that RIP1 could have a critical role in TRAIL/EMT-mediated apoptosis. *RIP1* was our focus gene in the following studies. RIP1 is important in controlling proteins involved in the process of NF-κB (survival), apoptosis, and necroptosis. Our hypothesis suggests that the overexpression or downregulation of RIP1 in the epithelial or mesenchymal cancer cell lines may have consequences in their response to TRAIL. Thus, we manipulated the morphology and EMT status of cancer cells and examine parallels with RIP1 expression. We treated these cells with TRAIL and examined their response.

In summary, as we know that mesenchymal cells are responding to TRAIL, but epithelial cells are not. Like any ligand, TRAIL binds to death receptors on the cell surface. The obvious question would be, are mesenchymal cells responding to TRAIL because they have a larger exposed cell

surface? To study this, we tried to acquire single-cell morphology (mesenchymal-like feature) in different epithelial cell lines and to investigate TRAIL response in these cells. This approach was accomplished by down regulating *CTNND1* (p120-Catenin protein). P120-Catenin has emerged as a master regulator of cadherin retention and stability at the cell surface. The stabilizing effects of p120-Catenin have been studied over the past years and researchers have shown that p120-Catenin is required to stabilized epithelial junctions in these cells. So, we knocked down p120ctn in two different epithelial cell lines (HT-29 and T47D) to acquire single-cell morphology and thus to examine their response to TRAIL. The result from this study indicates that the acquired single-cell morphology did not lead to any response to TRAIL.

Moreover, we aim to find whether the induction of EMT, upon TGF- β stimulation, would affect epithelial cells towards TRAIL response. We made an attempt to induce EMT in epithelial breast cancer cell lines (ZR-75-1) and (T47D). These cells were effectively transit from epithelial to mesenchymal phenotype. Again, we examined our gene of interest *RIPK1*, and we observed up-regulation of RIP1 in these cells. Remarkably, these cells when acquired mesenchymal feature, they responded to TRAIL, which were not responding to TRAIL when they were epithelial.

lastly, we induced MET by silencing a master EMT TFs (ZEB1) in the mesenchymal breast cancer cells (MDA-MB-231). MET was successfully induced in these cells, and they were transformed from mesenchymal to epithelial phenotype. In addition to this successful attempt, we looked at RIP1 expression in these cells. ZEB1 knockdown facilitated a significant change in RIP1 abundance. Upon TRAIL treatment, unlike control, we observed that MDA-MB-231 knockdown cells expressing less RIP1 and accordingly show no response to TRAIL.

In general, EMT manipulation was accomplished by different approaches aiming to get a different expression of RIP1 (upregulation in epithelial cells) by TGF-β and (down-regulation in mesenchymal cells) by knowing down ZEB1. Our results here showed a successful attempt of the hypothesis/ aims as TRAIL response was altered in these models. RIP1 abundance increased in epithelial cell lines (non-responders) and they showed response to TRAIL upon TGFβ–induced EMT. Whereas the mesenchymal cell line (responder) did not respond to TRAIL when we knock down ZEB1 as RIP1 went down. Perhaps, our data provide evidence that RIP1 is crucially involved in metastatic cells and TRAIL response.

Considering all the information above, we came to the conclusion that 1) EMT induces chemo-resistance and metastasis, 2) channeling TNF-induced NF-κB activation towards apoptosis can be used to selectively kill metastatic cells. 3) RIP1 plays a central role in controlling the cell's fate, survival or death in response to TNF stimulations. Based on all these findings we are proposing a novel biomarker/therapy axis. We suggest metastatic propensity of a cancer cell can be assessed by RIP1 expression and such cells can be eliminated using a TRAIL based therapy.

6.2 Potential future studies

Data presented in this thesis have highlighted the potential of EMT-induced RIP1 as a key mechanism in TRAIL sensitisation. However, significant additional work is required in order to further elucidate its role in cancer cells at a molecular and cellular level.

The limitations in the design of this study might also indicate that we currently know too little about RIP1 in general and that translates, in particular, lack of ideas as to how targeting RIP1 will apply to cancer therapies. However, this study provides evidence that metastatic/mesenchymal carcinoma cells overexpress RIP1 and this, at least, presents diagnostic significance in the form of a biomarker. We answered some unanswered questions to fill the gaps in the fields of EMT, TRAIL resistance and RIP1 in this project. We sincerely hope our findings will give new insight and allow proceed further in translation.

