The role of transmembrane mucin protein MUC1 in anoikis and in EGFR activation of human epithelial cancer cells

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The role of transmembrane mucin protein MUC1 in anoikis and EGFR activation of human epithelial cancer cells

MUC1 is a large, heavily glycosylated transmembrane mucin protein expressed on the apical membrane of normal epithelial cells. In epithelial cancer cells, however, MUC1 is overexpressed, abnormally glycosylated and loses its apical polarization, becoming expressed over the entire cell surface.

Galectin-3, a β-galactoside-binding protein expressed by many types of human cells, is a natural ligand for MUC1. Recent studies by ourselves and others have revealed that the interaction between galectin-3 and MUC1 induces MUC1 cell surface polarization and the exposure of underlying smaller cell surface adhesion molecules. This leads to increased cancer cell homotypic aggregation and cancer cell (heterotypic) adhesion to vascular endothelium.

Recently, mucin-1 (MUC1) was reported to be associated with epidermal growth factor receptor (EGFR) in epithelial cells. EGFR is a receptor tyrosine kinase involved in the regulation of multiple cellular process, including tumour proliferation and metastasis. Changes in MUC1, galectin-3 and EGFR expression have all, individually or in combination, been associated with poor cancer prognosis and increased tumour metastasis. Resistance of cancer cells to anoikis, a fundamental cellular process for maintaining tissue homeostasis, is a pre-requisite for metastasis. The aim of this study was to investigate the impact of MUC1 expression and the MUC1 interaction with galectin-3 and EGFR on EGFR activation and anoikis in epithelial cancer cells.

It was found in this study, that overexpression of MUC1 in epithelial cells prevents initiation of anoikis in response to loss of adhesion. This effect was found to be attributed to both MUC1 extracellular and intracellular domains with predominant effect from the MUC1 extracellular domain. Reduction of MUC1 *O*-glycosylation by stable shRNA suppression of core 1 gal-transferase (C1GT) reduced MUC1-mediated resistance to anoikis in human colon cancer cells HCT116 and SW620. It was also found that MUC1 expression enhanced EGF-induced EGFR activation in human breast and colon cancer cells. Both the MUC1 extracellular and intracellular domains contribute to EGFR activation, again with the predominant contribution from the MUC1 extracellular domain. Thus, binding of galectin-3 to the MUC1 extracellular domain induces MUC1 cell surface polarization and increases MUC1–EGFR interaction, leading to increased EGFR homo-/hetero-dimerization and activation.

These discoveries provide insight into the impact of MUC1 overexpression and MUC1 *O*-glycosylation on cancer cell behaviour in cancer progression and metastasis and may aid future development of novel therapeutic strategies for cancer treatment.

Abbreviation

AKT	Protein Kinase B
APAF 1	Apoptotic protease activating factor 1
AREG	Amphiregulin
BCA	Bicinchoninic acid
BCL2	B-cell lymphoma 2
BH3 domain protein	BCL2 homology domain 3
Bid	BH3 interacting-domain
t-Bid	Truncated Bid
BRCA 1	Breast cancer gene 1
BRCA 2	Breast cancer gene 2
BSA	Bovine serum albumin
BTC	Betacellulin
C1GT	Core 1 Gal-transferase
C3GnT	Core 3 GlcN Ac-transferase
CRD	Carbohydrate recognition domain
DAG	Diacylglycerol
DiL stain	1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate
DISC	Death inducing signalling complex
ECM	Extracellular Matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor(ErbB1)
EPI	Epigen
EREG	Epiregulin
ERK	Extracellular signal–regulated kinases
FAK	Focal adhesion kinase
FADD	Fas associated death domain
Fas-L	Fas ligand
bFGF	Basic fibroblast growth factor

FLIP	FLICE-like inhibitory protein
Gal	Galactose
Gal3F	Full form galectin-3 with both N and C terminal
Gal3C	Truncated galectin-3 without N terminal
GlcNAc	N-Acetyl glucosamine
GalNAc	N-acetyl-galactosamine
ppGalNAc-Ts	Polypeptide GalNAc-transferases
GRB2	Growth factor receptor binding protein 2
GSL-II	*Griffonia (Bandeiraea) Simplicifolia Lectin II*
HB-EGF	Heparin-binding EGF-like growth factor
HIF 1	Hypoxia-inducible factor 1
HIF 2	Hypoxia-inducible factor 2
HGF	Hepatocyte growth factor
HMOX 1	Heme oxygenase 1
HMOX 2	Heme oxygenase 2
cIAP 1	Cellular inhibitor of apoptosis protein 1
cIAP 2	Cellular inhibitor of apoptosis protein 2
ILK	Integrin-linked protein kinase
IP_3	1,4,5-triphosphate
MAPK	Mitogen-activated protein kinases
MEK 1	Mitogen-activated protein kinase kinase 1
MEK 2	Mitogen-activated protein kinase kinase 2
MYBL2	v-myb myeloblastosis viral oncogene homolog-like 2
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OMM	Outer mitochondrial membrane
PERK	Phosphorylated Extracellular signal–regulated kinases
PERP	p53-effector related to pmp22
PI	Propidium Iodide

PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PNA	Peanut Agglutinin
PON2	Paraoxonase 2
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
SH2	Src homology 2
sialyl-Tn	Neu5Ac β1,6GalNAcα
SMA	α-smooth muscle actin
sos	Son of sevenless
SOS1	Son of sevenless 1
SRC	Src homology and collagen
ST7	Suppressor of tumorigenicity protein 7
ST14	Suppressor of tumorigenicity protein 14
ST6GalNAc-T	GalNAc α 2,6 Sialyltransferase II
TGF	Transforming growth factor
TGF-α	Transforming growth factor-α
TKI	Tyrosine kinase inhibitors
TNF-α	Tumour necrosis factor α
TNF RI/ TNF RSF	Tumor necrosis factor receptor superfamily
mTOR	Mammalian target of rapamycin
TRAIL	TNF-related apoptosis-inducing ligand
XIAP	X-linked inhibitor of apoptosis protein
VNTR	Variable number tandem repeat
VVA	*Vicia Villosa Lectin*
ZEB1	Zinc finger E-box-binding homeobox 1
ZEB2	Zinc finger E-box-binding homeobox 2

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1 Introduction

1.1 Epithelial cancer

1.1.1 Epidemiology

Cancer can be defined as a disease where a group of cells disregard the normal rules of cell division and tissue homeostasis. Normal cells adhere to strict cellular signals that dictate whether a cell should divide, differentiate or undergo apoptosis. Cancer cells develop a certain degree of autonomy from these signals and are able to divide and proliferate independently. It is the uncontrolled cellular division, dissemination, survival and proliferation of these tumour cells that leads to poor prognosis and complications associated with cancer.

Epithelial cancers, also known as carcinomas, are currently the most prevalent cancer being diagnosed across Europe (1). In the UK alone, epithelial cancer originating in lung, bowel, breast and prostate together accounted for almost half (46%) of all cancer deaths in the UK in 2014 (2, 3). Tumour metastasis is the primary reason for cancer-related death. Although the mortality rate for all neoplasms has been falling over the last 2 decades, cancer-associated complications still remains the major cause of death in the UK (4).

1.1.2 Molecular basis of cancer

Nonlethal genetic damage is the initiator of carcinogenesis in the majority of epithelial cancers. Such genetic damage can generally be acquired from a variety of sources, such as carcinogens, radiation, viruses, tobacco smoking or it could be inherited in the germline. The genetic hypothesis of cancer implies that a tumour is

formed by the clonal expansion of a single precursor cell that has incurred genetic damage (5, 6). In the majority of tumours, four classes of normal regulatory genes are generally altered, which collectively confers growth and survival advantages of the tumour cells over normal cells. These are the growth promoting proto-oncogenes, the growth inhibiting tumour suppressor genes, genes that regulate programmed cell death (apoptosis), and genes that are involved in DNA repair (5).

Oncogenes are genes that promote autonomous cell growth in cancer cells. They are derived by mutations in proto-oncogenes and are characterised by the ability to promote cell growth in the absence of normal growth promoting signals (7). The mutant allele of a proto-oncogenes is considered dominant because it transforms cells despite the presence of a normal counterparts. Since 1970s, dozens of oncogenes have been reported and characterized, with human epidermal growth factor receptor 2 (HER-2/neu erbB-2), telomerase reverse transcriptase (hTERT), RAS and SRC being some of the major oncogenes reported in epithelial cancers (8, 9).

Tumor suppressor genes normally functions as an inhibitor against cancer development. Mutation leading to loss of function or insufficient expression of tumor suppressor genes leads to cancer progression. In contrast to oncogenes, which are generally dominant, the tumour suppressor family of genes are sometime referred as recessive oncogenes, as both alleles must be damaged to render a loss of protection against tumour transformation (10, 11). However, recently haplo-insufficiency has also been noted (12, 13), whereby loss of a single allele of a tumour suppressor gene has been shown to promote transformation. Key examples of

tumour suppressor genes include the retinoblastoma protein RB and p53. Other examples of tumour suppressor genes include ST7, ST14 and PTEN.

Genes that regulate apoptosis may be dominant or recessive (5). A wide variety of proteins control extrinsic and intrinsic apoptosis which will be discussed later. In short, there are sets of pro- and anti- apoptotic proteins whose activation and expression is tightly controlled in normal cells. In tumour cells, this balance is shifted towards promotion of anti-apoptotic protein. This is achieved via direct promotion in the activation of key anti-apoptotic proteins and/or suppression of key pro-apoptotic proteins. An example of this can be seen in seen in gastric cancer, where there is a downregulation in the expression level of the pro-apoptotic protein FAS, a key component of extrinsic apoptosis pathways (14) while at the same time there is an increase in the expression of anti-apoptotic FLICE-like inhibitory protein (FLIP), an inhibitor of FAS-mediated apoptosis (15).

Structural damage to DNA is remarkably common. Fortunately, normal cells have an in-house DNA repair mechanism that seeks out these structural damages to the DNA molecule and exerts a targeted fix. Mutations to genes responsible for DNA repair have been reported in a variety of cancers including breast, colon, pancreatic and prostate cancer (16-18). Such mutations do not directly transform cells by affecting proliferation or apoptosis. Instead, they have an indirect effect whereby they influence the ability of an organism to repair non-lethal damage to their genes including proto oncogenes, tumour suppressor genes and genes that regulate apoptosis. A prolonged disability in the DNA repair genes can predispose a cell to widespread mutations in the genome and eventually to neoplastic transformation.

Breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2) genes have been identified to play a key role in the DNA repair process, and their alterations are strongly associated with the morbidity of in a variety of epithelial cancers (17-19).

Carcinogenesis is a multistep process at both the phenotypic and the genetic level. The nonlethal genetic damage leads to initial mutation in the cells, which if not repaired, leads to mutation accumulation and acquisition of greater malignant potential. Even though most malignant tumours are initially monoclonal, by the time they are clinically detected, the tumour cells are extremely heterogenous (5). Tumour heterogeneity plays a key role in several acquired phenotypic attributes of a developing tumour such as excessive growth, local invasiveness and the ability to form distant metastasis – collectively termed the hallmarks of cancer

1.1.3 Essential alterations for malignant transformation in epithelial cancer

A key paper published by Douglas Hanahan and Robert Weinberg initially identified six biological capabilities acquired during the multistep development of human tumours (20). Termed hallmarks of cancer these capabilities include 1) self-sustained proliferative signalling without external stimuli, 2) insensitivity to growth inhibitory signals, 3) mechanisms to evade apoptosis 4) limitless replication potential leading to immortality, 5) ability to maintain sustained angiogenesis and neovascularization and, 6) the ability to invade and metastasize. In an updated publication in 2011 (21), Weinberg and Hanahan proposed four new hallmarks: 1) abnormal metabolic

pathways, 2) evading the immune system, 3) genome instability and 4) inflammation (21) leading to overall ten hallmarks of cancer.

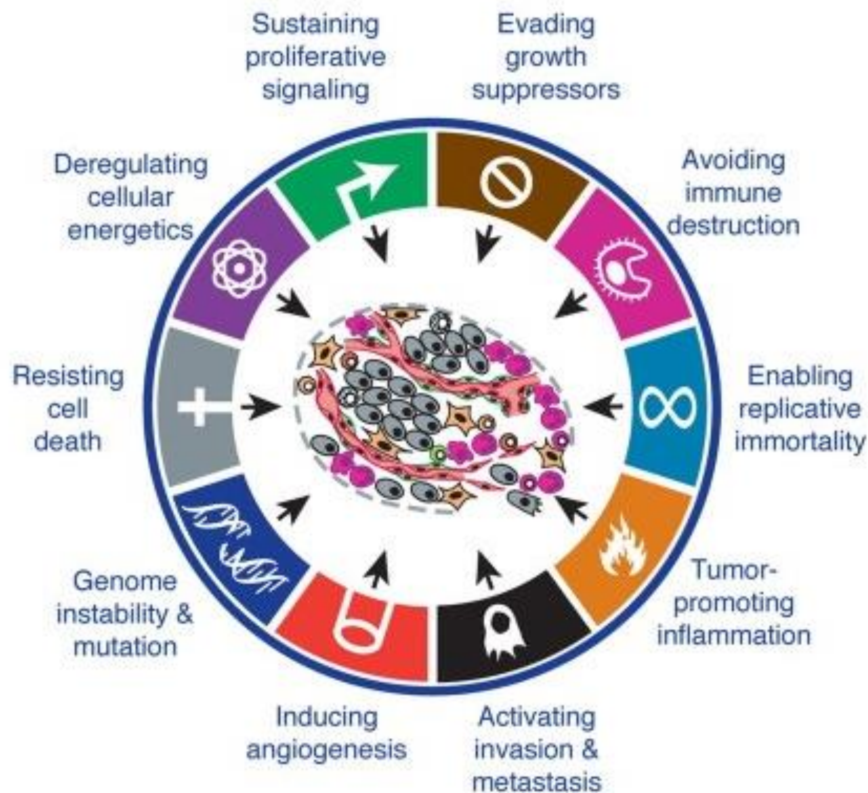


Figure 1.1: The ten hallmarks of cancer. The figure illustrates the ten biological capabilities acquired during the multistep development of human tumours and adapted from (21) (permission acquired from Cell, licence no 4296971476301)

Genome instability is the underlying cause for the acquisition of these hallmarks. Furthermore, conceptual progress in the last two decades has identified two emerging hallmarks of cancer— altered energy metabolism of tumour cells and the ability to evade immune-mediated destruction (21). Additionally, more and more research is pointing towards the significance of the tumour microenvironment and inflammation in the acquisition of tumour transformation and metastasis.