6.2.1 Future work and limitations

The main challenge we faced was the multi-faceted aspect of RIP1 as it contributes to survival, apoptosis and necroptosis. Therefore, simply overexpressing or knocking down RIP1 will not be sufficient or informative enough to come to a conclusion. The reasons and possible solutions to these problems are listed below.

1- RIP1 has emerged as a key regulatory molecule involved in regulating both cell death and cell survival. A study carried out by Kelliher *et al.* (1998) showed RIP1-deficient mice appear normal at birth but become runted and die postnatally due to extensive apoptosis [291]. So, silencing RIP1 completely cannot be our aim because the total lack of RIP1 is not compatible with life [292]. This means we could have failed if we attempted to knock down RIP1 in our models. Nevertheless, infecting mesenchymal carcinoma cells with a *RIPK1* targeting shRNA and selecting clones representing different expression levels of RIP1 seems like a viable option. Basically, our suggestion

is to attain a level of RIP1 expression that is much less than mesenchymal cells but comparable to epithelial ones. Obviously, treating these cells with TRAIL will give an answer about the direct involvement of RIP1 in TRAIL-induced apoptosis in mesenchymal carcinoma cells.

Alternatively, one can overexpress RIP1 in epithelial carcinoma cells and investigate TRAIL response. However, this approach also comes with potential problems as it is a well-known fact that RIP1 overexpression induces necroptosis. One way to tackle this problem can be by creating a tamoxifen-inducible system which will allow overexpression of RIP1 in an inactive state in which RIP1 can be made functional by the addition of tamoxifen. But again, tacking the problem, e.g., necroptosis versus apoptosis may be challenging. One can select clones expressing RIP1 at levels of mesenchymal carcinoma cells and perform TRAIL sensitivity related experiments. As both approaches require time beyond a PhD. study, they will be conducted later.

2- Here we propose for the first time that RIP1 is induced by EMT. We also showed that ZEB1 knockdown results in downregulation of RIP1. Therefore, we provided critical evidence that ZEB1, as an EMT-inducing TF, can activate RIP1.

As a future study, the mechanisms of how ZEB1 and other EMT pathways control RIP1 expression should be investigated. This can be achieved at multiple stages.

A) The first and maybe the key experiment is to see if *RIPK1* mRNA increases during EMT or RIP1 protein is stabilised. We are proposing to induce EMT by TGFb and check if *RIPK1* mRNA is increasing through a kinetics experiment as done for TG2. Also, we should check *RIPK1* mRNA levels in our MDA-231 ZEB1 knockdown cells. These experiments will indicate whether an EMT programme exerts a transcriptional or a post-translational control on RIP1 abundance.

B) In the case that *RIPK1* is transcriptionally regulated during EMT, we will be investigating whether *RIPK1* promoter has binding sites for EMT-TFs. For example, ZEB1 and ZEB2 bind to E-box motifs (CATGTG) along with other zinc finger transcription factors involved in EMT. If found positive, we will be performing luciferase reporter assays and chromatin immunoprecipitation experiments to show direct binding of transcription factors to *RIPK1* regulatory regions. These results will provide definite evidence that *RIPK1* is transcriptionally controlled as part of the EMT programme.