Better understanding of the widespread applicability of these concepts has a potential to identify new undiscovered mechanisms that can eventually lead to new means of treating human cancer. Keeping with the genetic instability noted in most cancer cells, previous work done by our lab and others has identified that epithelial cancer cells show an overexpression of cell surface mucin protein MUC1 (22-25). MUC1 overexpression has been shown to promote tumour cells ability to invade and metastasise (23, 25, 26), one of the hallmarks of cancer mentioned above. The thinking behind this thesis has been to explore if MUC1 expression allows tumour cells in acquisition of further tumour-associated phenotype.

1.2 Mucins

MUC1 belongs to the mucin family of proteins. The mucin family of proteins are large, heavily glycosylated and are often expressed by epithelia of respiratory, gastrointestinal and reproductive tracts (27). Over the years, the different mucin members have been categorised as secretory or membrane-bound mucins. Secretory mucins are primarily gel-forming as they coat the epithelial surface and function as a protective barrier for underlying mucosal surface. Membrane-bound mucins could potentially act as a second line of defence, however recently they have been shown to engage in signal transduction events (28, 29). Of the known mucins to date, the secretory family of mucins include MUC2, MUC5AC, MUC5B, MUC6 and MUC7, MUC9 and MUC19 whereas the membrane-bound mucins include MUC1, MUC3, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17 and MUC20. In addition to playing a physical barrier to underlying mucosa, mucins also have an essential role in lubrication, chemical sensing and molecular configuration of extracellular matrix

(ECM) as well as having a role in relaying and facilitating extracellular stimulus into cell signalling (30-32).

1.2.1 MUC1 structure

The *MUC1* gene encodes a single polypeptide chain which is autoproteolytically cleaved into two peptide fragments; the longer N terminal (MUC1-N) and the shorter C terminal (MUC1-C) (33, 34). Extracellularly the two subunits remain associated through stable hydrogen bonds. MUC1-N includes a proline, theronine and serine rich VNTR (variable number of tandem repeat) region encoded by highly polymorphic exons encoding for multiple 20-21 amino acid sequence repeats (35). In northern Europeans, VNTR is composed of 20-120 repeats with 40-80 repeats being most common (36).

In normal cells, MUC1 is extensively *O*-glycosylated and moderately *N*-glycosylated with glycosylation contributing to 50- 90% of total weight of MUC1. Based on cell type, the number of tandem repeats in the VNTR and the amount of glycosylation, the weight of MUC1 can vary between 250-500 kDa. The majority of MUC1 *O*-glycosylation happens in the serine and threonine rich VNTR region. There are 5 potential sites for MUC1 *N*-glycosylation, 4 of which reside in degenerate sequence of MUC1 which bears subtle sequence similarity to the VNTR region and one in the extracellular domain of MUC1-C (37) (Figure 1.2).

MUC1-C is short, comprising a 58-amino acid extracellular domain, a 28-amino acid transmembrane domain (TMD), and a 72-amino acid cytoplasmic tail (CT) (Figure 1A). The intracellular tail of MUC1 has 22 potential phosphorylation sites which have been

shown to have a key role in cell signalling (32, 38-40). Based on the *N*-glycosylation state of the ECD, the molecular weight of MUC1-C can vary from 17 to 25 kDa (37).

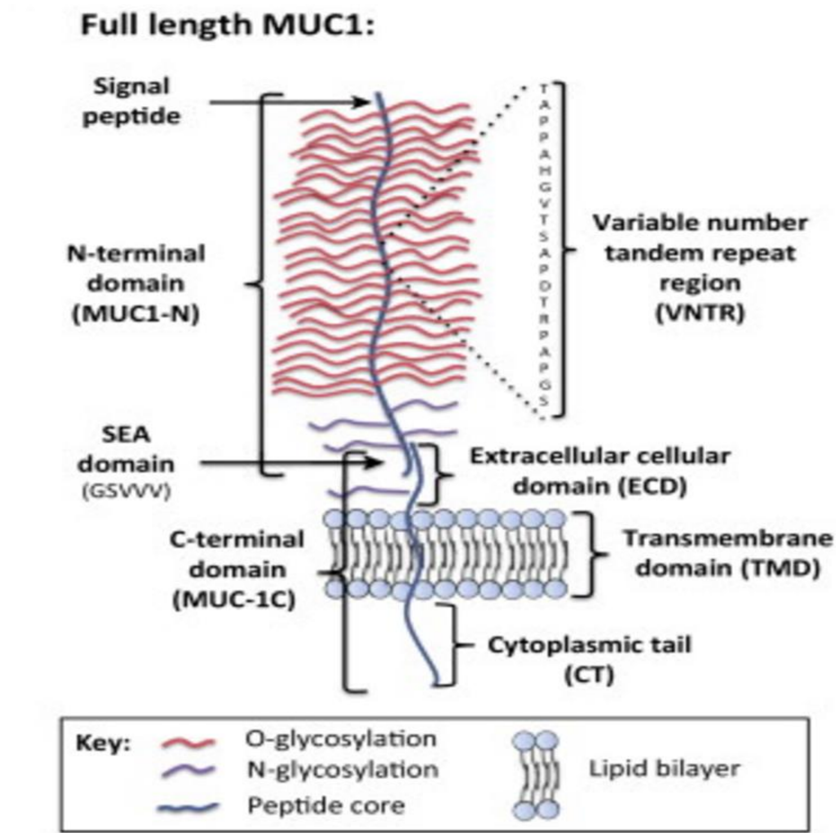


Figure 1.2: Schematic representation of structure of MUC1 adapted from (37) (permission acquired from Cell, licence no 4296980503660)

1.2.2 MUC1 glycosylation

The glycosylation pattern of MUC1 varies depending upon the tissue-specific expression of the glycosyltransferases (33). Potentially five types of glycans can be produced by a cell ranging from *N*-linked glycosylation, *O*-linked glycosylation,

phosphor-serine glycosylation, c-linked glycosylation and glypiation. Of these, the *N*-glycosylation and the *O*-glycosylation dominates in MUC1. The *N*-glycosylation of MUC1 is vital for protein folding, sorting, secretion and apical expression in polarized cells whereas *O*-glycosylation correlates with its biological properties (41). In normal cells, MUC1 is heavily glycosylated, with the peptide core masked by the sugar chains that shield it from undergoing proteolytic cleavage by environmental enzymes. Additionally, glycosylation also stabilizes mucins at the cell surface by preventing them from undergoing clathrin-mediated endocytosis (42).

The process by which MUC1 *O*-glycosylation occurs is well characterised, although relatively little is known about its regulation (43-46). The biosynthesis process starts with the addition of N-acetyl-galactosamine (GalNAc) to the serine (Ser) or threonine (Thr) residues, present extensively in MUC1 VNTR region, to form the initial *O*-linked GalNAcα-Ser/Thr structure (Tn antigen) catalysed by one or two of a large family of up to 20 distinct UDP-N-acetyl-α-D-galactosamine polypeptide GalNAc-transferases (ppGalNAc-Ts) (43, 47). This first step of biosynthesis for *O*-linked mucin type glycans is believed to start in an inter ER-Golgi compartment and finish in the Golgi apparatus. Following the formation of Tn antigen, the GalNAc residue can be modified by either addition of a Gal residue catalysed by the Core 1 Gal-transferase (C1GT) or with addition of a GlcNAc residue catalysed by the Core 3 GlcNAc-transferase (C3GnT) or with addition of a sialic acid residue by a sialyl-transferase (ST6GalNAc-T) to form Core 1 structure, Galβ1,3GalNAcα- [Thomsen-Friedenreich (TF) antigen], Core 3 structure of GlcNAcβ1,3GalNAcα- and Neu5Acβ1,6GalNAcα- (sialyl-Tn), respectively (48-52). The formation of sialyl-Tn terminates the sugar chain whilst the TF and Core 3 structures can be further acted on by other

glycosyltransferases in a stepwise fashion to yield up to 8 core complex glycosylation structures (53). It has recently been found that C3GnT, ST6GalNAc-T and C1GT directly compete with each other for accessibility to Tn structure and that suppression of one of these transferases leads to increased activity of others (54).

1.2.3 Physiological role of MUC1

Mucins have numerous functions in the body, especially in epithelial cells where their high degree of glycosylation provides lubrication, prevents dehydration and offers protection against proteolysis. Mucins in epithelial cells also have a role in protection against microbial challenge by acting as a physical barrier and inhibiting microbial access to the cell surface. (55, 56) Bacterial adhesins have been shown to bind mucin carbohydrate at the cell surface (57, 58), implicating a role of mucins as a barrier to potential infections.

Additionally, MUC1 in particular has been shown to modulate cell-cell and cell-ECM interaction (59, 60) and it has been proposed that MUC1 overexpression may promote metastasis via its interference with these interactions (61, 62). The number of tandem repeats in the MUC1 extracellular domain plays a crucial role in such interactions, as it has been shown that a reduction in VNTR makes MUC1 ineffective inhibitors of cell-cell and cell-ECM interactions (63, 64).

MUC1 intracellular domain, with its 22 potential phosphorylation sites, has been implicated in various cell signalling (65). Many intracellular signalling proteins have been reported to interact with the MUC1 intracellular domain, e.g. including PI3K,

Shc, PLC1, c-Src, Grb-2, p53, IKKβ, IKKγ, β-catenin, HSP90 and HSP70 (66). Interaction of the MUC1 cytoplasmic tail with β-catenin, son of sevenless (SOS) and Growth factor receptor-bound protein 2 (Grb2), has been shown to have a role in cell growth and wnt signalling in HEK 293 and gastric epithelial cells (67, 68).

MUC1 has also been shown to have a role in protecting the endometrium from microbial attack and MUC1 needs to be down-regulated to allow embryo implantation (69, 70). In MUC1 null mice, loss of MUC1 leads to chronic infection and inflammation of the uterus (69) yet blastocysts are only able to attach to the uterine wall in these mice when MUC1 overexpression is minimised (70). Indeed, MUC1 expression in mice is lost throughout the uterine epithelia by day 4 of pregnancy when the uterus is receptive to blastocyst attachment (71, 72). A similar effect is noted in the rabbit uterus, where although MUC1 expression is generally increased over the receptive phase, a local loss of MUC1 is noticed at implantation sites (73). *In vitro* studies indicate that human blastocysts produce factors that lead to local MUC1 loss on monolayers of human uterine epithelia (74).

Studies in tumour cells have highlighted that the type and the amount of MUC1 expressed in cancer cells modulate immune response to these cells. MUC1 is often less extensively glycosylated in tumour cells, exposing the physiologically inaccessible tumour specific epitopes that may trigger an immune response (75, 76). However, MUC1 has also been shown to supress immune surveillance of tumour cells (77, 78), highlighting both an immunostimulatory and an immunosuppressive function of MUC1.

1.2.4 MUC1 in epithelial cancers

In physiological conditions, MUC1 expression is limited to the apical surfaces of the cell and MUC1 itself is heavily glycosylated. In cancer cells, this apical polarisation is lost and MUC1 is overly expressed and abnormally (less) glycosylated; leading to the exposure of oncofoetal antigens which would normally be 'covered' by the sugar chain (79). A high level of MUC1-N at the cell surface is known to interfere with cell-cell and cell-extracellular matrix and is associated with increased malignancy (80, 81). Furthermore, since the typical length of the N terminal extends more than 10-fold beyond the length of a typical cell surface molecule, the over-expression of MUC1 in tumour cells causes it to act as a physical barrier to chemotherapeutic drugs and to the cells of the immune system.

1.2.5 MUC1 extracellular domain and altered glycosylation in cancer

MUC1 is a heterodimer that is co-translationally processed into two polypeptides, MUC1 extracellular domain (MUC1 ECD) and MUC1 transmembrane domain (MUC1-CD), which itself consists of a small stem region extracellularly, a transmembrane part, and a 72 amino acid long cytoplasmic tail (figure 1.2). The MUC1-ECD is linked to the stem region of MUC1-CD via non-covalent interactions. The MUC1 extracellular domain, as mentioned previously, is heavily glycosylated, and this glycosylation contributes to a significant part of the molecular weight of the entire MUC1 molecule.

Many epithelial tumours have been shown to exhibit aberrant *O*-glycosylation (82-84). The alteration in tumour glycosylation is mainly through three mechanisms. Firstly, neo synthesis or incomplete synthesis (85) may lead to increased expression of core 1 based structures, such as T, Tn or sialyl-Tn (86-88). These structures ae typically absent in healthy cells however in tumour cells increased expression of these truncated structure is driven mainly by alteration in expression, location and availability of molecular chaperone (Cosmc) of GalNAc-Ts (89-91). Secondly, hypermethylation of *Cosmc* gene also significantly contributes to aberrant *O*-glycosylation (92, 93). Finally, the relative positioning of the glycosyltransferases within the Golgi can lead to different *O*-glycosylation. Work by Kellokompu and colleagues (94, 95) and by Campbell and colleagues (96, 97) has shown that Golgi derangement occurs in epithelial cancers and can be mimicked by agents that block normal Golgi acidification, in both cases leading to increased formation of oncofetal carbohydrate antigens.

Phenotypically, overexpression of truncated mucin *O*-glycans is associated with increased aggressiveness and enhanced metastatic behaviour in a variety of cancers (87, 92, 98). These effects are, in parts, due to changes in the binding properties of key secreted and cell surface proteins that modulate interaction between tumour cells and the extracellular environment (e.g selectin and integrin) (98) and the direct effect on receptor-ligand interactions that alter signal transduction in affected cells. Re-expression of enzymes that maintain and extend the carbohydrate chain, such as *Cosmc* and core-3-synthase, seems to decrease these aggressive effects, as seen in pancreatic cancer cells (99).

Extracellularly, altered glycosylation significantly influences the interaction of tumor cells with their immediate surrounding. One example is the interaction with immune cells. Altered glycosylation of mucins often evokes an immune response in the body, as evident by increased circulating antibodies against various epitopes of MUC1 in patients with advanced metastasis (100, 101). Currently, these aberrant glycosylations are being explored as a potential target for immunotherapies, including the design of chimeric antigen receptor T cell against Tn on MUC1 (102, 103).

The altered glycosylation state of MUC1 also has a role in tumour metastatic spread. Expression of carbohydrate structures sialyl-Lewis^{X} and sialyl-Lewis^{A} on MUC1 enable the binding of MUC1 to both E-selectin and intercellular adhesion molecule (ICAM)-1 (104). As selectins play a critical role in immune cell rolling and extravasation from vasculature and subsequent trafficking through tissues, such interactions in MUC1 are also proposed to similarly have an effect in tumor cell extravasation, invasion, and metastasis (105).

Additionally, MUC1- ICAM1 interaction has been found to alter the metastatic phenotype of the cancer cell itself. It has been found that following interaction with ICAM-1, Src interacts with MUC1-CD, an interaction that promotes Src-mediated cytoskeletal rearrangement (106, 107). The Src family of nonreceptor tyrosine kinases have long been regarded as key mediators of metastatic progression (108). Interaction between MUC1 and Src induces pro-migratory Rac-1 and Cdc42-dependent actin reorganization at the sites of contact with endothelial cells, thereby

promoting an invasive phenotype in tumour cells (106). In addition, a truncated version of MUC1 consisting of only the external stem region, the transmembrane region and the transmembrane domain was shown to be sufficient to induce epithelial mesenchymal transition (EMT) in mouse mammary carcinoma cells, through interaction of MUC1 extracellular domain, ICAM1 and E-selectin (109). Collectively, these studies demonstrate that the MUC1 extracellular domain, through its altered glycosylation in cancer cells, can drive metastatic spread, as well as intracellular interactions that promote migratory behaviours.

1.2.6 MUC1 intracellular domain in epithelial cancer

The intracellular domain of MUC1, MUC1-CT, has been heavily studied for its potential role in tumour promotion. With 22 phosphorylation sites in the cytoplasmic tail, the intracellular domain of MUC1 forms a key kinase recognition site and binds to several kinases such as c-SRC (110) EGFR or (ErbB) family (111), glycogen synthetase kinase 3b (GSK3b) (112), and protein kinase C delta (113). As well as kinase interaction, the MUC1 cytoplasmic tail has been known to interact with key apoptosis proteins such as β catenin (114), heat shock proteins (HSP) such as HSP70 and HSP90 (115). MUC1-CT interaction with β catenin and p53 has been shown to prevent mitochondria- mediated apoptosis in response to DNA damage (116). MUC1-CT is also able to translocate to the nucleus in association with β-catenin (114) where it has been shown to supress e-cadherin expression, while promoting expression of epithelial mesenchymal transition (EMT) promotors Snail, Slug and Twist (117). As a result of this, β catenin association with cadherins at adherin junctions is

compromised and this, together with increased expression of EMT promoters, promotes MUC1-mediated anchorage-independent growth.

Aberrant glucose metabolism is a cancer hallmark that facilitates cell survival. MUC1-CT has been shown to mediate the expression of genes involved in glucose uptake and metabolism in orthotopic implantation models of pancreatic cancer (118). In this manner, MUC1-CT molecules can directly facilitate tumour cell survival and proliferation by upregulating glucose uptake and metabolism. While all the gene and protein targets for MUC1-CT are yet to mapped, its cytoplasmic interactions and nuclear localization have been correlated with poor prognosis, tumour-node-metastasis staging, increased EMT and lower survival.

MUC1 CT has also been linked with altered expression of growth factors such as connective tissue growth factor (CTGF), Platelet derived growth factor A (PDGF-A) and PDGF-B that promote tumour cell proliferation via MAPK and PI3K/AKT pathways (40, 119-122). In addition, recent research has indicated that MUC1-CT may also inhibit the apoptotic function of BAX through interaction with the BH3 domain (123), suggesting that over-expression of MUC1 may increase the pro-survival signal leading to anoikis resistance.

1.3 Anoikis

Anoikis is a Greek term, meaning “homelessness” or “loss of home”. First used in cell biology by Stephen M Frisch in 1994, the term refers to apoptosis induced by absent or inappropriate cell-cell/cell-matrix contact (124). One of the key aspect of

multicellularity is cellular growth and differentiation in correct context within a tissue to ensure tissue homeostasis. Cells within a tissue sense their location through specific interactions with the extracellular matrix (ECM) as well as with neighbouring cells. Cell death-induced via anoikis therefore, ensures tissue homeostasis by determining that any displaced cells undergo apoptosis; thereby preventing them from re-attaching at a secondary site and undergoing dysplastic growth. There are several cell surface molecules which sense cellular adhesion to cell and/or extracellular matrix and convert them into intracellular signals leading to eventual generation of pro- or anti-apoptotic signals. At any given time, the fate of a cell is dependent on which of these two, pro or anti-apoptotic signals, dominate. Anoikis therefore is a physiological process in development and tissue homeostasis and is often deregulated in disease (125, 126).

Anoikis has been described in many cell types; however, it now appears that not all cell types induce anoikis via similar pathways. Regardless of the pathways involved, the end result always leads to the activation of effector caspases and DNA fragmentation. In keeping with classical apoptosis, anoikis could either follow the intrinsic pathway, which involves the mitochondrial membrane and release of cytochrome C, or the extrinsic pathway, which is initiated at the cell surface via death receptors (126, 127). Both these pathways are heavily regulated by sets of proteins which, depending on their roles, can either be classified as pro- or anti-apoptotic proteins. The pro-apoptotic proteins involve the Bax, Bak, BoK (multi BH3 domain proteins) or Bid, Bik, Bmf, Noxa, Bad, Bim and Puma (BH3-only proteins) while Bcl-2, Bcl-XL and myeloid cell leukaemia sequence (Mcl-1) are the major anti-apoptotic proteins (128). In addition to these, several molecules have been discovered (Bad,Bix,

Noxa, Puma) which act as apoptotic sensitizers (129, 130). It is the activation, over-expression or inhibition of either set of these pro- and anti-apoptotic proteins that eventually determines the fate of the cell under anoikis conditions (131-134).

1.3.1 Intrinsic pathway

The intrinsic pathway is triggered in response to several pro-apoptotic signals, including DNA damage and endoplasmic reticulum stress (135), where the eventual executioner caspase (caspase-3, caspase-7) activation is due to increased mitochondrial membrane permeability (131, 135). In this pathway, following a pro-apoptotic signal, Bax and Bak translocate from the cytosol to the outer mitochondrial membrane (OMM), where their oligomerization leads to disruption of OMM integrity leading to increased OMM permeabilization leading in turn to increased cytochrome C release in the cytosol (136, 137). In addition to the OMM pore forming activity, both Bax and Bak have been shown to accelerate the opening of voltage dependent anion channels (138, 139), thus further destabilizing mitochondrial permeabilization and increasing cytochrome c release. Cytochrome c released in the cytosol interacts with pro-caspase-9 and cofactor apoptosis protease activating factor (APAF1) to form an 'apoptosome' (140). The apoptosome assembly cleaves pro-capase-9 into activated caspase-9 which subsequently leads to activation of effector caspase-3 and eventual proteolysis of caspase targets (140, 141)

The BH3 domain proteins play a critical role in the cascade of events leading up to intrinsic anoikis (142). Cell detachment from ECM leads to downward signalling and activation of Bid and Bim, termed "activators" of intrinsic anoikis (143). Bim is

normally sequestered in an inactive state in the dynein cytoskeletal complex. Loss of cell attachment leads to Bim release and accumulation in the mitochondria where it directly facilitates the formation of Bax/Bak oligomers (144). Cell detachment also leads to Bim accumulation through inhibition of its degradation; typically facilitated by integrin-mediated phosphorylation of Bim via ERK, PI3K and AKT (145, 146).

As well as anoikis activators, some of the BH3 group of proteins also act as anoikis "sensitizers". These proteins do not directly activate Bax and Bak oligomerization, nor do they contribute to Bim or Bid activation; yet they have a pro-apoptotic function as they inactivate the anti-apoptotic functions of the Bcl-2 group of proteins (129, 147, 148). Proteins such a Bad, Bik, Bmf, Noxa, Puma and Hrk are typical anoikis sensitizers (130, 131, 149). Their action ensures that the Bcl-2 members of anti-apoptotic proteins, which can inhibit Bax/Bak oligomerization on outer mitochondrial membrane as well as Bim and Bid activation, are inactivated; thus leading to the accumulation of pro-apoptotic signals.

Recent research has also pointed to the involvement of other members of BH3-only family in anoikis of different cell histotypes. For example, Noxa and Puma, transcriptionally regulated by p-53 have been shown to have an effect in fibroblast anoikis (150, 151). Furthermore in epithelial cells, the Bcl-2 modifying factor (Bmf) has been identified to have a similar role as an 'apoptosis sensitiser', where it neutralizes Bcl-2, leading to cytochrome C release and anoikis execution (151).

1.3.2 Extrinsic pathway

The extrinsic pathway also leads to anoikis execution. This differs from intrinsic anoikis, initially in the way the extrinsic pathway is activated and via the pathway's ability to recruit effector caspases without mitochondrial involvement. The signal is initiated when extracellular death ligands such as Fas ligand (FasL) or tumour necrosis factor α (TNF-α) bind to their respective receptors resulting in the assembly of death-inducing signalling complex (DISC) intracellularly (149). Through the action of Fas-associated death domain (FADD), DISC is able to aggregate several molecules of caspase 8 which then auto-activate themselves (152). Auto-activated caspase-8 then proteolytically activates caspase-3 and 7, leading to substrate proteolysis and cell death (143, 153) (type I extrinsic anoikis) or they can cleave the BH3-only protein Bid to form truncated-Bid (t-Bid) (154) which then has an effect on mitochondrial permeability and cytochrome c release (type II extrinsic anoikis). T-Bid therefore converges the extrinsic anoikis pathway towards the intrinsic pathway [Figure 1.3].

Loss of adhesion to the extracellular matrix leads to an increased expression of both the Fas and FasL, while FLIP, an endogenous inhibitor of Fas-mediated anoikis is reduced (155). Furthermore, the cell rounding following detachment can induce extrinsic anoikis mainly through relocatization and activation of Fas (156). Finally, activation of death receptor could be secondary to mitochondrial release thereby creating further crosstalk between intrinsic and extrinsic pathways (157).

Activation of caspase-3 is the common event of both the intrinsic and extrinsic anoikis pathways. Caspase 3 initiates the downstream proteolytic cascade and also has a role on reducing the effect of the pro-survival signal. Whether a cell undergoes anoikis

via intrinsic or extrinsic pathway depends on the cell type. With regards to epithelial cells, some studies have suggested that the initiating event is the activation of death receptor, as over-expression of a dominant-negative form of FADD, which blocks caspase-8 recruitment to DISC, inhibits anoikis (158, 159). However, in these studies extracellular inhibitors of death receptor (in the form of soluble extracellular portions of the receptor, which can sequester the ligand), failed to inhibit anoikis. Furthermore, the detachment-induced activation of caspase-8 was inhibited by Bcl-2 over-expression, suggesting that perhaps caspase-8 activation is dependent on mitochondrial membrane permeability and the release of cytochrome C.

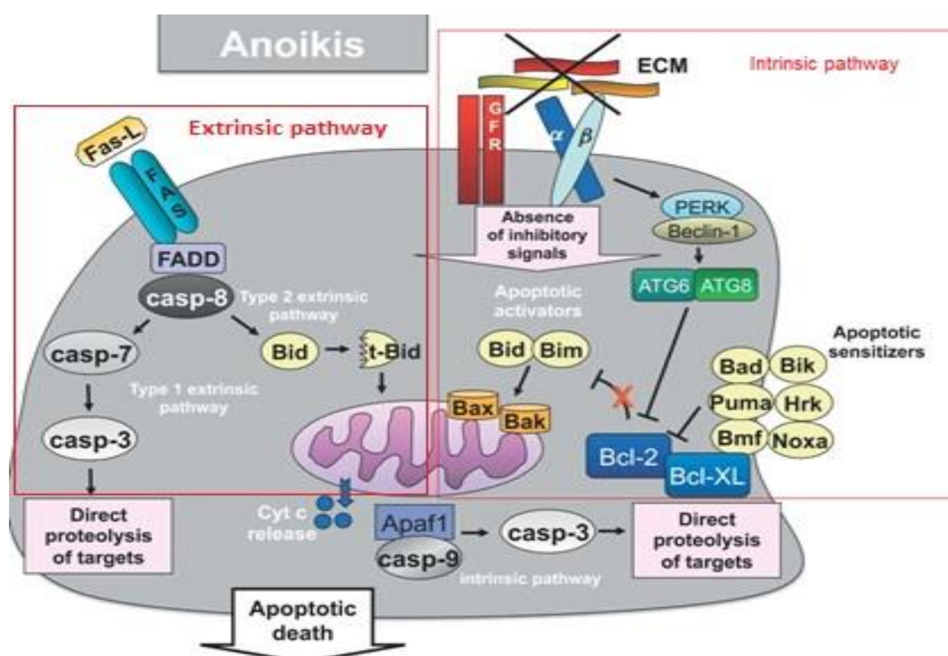


Figure 1.3: The intrinsic and extrinsic pathways of anoikis. Intrinsic pathway signal initiates at the cell surface, possibly due to breakdown of ECM contact with integrin. The net result is oligomer formation in the outer mitochondrial membrane and release of cytochrome C. The extrinsic pathway begins at the death receptor on the cell surface, which subsequently activates caspase- 8. Both intrinsic and extrinsic pathways lead to the activation of effector caspase, caspase-3, and direct proteolysis of targets and apoptotic death. Figure adapted from (131) (permission acquired from Journal of Pathology, licence no 4296980918248)

1.3.3 Physiological protection from anoikis

Not all cells undergo anoikis when detached. Certain cells, such as the epithelial cells under migration, mesenchymal cells (160), haematopoietic cells and leukocytes (161) evade anoikis physiologically via various mechanisms, some of which are discussed here.