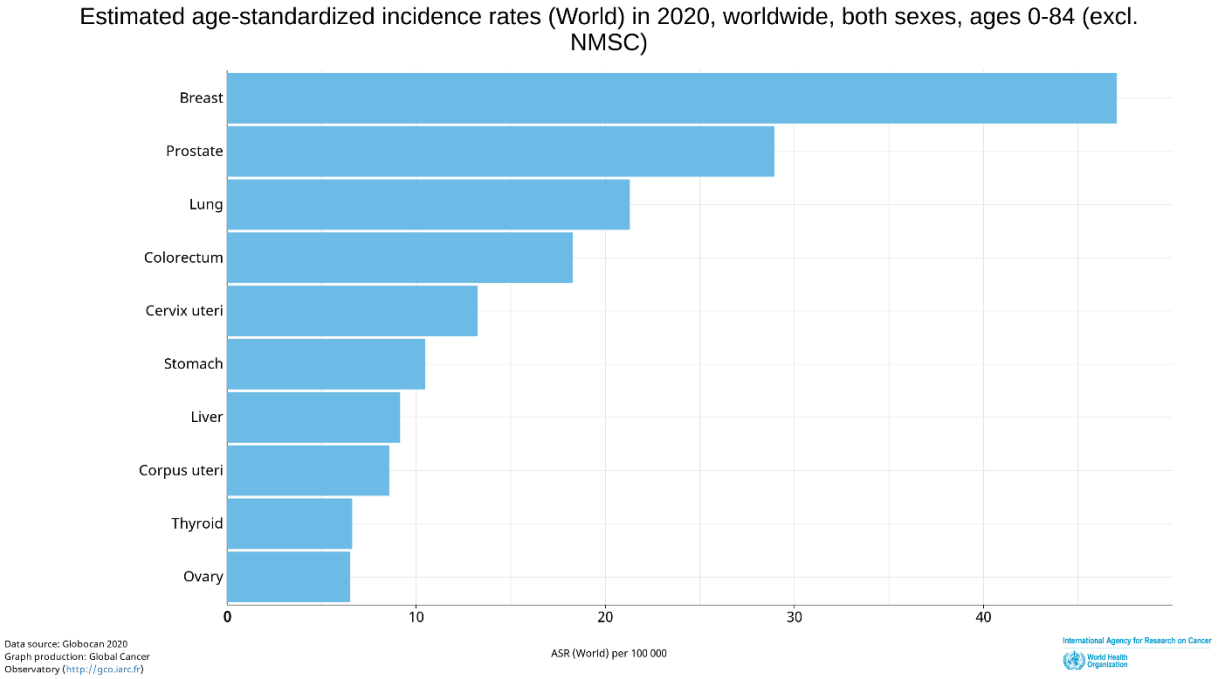
3- As RIP1 is also a key protein for cell survival through NF-κB activation**.** We believe RIP1 plays an important role in the survival of metastatic carcinoma cells. To study this in the future, we will check the nuclear presence of phospho-p65 (an indicator of NF-κB activity), and this can be performed by immunofluorescence or subcellular fractionation of the nucleus, to conclude about the NF-κB activity in metastatic carcinoma cells.

4- While RIP1 is a crucial adaptor protein for cell survival through NF-κB activation, researchers have acknowledged the different roles of RIP1 in opposing signalling pathways including TRAIL-mediated activation of apoptosis and necroptosis. RIP1 possesses a death domain and early experiments showed that artificial overexpression of RIP1 induces cell death. However, recent studies clearly demonstrate that RIP1 contributes to cell death, in a context-dependent manner. For example, when overexpressed alone, it induces necroptosis through phosphorylating RIP3. This activity can be overridden by overexpression of caspase 8, which, then transforms the pro-necroptotic activity to pro-apoptotic. These studies align with observations obtained in mice. Therefore, RIP1 is placed in a unique position to relay signals activated by different stimuli to different pathways. At low levels it signals to NFkb and work as a pro-survival protein. At higher levels it induces necroptosis. When directed by death receptor activation, it can contribute to apoptosis.