As mentioned already, cell adhesion to permissive ECM proteins and its immediate neighbouring cells are key determinant of anoikis initiation. The cell surface adhesion molecules such as integrins, cadherins and selectins monitor a cell's position with its immediate surrounding and play a key role in modulating key downstream effector molecules such as the FAK (162), Src kinase (163), PI3K/AKT (164), MPAK (165) and integrin-linked kinase (ILK) (166). Altered expression and improper activation of these molecules has been shown to interfere with anoikis (167-169), and it is therefore not surprising that some of these molecules are unregulated and have an enhanced activation in malignant cells (170-172). Formation of focal adhesion and subsequent activation of FAK is a key effect of integrin mediated surveillance of cell-ECM contact (149, 169, 173). Activated FAK affects multiple critical cellular processes such as cell survival, proliferation, motility and differentiation. The activated FAK also enables the recruitment of other scaffold and signalling molecules to the focal adhesion sites, consequently activating downstream cell survival signalling. PI3K and its downstream target protein kinase B (PKB/AKT) (174, 175), MAPK pathway (176), ILK (177, 178) and caveolin-1 have all been known to be activated, directly or indirectly by FAK; all of which influence anoikis regulation.

As well as cell-ECM contact, cell-cell contact mediated by cadherins are also able to support cell survival. Cadherins are a family of proteins that allow homo and heterotypic calcium-dependent anchorage between neighbouring cells (179, 180). This anchorage plays a crucial role in cell survival and ensures anoikis resistance. Indeed, it has been reported that blockage of E-cadherin binding induces anoikis (181, 182), while overexpression of β-catenin, a downstream regulator of cadherin signalling, elicits anoikis resistance in epithelial cells (183). Cadherins are able to promote cell survival in a PI3K/AKT dependent fashion (181) and are also able to indirectly interact with integrins (184, 185). Some integrins, notably α2β1 and α3β1, can localise to cell-cell contact and can mediate cell survival despite loss of ECM adhesion (186). In addition to cadherins, cell-cell adherion mediated by NCAM and L- and P- selectins have also been shown to trigger cell survival signalling such as FAK, Src, PI3K/Akt and MAPK, all of which can have can supress cell death (187-189).

Haemopoetic stem cells and leukocytes are unique because these are non-adherent cells, able to avoid anoikis. These cells can move in a protease independent manner across matrix barrier through adaptations of cell shape and being able to squeeze through small gaps. The weak interaction with ECM allows these cells to move in high velocity. Since these cells are unable to effectively use integrin mediated focal adhesions or cadherin mediated adherins junctions, they are unable to ensure a continuous pro-survival signal seen in adhering cells. In these cells, it is proposed that the anoikis resistance occurs mainly due to increased presence of several cytokines such as interleukin-2, (IL-2), IL-7, IL-15 and interferon-α which override the pro apoptotic signal from BH3 only proteins (149). Further support for this hypothesis comes from the fact that in quiescent T-cells, the withdrawal of these cytokines leads

to Bim accumulation, Bcl-XL downregulation and a final commitment to anoikis (149). The possible role of cytokines in anoikis regulation, emphasises the complexity of the pathways and highlights that multiple mechanism may be at play at any given time to determine the overall fate of a cell under anoikis conditions.

Epithelial cells avoid anoikis during cell migration. Migrating cells use mesenchymal motility that is characterised by cell elongation, ECM proteolysis and focal contact (190, 191). Integrins are heavily involved in this process as they activate focal kinase, which not only leads to PI3k activation and an eventual build up of pro-survival signals, but it also initiates mesenchymal motility (191, 192). Mesenchymal motility has been linked to pro-survival signals and epithelial mesenchymal transition (EMT) is one of the pre-requisites for metastasis (193-195).

Lastly, coordination between autophagy and anoikis resistance of epithelial cells is recently emerging (196-199). Following detachment, the detached cell show a reduction in EGFR expression (200) which, due to inhibition of pro survival PI3K and AKT pathways, leads to a marked reduction in ATP level (201). Autophagy in this context is a temporary solution to provide the necessary energy till the cell reattaches (196, 202). There are 4 ways a cell can undergo autophagy; macroautophagy, microautophagy, chaperone mediated autophagy and mitophagy. The key protein involved in this pathway is Beclin-1, which itself is an autophagic protein but also modulates the anti-apoptotic role of of Bcl-2 and Bcl-XL (203), and ERKs (204, 205). Autophagy allows epithelial cells to survive temporarily, given that they re-adhere onto the ECM in a timely fashion (206, 207), and it is likely a previously unrecognized tool used by circulating cancer cells to survive anoikis, thereby

facilitating tumour cell dormancy though nutrient recovery, as well as dissemination of metastases.

1.3.4 Anoikis resistance in cancer

Unlike normal cells, cancer cells do not require adhesion to ECM to grow and proliferate. This ability has important implications in metastatic processes where cancer cells must survive and migrate in the absence of proper ECM contact. In this scenario, anoikis resistance is a molecular prerequisite for the metastatic spread of cancer (149, 208).

The general strategy which allows early stage tumour cells to evade anoikis is to selectively activate pathways responsible for proliferation and cell survival, such as those transduced by PI3K (209, 210), MEK/ERK (211-213) and NF-kB (214). Depending on the tumour cell, this ability can be acquired either through a direct activation of the pathways themselves or via inactivation of pathway inhibitors. In some cases, tumour cells acquire an autocrine growth factor loop, whereby they are able to activate the growth factor in a ligand-independent manner, thus activating the pro-survival pathways (215, 216). Various growth factors such as basic fibroblast growth factor (bFGF) (217), HGF (218), EGFR (219) and TGF (220) have been shown to be abnormally activated in a variety of epithelial cancer cells to aid cell proliferation, survival and migration. Furthermore, tumour cells are also able to exploit the physiological anti-anoikis mechanisms, mentioned above, and are able to resist

anoikis by altering their integrin expression, undergoing EMT and adapting their metabolism through autophagy.

Given their role in promoting anti-apoptotic signals via activation of various kinases and ligand independent activation of growth factors, as mentioned above, a change in pattern of integrin expression have been reported in many anoikis-resistant cancer cells (169, 221, 222). Analysis of integrin expression profiles reveals that normal epithelial cells predominantly express integrins α2β1, α3β1 and α6β1 (133, 149, 223). Hyperproliferating epithelial cells over-express both αvβ5 and αvβ6 integrins (224, 225) and the anoikis-resistant squamous carcinoma predominantly over-expresses αvβ6 (226). This integrin switch to αvβ6 contributes to acquisition of an anoikis-resistant phenotype (227). Physiologically, integrin β6 is expressed during embryogenesis in a subset of epithelial cells of kidney, lung and skin but is undetectable in adults (224). However high level of integrin β6 has been reported in several types of carcinoma cells (228, 229). Along with β6, increased and altered expression of integrin β3 and integrin β4 have also been shown to resist anoikis and increase cancer cell invasiveness (230, 231).

A key physiological process exploited by tumour cells is epithelial-mesenchymal transition (EMT). During EMT cancer cells are able to activate pathways that leads to a downregulation of cell-cell adhesion molecules such as E-cadherins and γ-catenin while at the same time an increased expression of mesenchymal markers such as vimentin, fibronectin, α-smooth muscle actin (SMA), N-cadherin as well as to the activation of matrix metalloproteinases (149). This enables the epithelial derived cells with a motile and highly invasive phenotype, ultimately facilitating metastatic

spread. It is now generally accepted that anoikis resistance is correlated with the acquisition of the mesenchymal phenotype (193, 195, 232, 233). This is possible because the key players in EMT such as Snail (234), ZEB1/2 (235), Twist (236, 237), NF-kB(238) and HIF1/2 (239) are collectively able to upregulate the pro survival signal (such as upregulation of Bcl-2 family proteins and increased activation of PI3K/AKT) while downregulating the pro-apoptotic proteins such as p53-effector related to pmp22 (PERP), p21, Bim, Bax and Noxa (149, 234).

Compelling evidence suggests that oxidative stress and hypoxia experienced by rapidly diving cancer cells further contributes to anoikis resistance (239-241). Caveolin-1, an activator of the AKT pathway, has been reported to be upregulated in increased hypoxic state and has been implicated to have a direct role in anoikis resistance and anchorage-independent growth (242-244). Furthermore oxidative stress has been shown to modulate the activation of Akt and MAPK signalling pathways (245, 246), as well as the activity of redox sensitive transcription factors such as NF-kB, HIF-1/2, p53, AP-1, Nrf2, thereby contributing to increased pro-survival signals (247, 248). Furthermore, hypoxia promotes EMT in a variety of carcinoma cells including melanoma, breast, prostate and colon cancers (249, 250), thereby further assisting anoikis resistance.

As well as the examples given above, there are many molecules and proteins such PTEN (251), Ras (252), Tyrosine kinase receptor B (253), ZEB transcription factor (254), that have been identified as having a role in anoikis resistance. The majority of these proteins/molecules work by either promoting the pro-survival signals via mechanisms already described (whereby they facilitate anoikis resistance by either

increasing the Bcl-2: Bax ratio, having an effect on the PI3k/AKT pathway or simulating the pro-survival kinases) or by inhibiting the pro-anoikis pathway.

1.3.5 MUC1 and its potential role in anoikis resistance and metastasis.

MUC1 overexpression and abnormal glycosylation is a key feature of many metastasising epithelial tumour. Our previous work has shown that MUC1 overexpression leads to the initial escape of the metastasising cell from the primary tumour and its interaction with its ligand, galaectin-3, leads to MUC1 cell surface polarization. This exposes the underlying adhesion molecules and leads to both tumour cells homotypic aggregation and heterotypic adhesion. Homotypic aggregation leads to the formation of tumour emboli which prolongs the survival of metastasising tumour cells in circulation, enhances their physical trapping in the micro-vasculature and thus increases metastatic spread of cancer cells to remote sites. MUC1 interaction with its ligand galectin-3 therefore may represent an effective therapeutic approach for preventing tumour metastasis.

1.4 Galectin-3

MUC1 is a ligand for Galectin-3 and MUC1-galectin-3 interactions have been associated with tumour metastasis in several epithelial cancers. Galectin-3 belongs to galectin family of β-galactoside binding lectins that contain one or two highly conserved carbohydrate domains (CRD) that recognise galactose-terminated glycans (255). To date there are 15 known galectins categorised into three groups based on

their structural differences and the number of CRD (256). Prototype galectins include galectins 1,2,5,7,10,11,13,14 and 15. They are classed as such based on the presence of a single CRD per subunit. Tandem repeat galectins, which includes galectins 4, 6, 8, 9 and 12 contain two CRD joined by a linker peptide. Galectin-3 is the only known member of the chimera-type galectin and contains one CRD at its C terminal connected to an extended and flexible N-terminal. The N-terminal of galectin-3 is responsible for galectin-3 multimerization upon galectin-3 contact with a multivalent ligand.

Of the known galectins, galectin-3 is perhaps the most studied. It was first characterized as a 32 kDa antigen on the surface of murine macrophages (257). As a multifunctional protein with varied expression in cancer and pre-cancerous conditions, varied presence at cellular locations (cell surface, nucleus, cytoplasm, mitochondria and endosomal compartments) (258) and direct involvement in tumorigenesis (259), cancer progression and metastasis (260); galectin-3 has generated significant interest in cancer research over the past decade.

1.4.1 Galectin-3 structure

The structure of galectin-3 is highly conserved (261) and typically comprises three structurally distinct domains: a highly conserved 12 amino acid long short N-terminal domain (ND), a proline- and glycine-rich long ND of 130 amino acid and a CRD domain of roughly 130 amino acids (262, 263) . The short ND may have a role in apoptosis and secretion as deletion of short ND blocks galectin-3 secretion (264) while

mutation of the highly conserved Ser6 affects galectin-3 anti-apoptotic signalling activity (265). The long N-terminal domain of galectin-3 normally consists of 7-14 repeats of a 9 amino acid sequence Pro-Gly-Ala-Tyr-Pro-Gly-X-X-X and due to its homology to collagen α1 (II) chain, is sometimes referred to as collagen like-ND (266). The long ND of galectin-3 is essential for galectin-3 multimerization and is sensitive to proteolysis by matrix metalloproteinases 2 and 9 (267, 268).

The C-terminal domain of galectin-3 contains the CRD structure responsible for lectin activity. It typically forms a globular structure with five- and six-stranded β-sheets arranged in a β-sandwich (269). NWGR is a particularly interesting amino acid sequence found within the CRD. This motif is highly conserved within the BH1 domain of the B-cell lymphoma 2 (Bcl2) family proteins and is responsible for anti apoptotic properties in both Bcl-2 and galectin-3 (270), through its interaction with apoptosis regulator Bax (271) .

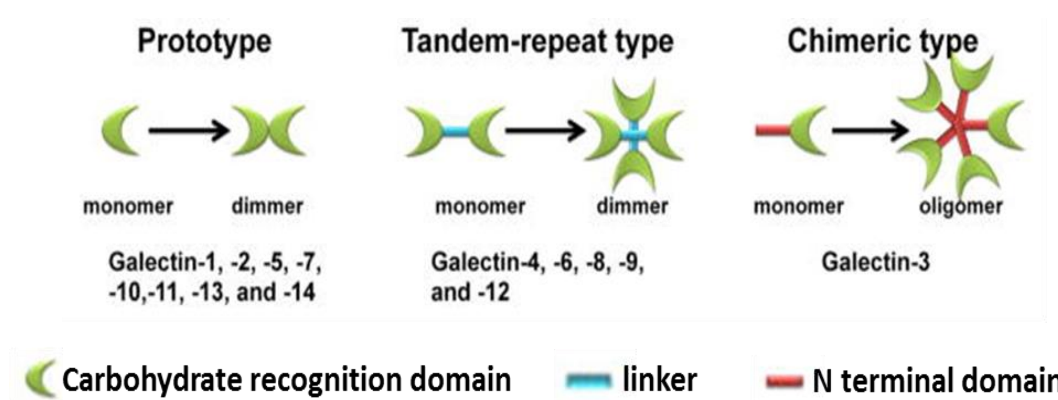


Figure 1.4 The structure of galectin family members. The galectin family members are divided into three types: the prototype with one carbohydrate recognition domain (CRD), the tandem-repeat type with two CRDs connected by a non-conserved linker, and the chimeric type with one CRD and a non-lectin N-terminal domain (ND). Some galectins can self-associate into dimers or oligomers.

1.4.2 Carbohydrate-binding properties and ligands of galectin-3

Although all galectins bind to β-galactoside, they possess a different binding affinity to different galactose-containing structures. For example, galectins bind 5-10 more strongly to N-acetyllactosamine (Galβ1,4GlcNAc) than to lactose (272-275). Interestingly, a striking difference was observed between galectin binding towards TF-disaccharide. Preliminary data from our lab and others have shown that galectin-3 has up to a 100 fold higher affinity to interact with TF antigen compared to galectin-1 (276). This could potentially be due to the different tertiary structure of galectin-3 following initial interaction with its carbohydrate ligand. Galectin-3 is present in cytosol and in ECM and has been shown to interact with a variety of proteins including laminin (277, 278), fibronectin (279), α1β1 integrin (279), Bcl-2 (280), and β-catenin(281, 282) via both protein-protein and protein-carbohydrate interactions.

1.4.3 Galectin-3 role intracellularly and extracellularly

Although much recent research has highlighted the link between overexpression of galectin-3 and tumorigenesis, galectin-3 also has a significant role physiologically. Galectin-3 regulates normal development through regulating cell proliferation, apoptosis, cell adhesion and angiogenesis (283-285). Physiological expression of galectin-3 has been linked to development and embryogenesis in both human (286) and mouse (287). In the initial stages of mouse embryogenesis galectin-3 first appears at 4th day of gestation followed by its expression in the notochord cells between 8.5 and 11.5 days of gestation (287). In later stages, galectin-3 expression is

tissue-specific and expressed primarily in cells undergoing rapid growth such as epidermis, endodermal lining of the bladder, larynx and oesophagus (262). In humans , galectin-3 is mainly expressed in epithelial cells such as small intestine (288), colon (289), cornea (290), kidneys (291), lungs (292), thymus (293), breast (294) and prostate (295). The expression of galectin-3 is also detected in ductal cells such as salivary gland (296), pancreas (297), kidneys (298) and hepatocellular biliary ducts (299).

Galectin-3 can shuttle between the cytoplasm and nucleus. Cytoplasmic galectin-3 has the ability to bind to Bcl-2 protein family and inhibit apoptosis (270). The NWGR anti-death domain, mentioned previously, is crucial for this antiapoptotic property as it shows a strong homology with the groove BH1 motif interface of the Bcl-2 family proteins (300). Cytoplasmic galectin-3 can also interact with activated K-Ras and affect Ras-mediated Akt signalling. Nuclear galectin-3 has been shown to promote pre-mRNA splicing as well as regulating gene transcription by enhancing transcription factor association with gene promotor sequence (301, 302).

Extracellularly, galectin-3 normally exists as a monomer (303) but it has been known to form homodimers by self-association through its CRDs in the absence of a ligand (304). In the presence of excess ligand however, galectin-3 can polymerise through interaction with its ND to form a pentameric structure (304, 305). Such multimerization cross-links its cell surface ligands and triggers the initiation of cell surface molecule-associated signalling (306, 307). Physiologically, galectin-3 is expressed in a wide variety of cells including immune, epithelial and sensory neuron cells (262, 308). Its absence, as experimented in galectin-3 knockout mice, has shown

a wide variety of phenotype including accelerated glomerular injury (309), attenuated inflammatory responses (310) and premature senescence without oncogenic stress (311); suggesting that galectin-3 has a much wider physiological function in body.

1.4.4 Galectin-3 in cancer

Galectin-3 overexpression is a common feature of several epithelial cancers including breast cancer (312), colon cancer (313) and melanoma (314, 315). There is also a general shift in location of galectin-3 with increased presence in cytoplasm (316, 317) and up to 30-fold increase in the circulation (318, 319). There is strong evidence that links increased expression and altered localization of galectin-3 to a broad range of cancer progression (320), including transformation, apoptosis resistance, adhesion, invasion, metastasis and angiogenesis at the secondary tumour site, some of which will be briefly covered here.

1.4.4.1 Galectin-3 in tumour transformation

Multiple research groups have shown that galectin-3 overexpression is linked to increased neoplastic transformation (306, 321-323). Suppression of galectin-3 in a highly malignant human breast carcinoma cells resulted in altered morphology and increased cell death via anoikis and supressed tumour progression in immunologically supressed mice (322). Similarly, galectin-3 knockdown in human thyroid papillary carcinoma led to inhibition of anoikis resistance (324). Furthermore,

transfection of galectin-3 cDNA into normal thyroid follicular cells led to increased anoikis resistance and serum-independent growth (324). These results suggest that increased expression of galectin-3 is highly associated with a tumorigenic phenotype in breast and thyroid cells.

The molecular mechanism of the galectin-3-mediated tumorigenic phenotype is speculated to be due to a galectin-3 effect on oncogenic K-Ras (321). Galectin-3 has been shown to increase K-Ras availability at the plasma membrane, leading to continuous activation of Ras-dependent PI3K and Raf-1 activation (321). As well as this, the complex interaction of galectin-3 with BCL-2 has been linked to promotion of cell survival and tumour progression (325, 326). Lastly, galectin-3 interaction with β-catenin leading to enhanced expression of cyclin D and c-MYC (256, 281) has also been shown to promote cell cycle progression (262).

1.4.4.2 Galectin-3 in apoptosis

Galectin-3 has been shown to be both an apoptotic promotor and an apoptotic inhibitor. The overall fate of the cell depends on the precise location of galectin-3 intracellularly. Cytoplasmic galectin-3 has been shown to interact with synexin (325). Galectin-3/synexin interaction leads to galectin-3 translocation to mitochondria, where its direct interaction with Bcl-2 stabilises mitochondrial membrane potential and blocks cytochrome C release(325, 326). Reduction of cytochrome C release protects the cells from apoptosis via intrinsic and indirect extrinsic apoptosis pathways. Nuclear galectin-3, on the other hand has been shown to promote apoptosis (327, 328). The precise mechanism of the nuclear action of galectin-3 is yet

to be decoded but it has been suggested that galectin-3 associates with the nuclear apoptosis-associated protein Nucling (329).

1.4.4.3 Galectin-3 in tumour metastasis

Tumour cell breaking from the primary tumour and into circulation, adherence to the endothelial cells and invasion through this endothelium to seed at the secondary tumour site are all an essential prerequisite to metastasis. Galectin-3 has been linked to all of these stages. Initially, overexpression of galectin-3 at the primary tumour site has been shown to enhance cell-ECM adhesion and facilitate tumour cells escaping the primary tumour site (307), attributed partly due to galectin-3 interaction with a range of ECM glycoproteins such as fibronectins, collagen IV, elastin, and laminin (262). Circulating galectin-3, via its interaction on cell surface glycans of tumour cells have been linked to promote tumour cell aggregation and formation of tumour emboli (25). Formation of tumour emboli further assists metastasising tumour cells to evade anoikis (330). Galectin-3 interaction with TF antigen on MUC1, discussed later, has been shown to lead to cancer cell adhesion to the endothelium. Finally, galectin-3 interaction with growth factor receptors such as epidermal growth factor receptor (EGFR) and transforming growth factor-β receptor (TGFβR) is believed to also contribute to the increased invasiveness of tumour cells.

Neovascularisation is another essential requirement for tumour emboli to develop into a secondary tumour (306). Galectin-3 has been shown to have a key role in angiogenesis and neovascularization at the secondary tumour site (331) via its interaction with integrins and glycans (332), leading to eventual activation of focal

kinases. Activated focal kinases modulate vascular endothelial growth factor (VEGF)- and basic fibroblast growth factor (bFGF) leading to neovascularization and angiogenesis at the secondary tumor site (333). Recent research has also reported galectin-3 interaction with aminopeptidase N/CD13, which has been linked to early stages of angiogenesis (334).

1.4.5 MUC1 and galectin-3 interactions in cancer

The circulating galectin-3 level in patients with advanced metastasis has been found to be up to 30-fold higher than normal (335, 336). Work done in our lab has shown that galectin-3 binds to the oncofoetal Thomsen-Friedenreich (galactoseβ1,3 N-acetylgalactosamineα, TF) antigen on the transmembrane mucin MUC1 expressed on tumour cells (79). The multimerization of galectin-3 together with its association with TF antigen on MUC leads to MUC1 cell surface polarization, thus revealing the previously covered underlying cell surface adhesion molecules (337). Exposure of underlying adhesion molecules leads to galectin-3 mediated tumour cell homo- and hetero- typic aggregation, leading to increased formation of tumour emboli and increased tumour-endothelium adhesion, respectively (25). Formation of tumour emboli suppresses anoikis by preventing anoikis initiation, whereas tumour-endothelium adhesion leads to tumour invasion and eventual formation of secondary tumour.

As MUC1 is associated with EGFR in epithelial cancer cells (338, 339), the effect of galectin-3 binding to MUC1 on cancer cell-cell interactions led us to examine the impact of their interaction on EGFR activation in epithelial cancer cells.

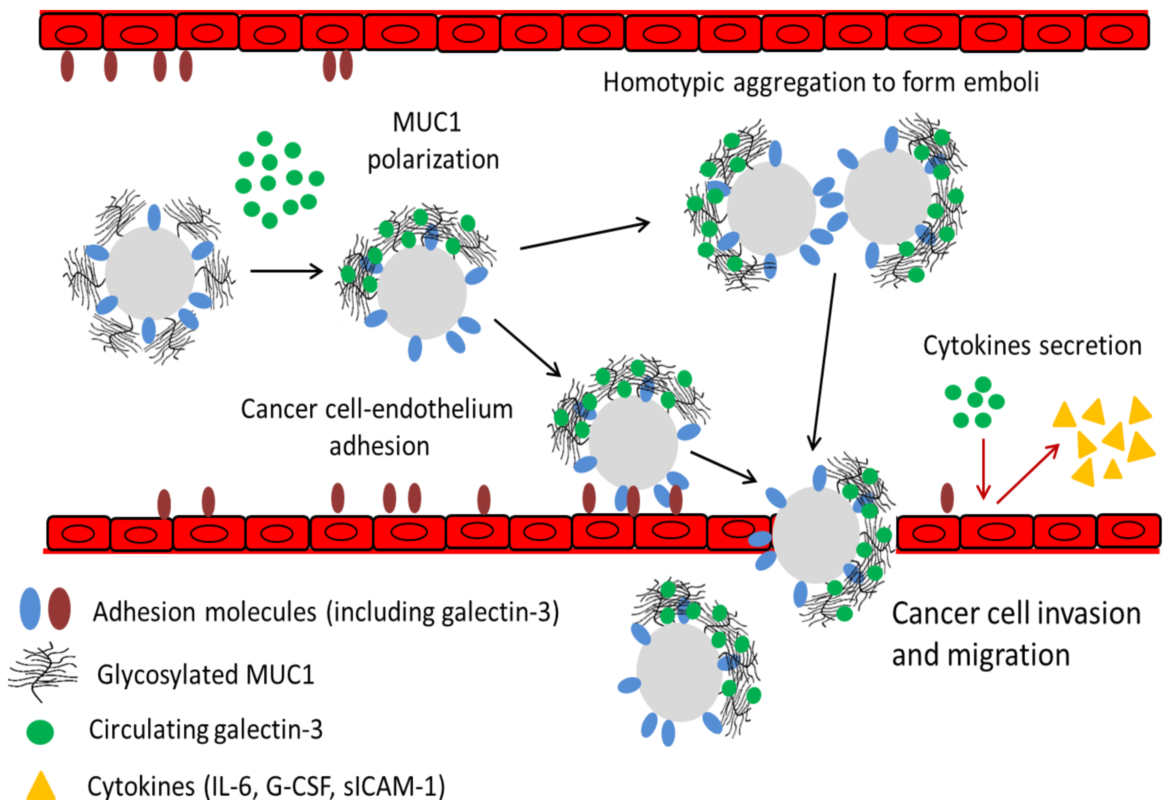


Figure 1.5: Galectin-3 MUC1 interaction. Galectin-3 concentration is greatly increased in the circulation of cancer patients. interaction of circulating galectin-3 with cancer-associated MUC1 causes MUC1 cell surface polarisation and consequently, exposure of small adhesion molecules including surface-associated galectin-3, which enhance cancer cell-endothelium adhesion, followed by migration through the endothelium.