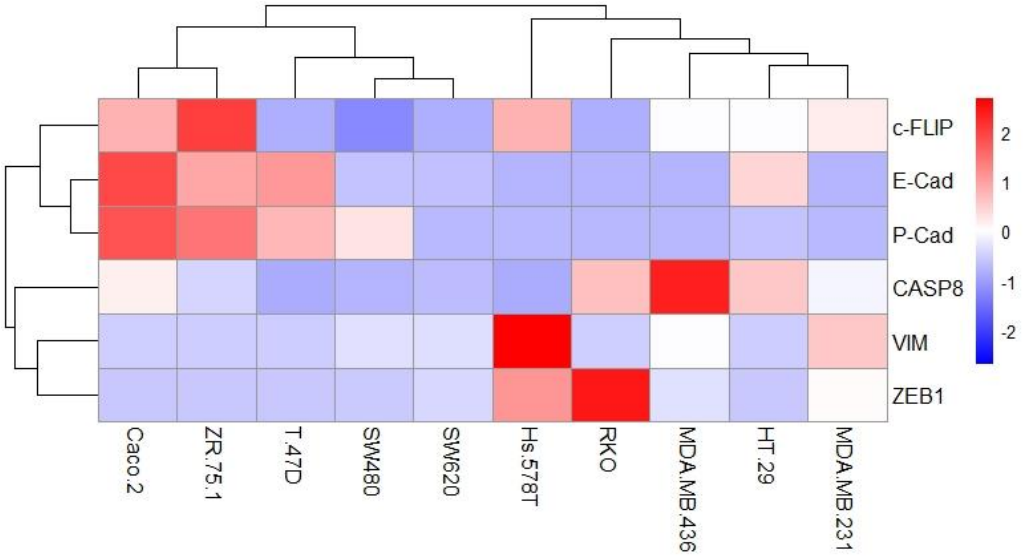
   We believe that RIP1 have a key role, other than facilitating NF-κB signaling and caspase-8 dependent apoptosis is being involved in regulated necroptosis. This process is activated by TNF receptors and executed when caspase-8 is inactive. The point of no return in necroptosis is when mitochondria rupture in a RIP1/RIP3/MLKL/DRP1 dependent manner. We believe this may be an alternative means (other than NF-κB activation) that mesenchymal carcinoma cells become resistant to apoptosis. We found that mesenchymal carcinoma cells have more RIP1 which may lead to changes in mitochondria dynamics in terms of mitochondria shape, size, function and polarity in the absence of TNF activation. Our initial findings in this regard suggest that cells expressing more RIP1 have more mitochondria in terms of mass. We started investigating this topic by electron microscopy for mitochondria in selected epithelial and mesenchymal cells. However, this experiment was stopped due to the pandemic and the data are not presented in the thesis as it is not fully completed. Nevertheless, this aspect might be worth investigating by mitochondrial probes, electron and confocal microscopy, cellular fractionation of mitochondria as techniques to study in the future.

Appendix

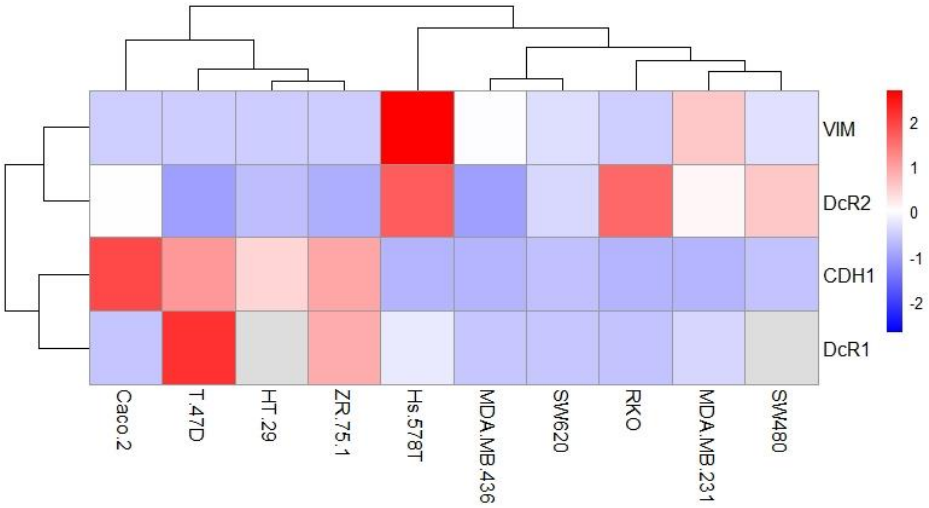
Supplementary figures:



Supplementary figure 1. Cancer statistics for the year 2020. [3]



Supplementary figure 2. RNA-expression analysis identified gene expression profile of c-FLIP expression along with EMT markers. The BC and CRC cell lines in the CCLE cohort of Gene Atlas database [260] were probed for the expression of c-FLIP, E-Cad and P-Cad (epithelial markers), VIM and ZEB1 (mesenchymal marker), CASP8 (Caspase-8).



Supplementary figure 3. RNA-expression analysis identified gene expression profile of TRAIL decoy receptors in parallel with EMT markers. The BC and CRC cell lines in the CCLE cohort of Gene Atlas database [260] were probed for the expression of *CDH1* (epithelial marker), *VIM* (mesenchymal marker). *DcR1* (decoy receptor1), *DcR2* (decoy receptor2). The expression of *DcR1* was most prominent only in the TRAIL-resistant T47D cell line but not in other epithelial cell lines Caco-2 and HT29 cell lines. *DcR2* was significantly expressed in the highly TRAIL- mesenchymal cell line Hs578T and RKO. The presented data indicates no correlation between TRAIL sensitivity/ EMT status and the presence of TRAIL decoy receptors *DcR1* and *DcR2*.