1.5 EGFR

A key cell surface protein associated with MUC1 is Epidermal growth factor receptor 1 (EGFR/ERBb1). EGFR is a heavily studied cell surface protein which is known to overexpressed and/or abnormally activated in cancer cells. Abnormal (increased) activation of EGFR allows cancer cell to evade growth suppression signals and allows them to proliferate – hence allowing the tumour cells to acquire two of the major hallmarks of cancers, mentioned earlier.

EGFR is a member of the ErbB family of receptor tyrosine kinases that includes EGFR/ ErbB1 (Her1), ErbB2 (Her2/c-Neu), ErbB3 (Her3) and ErbB4 (Her4) (340). EGFR receptors consist of a glycosylated extracellular domain, a hydrophobic transmembrane domain, a juxtamembrane domain adjacent to the inner leaflet of the plasma membrane, and a C-terminal kinase domain (341). The ErbB/Her receptor which includes the epidermal growth factor receptor, are among the most studied cell signalling families in biology (342). The receptor was first identified and characterised by Stanley Cohen in the 1980s (343). Cohen *et al* found the 170 kDa polypeptide chain that contained both the EGF binding activity and protein kinase activity (344).

The last two decades have seen significant research into EGFR-related signalling in cancer progression (219, 345-348). It is now generally accepted that increased EGFR activation and expression leads to a poor prognosis in cancer. Not surprising, EGFR is now a therapeutic target in the fight against cancer.

1.5.1 EGFR Structure

The EGFR is synthesised as a 1210 residue precursor that is further cleaved at the N-terminal to form the mature 1186 residue long transmembrane EGFR. (349) From N-terminal to C-terminal, the EGFR consists of an extracellular ligand binding and a dimerization arm (exon 1-16) a hydrophobic transmembrane domain (exon 17) and the intracellular tyrosine kinase and c-terminal domain (exon 18-28) (341).

The extracellular region of EGFR is composed of 621 amino acids and is subdivided into 4 domains 1-4. Domain 1 and 3 are leucine rich fragments that participate in ligand binding, whereas non-ligand interacting domains 2 and 4 are cysteine-rich. Domain 2 forms the homo- or hetero-dimer with the corresponding domain in other members of ErbB family. Domain 4 links the extracellular region of EGFR to the transmembrane domain and can also form a disulphide bond with domain 2.

The transmembrane domain of EGFR is a 23-amino acid long hydrophobic single pass membrane structure, that anchors receptor to the membrane (350). The transmembrane domain of EGFR has been hypothesised to play a role in receptor dimerization (351), especially in heterodimerization with ErbB2, as mutation to transmembrane domain in ErbB2 has been shown to augment dimerization (352).

The intracellular domain of EGFR is 542 amino acids long and consists of a juxtamembrane segment, a tyrosine kinase domain and a C-terminal tail (353). The tyrosine kinase domain can be further divided into an N-lobe consisting mainly of β sheet structures and C-lobe containing mainly α-helical structure, with an ATP binding site located between the two lobes (354). Transautophosphorylation relies on the interaction of N lobe from one receptor to C lobe of another (355). The C-

terminal tail also includes various tyrosine residue, which when phosphorylated, allows anchoring of a variety of intracellular proteins to the activated receptor.

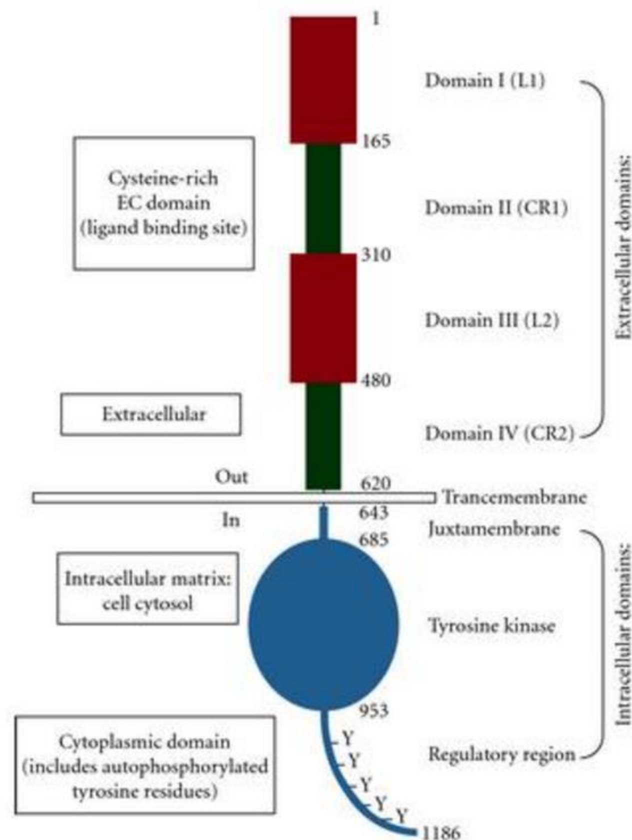


Figure 1.6: Basic structure of EGFR revealing relavent domain adapted from (356). (Permission under Creative Commons Attribution License)

1.5.2 EGFR ligands and receptor dimerization

Human EGF is a 6 kDa protein made up of 53 amino acid which was identified as the first ligand to bind and phosphorylate EGFR (357). Aside from EGF, six other ligands have been described to also bind to EGFR. These include transforming growth factor-α (TGF-α), amphiregulin (AREG), epiregulin (EREG), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), and epigen (EPI). The ligand EGF, TGF-α, and

amphiregulin are specific for EGFR only (358), whereas BTC, HB-EGF, and EREG have been shown to have a dual specificity for both EGFR and ERBB4 (219). Regardless of the ligand, the initial stages of receptor activation is the same, with ligand binding leading to receptor dimerization, receptor transautophosphorylation and recruitment of various signalling and adapter proteins. This is followed by EGFR receptor internalization and trafficking to the early endosome. Despite these similarities, the activation of EGFR by different ligand has been shown to cause distinct biological activity (359, 360). For example, Sweeney *et al* showed that although EGF and neuregulins (NRG) can both lead to downstream activation of ERK1/2, EGF mediated signalling normally leads to the formation of EGFR and ERBB2 heterodimer and simulation of PKC, whereas NRG mediated simulation normally leads to ERBB2 and ERBB3 heterodimers and preferentially simulation of AKT (361). The exact mechanism as to how different ligands bring about different biological effects remain undiscovered.

Ligand binding and EGFR dimerization are crucial to ensure EGFR activation. However, it was a matter of controversy as to which step occurs first. It is now generally accepted that ligand binding to EGFR precedes EGFR dimerization. Work done by Yarden and Schlessinger has shown that prior to ligand binding, domain II of EGFR is folded into domain IV via disulphide bond, in a confirmation that inhibits receptor dimerization (362, 363). Ligand binding to domain I and III of EGFR leads to domain rearrangement that exposes the dimerization arm in domain II, leading to a more stable confirmation (364, 365). Dimerization of domain II is followed rearrangement of transmembrane and juxtamembrane segment which promotes increased kinase activity. The EGFR dimer is an asymmetric dimer pair, so that the c-

terminus of the activating kinase inserts into the active site of receiving kinase, resulting in transautophosphorylation (355). This is different from other receptor tyrosine kinases, where conformational changes following dimerization induces receptor monomers to activate itself.

1.5.3 EGFR signalling

Of the four family members, EGFR is known to signal the largest number of unique signalling pathways including the ERK/MAPK, PI3K-AKT, SRC, PLC-γ1-PKC, JNK, and JAK-STAT pathways. As these pathways are generally interlinked, the activation of EGFR simulates an entire signalling networks that are heavily involved in tumour growth, proliferation, differentiation, migration and inhibition of apoptosis. Many of the proteins involved in EGFR signalling transduction, as well as EGFR itself, is the subject of pharmaceutical targeting in malignancies.

EGF binding to EGFR leads to receptor transphosphorylation of multiple tyrosine residue on the intracellular C-terminal tail. Tyrosine residue Y703, Y920, Y992, Y1045, Y1068, Y1086, Y1148, and Y1173 on intracellular C-terminal tail have all been identified to be phosphorylated following EGF addition to the cell. In addition to these, there are additional residues phosphorylated by other kinases which interestingly appear downstream in the EGFR activation cascade. For example, Y845 is phosphorylated by c-SRC (366), and T654 is phosphorylated by PKC (367). The newly phosphorylated tyrosine residues act as potential docking sites for proteins

harbouring phosphor-tyrosine-binding residues, such as those with Src Homology 2 (SH2) and phosphotyrosine binding (PTB) domains (368).

The receptor transphosphorylation and binding of key binding partners to phosphorylated tyrosine residues allows EGFR and the other ErbB family members to amplify the signal which eventually results in cells proliferation, survival, and migration. The main pathways activated in response to EGF ligand binding to EGFR includes the RAS-RAF-MEK-ERK-MAPK Pathway, PI3K-AKT-mTOR pathway and phospho-lipase C-γ (PLC-γ) pathway.

1.5.3.1 RAS-RAF-MEK-ERK-MAPK Pathway

Following receptor transphosphorylation, residues Y1068 and Y1086 play a key role in initiating RAS-ERK/MAPK pathways by binding to growth factor receptor binding protein 2 (GRB2) (369-371) and by recruiting Src homology and collagen (SRC) (372-374). These two adaptor proteins act as a signal amplifier for ligand-activated EGFR to activate complex intracellular biological pathways. Upon binding to EGFR, SHC is phosphorylated and acts a phosphorylation site for GRB2 (373, 375). GRB2 then binds to son of sevenless 1 (SOS1) which subsequently activates RAS (376-379). RAS can then activate RAF1, which has been shown to directly activate MEK1/2 by phosphorylation at serine residue 217 and 221 (380-383). MEK (mitogen-activated protein kinase kinase-MAPKK) 1/2 forms a rare class of tyrosine and threonine/serine dual specificity kinases that activate ERK 1/2 (384). ERK 1/2 then phosphorylates multiple substrates to induce various biological responses. Ras is also known to

activate the PI3K pathway (discussed below) in addition to the MEK/ERK pathway, demonstrating a role for signal crosstalk in cancer progression (385).

1.5.3.2 PI3K-AKT-mTOR pathway

PI3K is a major downstream activator of EGFR. Following activation by EGF simulation PI3K phosphorylates the 3-OH group of the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP_3) (386-388). Although PI3K can binds directly and indirectly to members of ErbB family (389-391), the major signal transduction with from PI3K is due to its ability to phosphorylate 3-OH group of the membrane lipid PIP_2 to form PIP_3. PIP_3 is a potent second messenger and a predominant mediator of PI3K activity (392). The strong signalling potential of PIP_3 is highlighted by the fact that the PI3K antagonist PTEN, which dephosphorylates and limits the activity PIP_3, is frequently inactivated in cancer (393). PIP_3 also leads to the recruitment and activation of AKT (also known as Protein Kinase B or PKB) that has an impact on cell survival, proliferation, metabolism, growth and migration. One of the most important AKT pathways is to signal mTOR (mammalian target of rapamycin) which is well known to regulate cell growth and autophagy. In terms of cell survival, AKT functions in an anti-apoptotic manner by directly interacting with components of cell death machinery. AKT directly phosphorylates BAD (394) and caspase-9 (395) which supresses their pro apoptotic action. Indirectly, AKT has also been shown to reduce the expression of pro-apoptotic protein BIM (396) and downregulate P53 (397-399).

1.5.3.3 Phospho-lipase C-γ (PLC-γ) pathway

PLC-γ1 (Phosphoinositide phospholipase C – isozyme γ1) directly binds to activated EGFR at Y992 and Y1173 in the cytoplasmic tail (400-403). PLC-γ1 can also be recruited to the plasma membrane by binding to PIP_3, formed in response to EGF stimulation (404, 405). Once translocated near the plasma membrane and activated, PLC-γ1 hydrolyses PIP_2 into free intracellular 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces intracellular calcium release which converges with the DAG pathway to activate protein kinase C (PKC). Activated PKC has a whole host of cellular substrate including EGFR, RAF-1, H-RAS, p21, BAD, and BCL-2 (406); all of whom collectively promote proliferation and apoptosis resistance.

Because the signalling cascade activated following EGFR activation is quite potent, physiologically, the signal is quickly quenched through receptor degradation. Following ligand receptor interaction, EGFR undergoes clathrin-mediated endocytosis (407, 408). These early endosomes can either be recycled back to the cell surface, or be degraded in lysosome. The fate of the EGFR receptor following endocytosis has important consequences for cell's biological output, with the recycling pathway favouring cell proliferation whilst degradative and docking with lysosomes correlating with normal cellular homeostasis (409). Atypical trafficking pathways to the nucleus (410, 411) and mitochondria (412) have also been described and are proposed to favour survival, but the transport mechanisms are not well established.

1.5.4 EGFR in epithelial cancer

EGFR is physiologically expressed with its expression and ligand availability tightly regulated to ensure that cell proliferation matches tissue requirements for homeostasis. In the tumour state however, EGFR activation is increased, either due to increased expression and mutation of the EGFR itself or due to increased availability of the EGFR ligand (413, 414). EGFR is an indicator of poor prognosis in gastric cancer (415) , head and neck squamous cell carcinoma (416) , colorectal cancer (417), breast cancer (418), renal cell carcinoma (419), medulloblastoma (420), non-small cell lung cancer (421), and prostate cancer (422) among others.

As EGFR is a powerful inducer of signals leading to phenotypes that are important in development, such as proliferation, survival, and motility, EGFR is often exploited in cancer. Mutation in the kinase and ligand binding domain of EGFR promoting continuous receptor activation has been noted in lung cancer and gliomas (340). In breast cancer, the EGFR is overexpressed and overactivated but it is not mutated (423). Aberrant expression of TGFα or nuclear localization of EGFR by tumours typically confers a more aggressive phenotype and is often an indicator of poor prognosis in several epithelial cancers (424). Not surprisingly, EGFR has emerged as a principal target for therapeutic intervention.

1.5.5 Interaction between MUC1 and EGFR

Recently several studies have pointed to a potential link between MUC1 and EGFR (338, 425). EGF is the primary ligand for EGFR during lactation (339, 426, 427), and

MUC1 is highly expressed during this period of mammary development (428). In addition, it has been shown that EGFR and MUC1 interact at the apical cell surface of lactating mammary gland (425). Furthermore, in the same study by Schroeder *et al*, Immunoprecipitation experiments revealed that full-length transgenic MUC1 physically associates with all four erbB receptors, and co-localizes with erbB1 in the lactating gland (425).

MUC1-C is also associated with other tyrosine kinase receptors such as ErbB2 to 4, fibroblast growth factor receptor-3, platelet-derived growth factor receptor b and MET, with resultant downstream signalling (429). Given the role of growth factor receptor in promoting pro-survival signal, it is possible that in cancer cells over-expression of MUC1 coupled with increased expression and activation of growth factor receptors promotes pro survival signal, EMT, survival and anoikis resistance in suspension conditions.

Activated EGFR have also been shown to associate with MUC1-CT directly and translocate to nucleus. In the nucleus, MUC1-CT and EGFR complex binds to cyclin D1 and v-myb myeloblastosis viral oncogene homolog-like 2 (MYBL2) promotors that enable G1/S phase gene expression (338). Activated EGFR has also been shown to drive high level MUC1 expression in multiple cell lines of uterine adenocarcinoma and pancreatic cancer origins (430). In some cells, addition of exogenous EGFR ligands (EGF or HB-EGF) elevates MUC1 levels while addition of the EGFR tyrosine kinase inhibitor, AG1478, reduces MUC1 levels (430). Recently a reciprocal action, with MUC1 presence simulating EGFR expression, has also been reported in endometrial cancer (339).

1.6 Potential for interaction between galectin-3, MUC1 and EGFR

It follows that there is a considerable potential for interactions between galectin-3, MUC1 and EGFR to underlie many of the changes that are essential to cancer progression and metastasis. Previous work in our lab has identified complex interaction between MUC1 and galectin-3 leading to the formation of tumour homotypic aggregation and heterotypic adhesion (25). Recent work by various research groups has identified a potential interaction between MUC1 and EGFR suggesting a much broader role of MUC1 in tumour progression (338, 339). Exploration of these interactions is the principal aim of the work described in this thesis

1.7 Hypothesis

MUC1 intracellular and extracellular domains may contribute differently to MUC1-mediated resistance to anoikis of epithelial cancer cells

MUC1-galectin-3 interaction may influence EGFR activation of epithelial cells

1.8 Aims

To investigate the relative contributions of MUC1 extracellular domain and intracellular domain to MUC1-mediated epithelial cancer cell resistance to anoikis.

To assess the influence of O-glycosylation of MUC1 extracellular domain on MUC1-mediated cell resistance to anoikis.

To investigate the impacts of expression of MUC1 and its interaction with galectin-3 on EGFR activation and signalling in epithelial cancer cells

2 Materials and Methods

2.1 Materials

Proteome profiler human apoptosis array kit (ARY 009), full- length recombinant galectin-3 (1154-GA/CF, carrier free), and anti-CD44 (BBA10), anti-integrinβ1 (MAB17782), anti-E-cadherin (MAB1838), anti-Fas (AF2267) and anti-Fas ligand (AF126) antibodies were from R&D Systems (Abingdon, UK). The Caspase 3/7 Glo® kits and Caspase-8 Glo® kits were obtained from Promega (Southampton, UK). Recombinant Fas-L, anti-EGFR antibody (500-p306), and recombinant human EGF (AF-100-15) was from PeproTech (London, UK). FITC-Annexin-V/PI apoptosis detection kit was from Cambridge Biosciences (Cambridge, UK). Biotinylated peanut agglutinin (PNA) and biotinylated *Vicia villosa* agglutinin (VVA) were purchased from Vector Laboratories, (Peterborough, UK). FITC-conjugated anti-mouse antibody (115-095-146) was purchased from Jackson Immunoresearch Labs, (West Grove, PA, USA). Metafectene was from Biontex Laboratories (München, Germany). B27.29 anti-MUC1 antibody was kindly provided by Dr Mark Reddish (Biomira, Edmonton, Canada), anti-MUC1 antibody 214D was kindly provided by Dr John Hilkens (Netherlands Cancer Institute, Amsterdam, NL) and CT2 anti-MUC1 antibody was kindly provided by Prof Sandra Gendler (Mayo Clinic, AR, USA). shRNA plasmid DNA for Core 1 Gal-transferase (SHCLND-NM_020156-C1GALT, TRCN0000289384), control shRNA (SHC002v, non-target shRNA), non-enzymatic cell dissociation solution (NECDS) and Lapatinib were purchased from Sigma-Aldrich (Dorset, UK). Antibodies against p-EGFR (SC-23420), EGFR (SC-03), p-ERK1/2(SC-7383), ERK1/2 (SC-94) and Protein A/G plus agarose beads were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-EGFR antibody used in confocal

microscopy and for immunoprecipitation (DB81) was purchased from New England Bio-Labs (Hitchin, UK). Bis(sulfosuccinimidyl) substrate (BS3) cross-linker, Alexa Fluor 643 conjugated anti-rabbit antibody, FITC conjugated avidin and chemiluminence detection kits were from Life Technologies (Paisley, UK). Anti-actin (M0851) and peroxidase-conjugated secondary antibodies were from Dako (Glostrup, Denmark). Mouse IgG1 monoclonal (ab81032) and rabbit IgG monoclonal antibodies were purchased from Abcam (Cambridge, UK).

2.2 Medium

All cell culture media were purchased from Sigma-Aldrich, unless specified.

- A) Dulbecco's Modified Eagle's medium (DMEM)

  1. Complete culture medium contains 10% fetal calf serum (FCS), penicillin 100 U/ml, streptomycin 100 µg/ml and glutamine 2 mM.
  2. Serum-free DMEM contains 0.5% bovine serum albumin, penicillin 100 U/ml, streptomycin 100 µg/ml and glutamine 2 mM.
  3. Antibiotics-free and serum-free DMEM contains glutamine 2 mM

The cell lines HCA1.7+, HCA1.7-, HTD, ACA19+, ACA19-, ADT, SW620 and fibroblasts calls with variable numbers of tandem repeats were cultured in DMEM.

- B) McCoy's 5A

  1. Complete culture medium contains 10% (FCS), penicillin 100 U/ml, streptomycin 100 µg/ml and glutamine 2 mM.

2. Serum-free McCoy's 5A contains 0.5% bovine serum albumin, penicillin 100 U/ml, streptomycin 100 μg/ml and glutamine 2 mM.
3. Serum free and Antibiotics free McCoy's 5A contains glutamine 2mM

The cell lines HCT116 MUC1 full, HCT116 MUC1 neo, HCT116 MUC1 Δ CT, HCT116 MUC1 Δ TR were cultured in McCoy's 5A.

- C) Endothelial growth media (EGM) supplemented with 3mg/mL bovine brain extract (BBE), 10 mg/mL hEGF, 1 mg/mL hydrocortisone, 2% FBS, 50 mg/mL gentamycin and 50 μg/mL amphotericin-B (EGM bullet kit, Cambrex Bio Science, UK) were used for the culture of human umbilical vein endothelial cells (HUVEC).

2.3 Cell lines

HUVECS: Human umbilical vein endothelial cells (HUVECS) were obtained from Cambrex Bio Science Wokingham Ltd (Wokingham, UK). HUVECS were cultured in endothelial growth media (EGM Bullet Kit, Cambrex Bio Science, UK). Less than 6 passage cells were used in all the experiments. The culture medium was changed every 2 days.

MUC1 transfection of HBL-100 human breast epithelial cells and human melanoma A375 cells with full-length cDNA encoding MUC1 and the subsequent selection of the MUC1-positive transfectant HCA1.7+ (from HBL-100) and ACA19+ (from A375), and the negative revertant HCA1.7− (from HBL-100) and ACA19− (from A375) was conducted as described previously (431). The cell lines were last authenticated by

DNA profiling (DNA Diagnostics Center, London, UK) in May 2014. MUC1 transfection of A375 cells with cDNA encoding only the MUC1 extracellular and transmembrane domains and subsequent selection of the MUC1-positive transfectant ATD and fibroblast cells transfected with zero (parental), eight, fifteen and thirty-six variable number of tandem repeats (VNTR) was kindly provided by Dr. John Hilkens (Netherlands Cancer Institute, Amsterdam, NL).

The MUC1-negative human colon cancer HCT116 and MUC1-positive human colon cancer SW620 cells were obtained from European Collection of Cell Culture (Salisbury, UK) and were cultured in McCoy's5A medium and DMEM, respectively. The cell lines were last authenticated by DNA profiling (DNA Diagnostics Centre, London, UK) in 2014. MUC1-expressing HCT116MUC1-F3 and MUC1-negative HCT116MUC1-neo cells were obtained by stable transfection of HCT116 cells with MUC1-expressing or control vectors by other lab members as described previously (431, 432). MUC1 expression vectors for full-length MUC1, the extracellular domain-depleted MUC1 (MUC1ΔTR), the cytoplasmic domain-depleted MUC1 (MUC1ΔCT) and control vector were kindly provided by Prof Tony Hollingsworth (University of Nebraska Medical Centre, Omaha, USA).

Table 2.1 Summary of cells used in this thesis

Cell name	**Property**
HCA1.7+	Human breast epithelial HBL100 cells stably transfected with full length MUC1. (62)

HCA1.7-	Human breast epithelial HBL100 cells stably transfected with control vector. (62)
HTD	Human breast epithelial HBL100 cells stably transfected with MUC1 with mutant MUC1 Δ CT.. (62)
ACA19+	Human melanoma cells A375 stably transfected with full length MUC1. (62)
ACA19-	Human melanoma cells A375 stably transfected with control vector. (62)
ATD	Human melanoma cells A375 stably transfected with mutant MUC1 Δ CT. (62)
HCT116 MUC1 full	Human colon cancer HCT116 cells stably transfected with full length MUC1. (433)
HCT116 MUC1 neo	Human colon cancer HCT116 cells stably transfected with control vector. (433)
HCT116 MUC1 Δ CT	Human colon cancer HCT116 cells stably transfected with MUC1 without MUC1 intracellular domain. (433)
HCT116 MUC1 ΔTR	Human colon cancer HCT116 cells stably transfected with MUC1 without MUC1 extracellular domain. (433)
HCT116 MUC1 F3	Human colon cancer HCT116 cells stably transfected with full length MUC1.

F3 sh-con (F2)	Human colon cancer HCT116 cells stably transfected with full length MUC1 subsequently transfected with control vector for C1GT, variant F2.
F3 sh-con (C8)	Human colon cancer HCT116 cells stably transfected with full length MUC1 subsequently transfected with control vector for C1GT , variant C8.
F3 sh-C1GT (E7)	Human colon cancer HCT116 cells stably transfected with full length MUC1 subsequently transfected with C1GT knockdown shRNA, variant E7
F3 sh-C1GT (B7)	Human colon cancer HCT116 cells stably transfected with full length MUC1 subsequently transfected with C1GT knockdown shRNA, variant B7
Neo sh-con 1	Human colon cancer HCT116 cells stably transfected with control vector subsequently transfected with control vector for C1GT, variant con 1
Neo sh-con 2	Human colon cancer HCT116 cells stably transfected with control vector subsequently transfected with control vector for C1GT, variant con 2
Neo sh-C1GT 3	Human colon cancer HCT116 cells stably transfected with control vector subsequently transfected with C1GT knockdown shRNA , variant C1GT 3

Neo sh-C1GT 4	Human colon cancer HCT116 cells stably transfected with MUC1 negative control vector subsequently transfected with C1GT knockdown shRNA, variant C1GT 4
SW620	Metastasizing colon cancer cells naturally overexpressing MUC1
SW620 sh-con	Metastasizing colon cancer cells naturally overexpressing MUC1 stably transfected with control vector for C1GT
SW620 sh-C1GT	Metastasizing colon cancer cells naturally overexpressing MUC1 stably transfected with shRNA for C1GT knockdown

2.4 Detachment of cells with trypsin or non-enzymatic-cell dissociation solution.

Enzymatic cell sub-culture was performed using 0.25% Trypsin-EDTA. Trypsin acts by cutting amino acids, specifically lysines or arginies, on their c-termini unless these amino acids are followed by proline. EDTA is frequently included in the trypsin solution for its function as divalent cations chelator. By removing calcium from a solution with cells, cadherins which hold cells to each other, are broken and cells separate from each other as well as from the surface of the tissue culture plastic.

Non-enzymatic cell dissociation solution (NECDS) was used to detach cell for anoikis assays and to assess the accessibility to cell surface molecules. NECDS are

membrane-filtered, isotonic, and enzyme-free solution of salts, chelating agents, and cell-conditioning agents in calcium-free and magnesium-free phosphate-buffered saline (PBS). They ensure a gentle dissociation of mammalian cells without partly digesting cell surface molecules.

Sub-confluent cells were washed with PBS before 1 ml of either 0.25% trypsin EDTA (for sub-culture) or NECDS (for flow cytometry and anoikis experiments) was added to detach the cells. The cells were briefly incubated at 37^0C for 5-10 minute until the cells became detached from the flask. After gentle tapping and pipetting cells up and down to ensure single cell suspension, 10 ml of pre-warmed complete culture medium was added to the cells to inhibit the effect of trypsin or NECDS. FCS in complete media contains protease inhibitors, such as α1-antitrypsin and α2-macroglobulin which inhibits trypsin activity. FBS also contains a high concentration of calcium which has a neutralizing effect on chelating agents found in NECDS and EDTA.