Supplementary table 1. Recipe for resolving SDS- PAGE gels used in western blotting

10ml Running gels				
Constituent	***8%***	***10%***	***12%***	***15%***
dH_2O (ml)	4.6	4.0	3.3	2.3
30% acrylamide mix (ml)	2.7	3.3	4.0	5.0
1.5M Tris (pH 8.8) (ml)	2.5	2.5	2.5	2.6
10% SDS (w/v) (µl)	100	100	100	100
10% Ammonium persulphate (APS) (w/v) (µl)	100	100	100	100
TEMED (µl)	6	4	4	4

4ml Stacking gel	
Constituent	
dH_2O (ml)	2.7
30% acrylamide mix (ml)	0.67
1 M Tris (pH 6.8) (ml)	0.5
10% SDS (w/v) (µl)	40
10% Ammonium persulphate (APS) (w/v) (µl)	40
TEMED (µl)	4

Supplementary table 2 List of in-house buffers used in the project.

Buffer Name	*Buffer Components*	*Supplier*
30% (w/v) 37.5:1 Acrylamide : Bis-acrylamide solution	37.5 g Acrylamide (Fisher) 1 g Bis-Acrylamide (Fisher) Dissolved in dH2O to a final concentration of 30%	In house or Geneflow
30% (w/v) 29:1 Acrylamide : Bis-acrylamide solution	29.0 g Acrylamide (Fisher) 1 g Bis-Acrylamide (Fisher) Dissolved in dH2O to a final concentration of 30%	Geneflow.
1.5M Tris buffer (pH:8.0 and pH:8.8)	182.25g Tris (Fisher). dH2O Adjust pH to 8.0, adjust volume to 1 litter Adjust pH to 8.8, adjust volume to 1 litter	In house
1M Tris buffer (pH:6.8)	121.1g Tris-base (Fisher) dH2O Adjust pH to 6.8, adjust volume to 1 litter	In house
10% APS	1 g APS (Fisher) Adjust volume to 10 ml with dH2O Store at 4ºC for several weeks	In house
10% SDS	1 g SDS (Fisher) Adjust volume to 10 ml with dH2O Store at RT	In house

Supplementary table 2 List of in-house buffers used in the project.

Buffer Name	*Buffer Components*	*Supplier*
5 X SDS-PAGE buffer	dH20 0.5 M Tris (pH 6.8) ----------12.5% Glycerol (Sigma)----------10% 10% SDS (w/v) ----------20%	In house
5 X SDS-PAGE Gel Loading buffer	dH20 0.5 M Tris (pH 6.8) ------12.5% glycerol----------10% 10% SDS (w/v)----------20% 2β- Me----------5% 0.05%(w/v) BPB----------5%	In house
10 X Tris-Glycine-SDS (TGS)	0.25M Tris 1.92M Glycine 1% SDS dH2O	Geneflow/In house
10 X Tris-Glycine-Methanol (TGM)	0.25M Tris 1.92M Glycine 20% Methanol (Fisher) dH2O	Geneflow/ In house
10 X TBS	100 mM Tris (pH 8.0) 1.5 M NaCl dH2O	In house
1 X TBS-T (1 litter)	100ml 10X TBS (Geneflow) 900ml dH2O 0.1% Tween®20 (Sigma)	In house
4% Dried skimmed milk in TBS-T	2g dried skimmed milk (Marvel) 50 ml 1X TBS-T	In house
2.5% BSA in TBS-T	1.25g BSA (Fisher) 50 ml 1X TBS-T	In house
PBS	125mM NaCl (Fisher) 16mM Na2HPO4.7H20	In house

Supplementary table 2 List of in-house buffers used in the project.

Buffer Name	*Buffer Components*	*Supplier*
	10mM KH_2PO_4 HCL to Adjust pH 7.3-7.6	
PBS-T	1 x PBS buffer 0.1%-1.0% Tween-20	In house
Ponceau S	5 % (w/v) Ponceau S 10 % Glacial Acetic Acid (Sigma) dH_2O Store in dark at RT	
0.05 M EDTA (dispdiumethylenediaminetetraacetate)	8.61 g EDTA (Sigma) 80 ml dH_2O Adjust pH to 8.0, Adjust volume to 100 ml	In house
4% paraformaldehyde (v/v)	4ml paraformaldehyde (Fisher) 96 ml dH_2O	In house

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