2.5 Cell counting

Cell numbers were counted using a haemacytometer.

2.6 Electrophoresis and Immunoblotting

Cell lysis: Up to 90% confluence cells in a T25 flask were washed with PBS 10 ml twice. One ml/T25 flask of sodium dodecyl sulphate (SDS) sample buffer was added and

incubated for 20 minutes at room temperature. The cell lysates were collected into 1.5 ml tubes and kept at -80^{0}C until use.

Table 2.2 SDS-sample buffer.

	X 2 Concentration	X 4 concentration
0.5% Tris-HCL	2.5ml	0ml
glycerol	1.0ml (20%)	2ml
B-Mercaptoethanol	0.5ml (10%)	1ml
20% SDS	1.0 ml	2ml
1% Bromophenol blue	50 µl	0.1ml

Table 2.3 Gel preparations separating gel

	4%	7.5%	10%	15%
Deionized Water	6ml	4.85ml	4.2ml	2.35ml
1.5M Tris-HCL resolving gel buffer	2.5ml	2.5ml	2.5ml	2.5ml
10% SDS	100 µl	100 µl	100 µl	100 µl
30% acrylamide (acrylamide to bis acrylamide ratio 29:1)	1.35ml	2.5ml	3.33ml	5ml
TEMED	5µl	5µl	5µl	5µl
10% ammonium persulfate	50µl	50µl	50µl	50µl

Table 2.4 Gel preparations stacking gel

	3.75%	4%
Deionized water	3.09 ml	3.05ml
0.5M Tris-Hcl stacking gel buffer	1.25ml	1.25ml
10% SDS	50µl	50µl
30% acrylamide (acrylamide to bisacrylamide ratio 29:1)	0.625ml	0.665ml
TEMED	10µl	10µl
10% ammonium persulfate	50µl	50µl

After the glass plates and spacers (1.0 mm or 1.5 mm) thick were assembled, the running gel was poured to about 1cm below the wells of the comb (~4.8 ml or 9.6ml for 1.0 mm and 1. 5mm, respectively). Then 200µl water saturated butanol-1 was added on top of the gel. When the gel had set (~40 minute), the butanol was poured off and the gels were rinsed with about 2ml deionised water 3 times. Stacking gel was added and the 10 well or 15 well combs were inserted immediately. When the stacking gel had set, the glass plates were placed in a gel rig and immersed in running buffer. Prior to running the gel, the wells were flushed out thoroughly with running buffer.

Table 2.5 Running buffer

Tris-Base	30.67 grams

Glycine	64.04 grams
SDS	2.2 grams
Make up with dH_2O	4L

Running the gels

The samples were heated at 100^{0}C for 10 minutes before being loaded to the gels. The gels were run at 60 volts for 10 minutes before voltage was increased to 100 volts for 90-120 minutes. The duration of gel run varied depending on gel percentage. The proteins separated on the gel were transferred to nitrocellulose membrane using a transfer buffer containing following ingredients

Table 2.6 Transfer buffer

Tris-Base	12.12 grams
Glycine	57.65 grams
Methanol	800 ml
Make up with dH2O	4L

The gel was sandwiched as negative pole (black) – sponge- filter paper- gel- nitrocellulose membrane-filter paper-sponge- positive pole (white) and transferred at 100 volts for 1 hour.

Immunoblotting

The membrane was blocked in blocking buffer (1% BSA in PBS or TBS and 0.05% Tween-20) for 1 hour at room temperature or at 4^0C overnight. Following blocking, the primary antibodies were mixed in blocking buffer at a concentration specified in the results section before being added to the membrane for one hour at room temperature. The blot was washed 3 times with 50 ml of 0.05% Tween 20 in PBS or TBS before being rolled on a rolling machine for 10 minutes between washes. After removal of the solution, secondary antibody was diluted in blocking buffer before being added at to the membrane for 1 hour at room temperature. After 3-5 washes with 0.05% Tween 20 in PBS 100ml each time, the binding was visualized using luminol-based enhanced chemiluminescence (ECL) HRP kit.

2.7 Assessment of cell anoikis

Anoikis conditions were created by triple coating 96 well and 6 well plates with 2-hydroxyethyl methacrylate (poly-HEMA). Poly-HEMA is a polymer that forms a hydrogel in water and prevents cell adhesion, thereby creating anoikis conditions. Briefly, poly-Hema Crystals were dissolved in 95% ethanol to make a 10mM solution. 200 µl or 1 ml of this solution as added to each well of a 96 well plate or a 6 well plate, respectively and the plates were allowed to incubate overnight in sterile environment. The coating process was repeated 3 times to ensure that poly-HEMA coated the bottom and the side of the wells used for anoikis assessment.

2.8 Caspase 3/7 activity assay

The Caspase-Glo® Buffer was thawed and equilibrated prior to use. The Caspase-Glo® substrate was equilibrated to room temperature prior to use. All of the Caspase-Glo® Buffer was transferred into Caspase-Glo® substrate to reconstitute the lyophilized enzyme/substrate mixture. This formed the Caspase-Glo® Reagent.

All the caspase 3/7 assays in this study were done at a 0 or 24-hour time-points, unless specified. The cells were plated at a concentration of 1 X 10^4 cells/ml in a flat white (block) transparent 96 well plate. For 0-time point, assessment was done on cells under adhesion conditions and for 24-hour time points the cells were plated in a poly-HEMA coated plate. Prior to the addition to cells, Caspase-Glo® Reagent was mixed gently vortexing, swirling or by inverting the contents to obtain a homogenous solution. One hundred µl of this solution were added to cells in media in a 1:1 ratio. The control wells with medium only were used to obtain a value for background luminescence. Contents were mixed for 2 minutes on an orbital shaker to lyse the cells. The plate was allowed to incubate at room temperature for 30 minutes to equilibrate. A luminescence reading was taken after 30 minutes using Tecan infinite 200.

Application:	Tecan i-control, 1.3.3.0
Device:	infinite 200
System:	MED17-86467C
Plate:	Corning 96 flat bottom transparent polystyrol
Mode:	Luminescence

Attenuation: none

Integration time: 500 ms

Settle time: 0 ms

2.9 Annexin V/ PI cell surface binding analysis

The cells were released by NECDS and were cultured in either a 6 well cell culture plate (0 time point) or a 6 well poly-HEMA coated cell culture plate (24 hour time point). Approximately 2.5 x 10^5 cells were collected by centrifuging the cell at 330 x g for 5 minutes. The cells were then resuspended in 500 μl of 1X binding buffer (from Biovision) at a concentration of 5 X 10^5 cells/ml. Five μl of annexin V-FITC and 5 μl of propidium iodide (PI 50 μg/ml) were added into 0.5 ml cell solution incubated at room temperature for 5 minutes in the dark before analysis of annexin-V cell surface binding by flow cytometry (Ex= 488 nm, Em= 530 nm) using FITC signal detector (usally FL1) and PI staining by the phycoerythrin emission signal detector (usually FL2).

Cells without staining and cells stained with either annexin V-FITC or propidium iodide (PI) were used as control to identify the position of non-staining live cells (bottom-left panel). Annexin-V FITC (bottom right panel) and PI (upper right panel) labelled cell population in the bivariate correlation plot. Annexin-V FITC positive and PI negative (early apoptotic, at the bottom right of the bivariate correlation plot) and annexin-V positive and PI positive (late apoptotic, at the top right in the bivariate correlation plot) cells are considered as apoptotic cells.

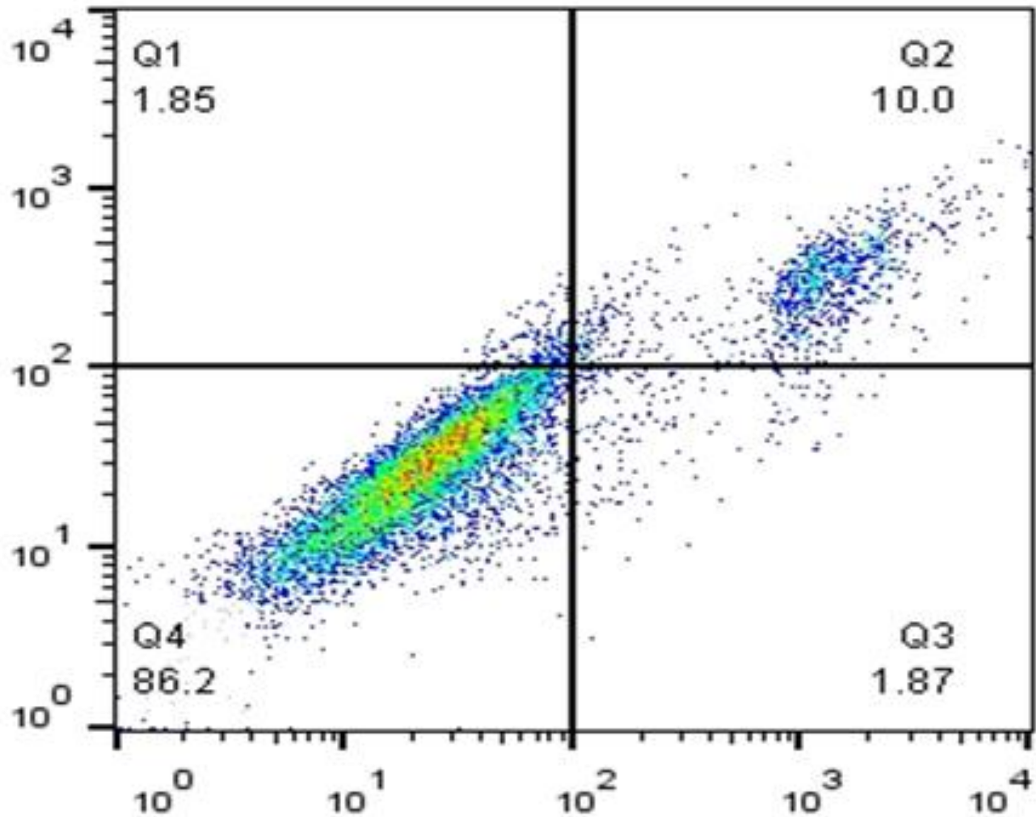


Figure 2.1: Annexin-V and PI analysis of F3 sh-C1GT (E7) at 0 hour. The analysis shows that at 0 time point 86.2% of cells are alive (bottom left quadrant), 10% of cells are undergoing late apoptosis (top right quadrant) and 1.87% of cells are undergoing early apoptosis (bottom right quadrant).

2.10 Apoptosis array

Proteome Profiler™ Human Apoptosis Array Kit (Ary009) was purchased from R&D systems. HBl100 breast epithelial cells transfected with either the full length MUC1 (HCA1.7+), no MUC1 (HCA1.7-) or MUC1 Δ CT (HDT) along with HCT116 cells transfected with either Full length/ neo/ Δ CT or Δ TR MUC1 were cultured at 1 ×

10^5 cells/ml in serum-free DMEM for 24 h in poly-HEMA-coated plates at 37 °C. The suspended cells were collected, lysed and protein amount was measured using a bicinchoninic acid assay (BCA) protein assay kit. The cells were collected and lysed with lysis buffer (provided by the Human Apoptosis Array Kit, R&D Systems) at 4 °C for 30 min. After centrifugation at 14 000 x g for 5 min, the supernatants were obtained and 500 μg proteins from each sample were applied to the Human Apoptosis Array as described by the array kit. Each array contains 35 apoptosis-related proteins, each in duplicate (Bad, Bax, Bcl-2, Bcl-x, pro-caspase-3, cleaved caspase-3, catalase, cIAP-1, cIAP-2, claspin, clusterin, cytochrome c, TRAIL R1/DR4, TRAIL R2/DR5, FADD, Fas/TNFSF6, HIF-1α, HO-1/HMOX1/HSP32, HO-2/HMOX2, HSP27, HSP60, HSP70, HTRA2/Omi, livin, PON2, p21/CDNK1A, phosphor-p53(S15), phosphor-p53(S46), phosphor-p53(S392), phosphor-Rad17(S635), SMAC/Diablo, surviving, TNF R1/TNFRSF1A and XIAP). The density of each apoptosis-related protein in the array was quantified by ChmiDoc XRS Imager (Bio-Rad, Hertfordshire, UK).

2.11 Flow-cytometry to access the cell surface expression of MUC1, E-cadherin, Integrin β1, FAS and CD44

Sub-confluent cells were released by 1 ml NECDS solution and were washed twice with 10 ml PBS. Following the removal of supernatant, the cell pellets were re-suspended in 5 ml of 2% paraformaldehyde and cells were fixed at room temperature for 15 minute. After washing the cells twice with PBS and centrifugation at 1000 x g for 5 minute, the supernatants were removed and the cell pellets were incubated with 5% goat serum (10^6 cells/ml) for 30 minutes at room temperature on the roller

mixer. After removal of the supernatant following centrifugation at 1000 x g for 5 minute, the cells were re-suspended into 10^6 cells/ml in 1% goat serum in PBS and divided 1ml/tube in 1.5ml eppendorf tubes. Biotin-PNA (2 µg/ml), biotin-VVA (2 µg/ml) or antibodies against MUC1 extracellular domain B27.29 (1 µg/ml), E-cadherin (0.5 µg/ml), CD44 (0.5 µg/ml), integrinβ1 (0.5 µg/ml), Fas (0.5 µg/ml) or control mouse IgG were added to the cells and the solution was incubated at room temperature for 1 hour on a roller (or overnight at 4^0C). After washing twice with PBS, fluoroscein-conjugated secondary antibodies (1:400 in 1% goat serum in PBS) or fluoroscein-avidin (1:400 in 1% goat serum in PBS) were applied for 1 hour at room temperature. After three washes with PBS, the cells were re-suspended in PBS in 0.5ml/tube. The cell surface expression of MUC1, E-cadherin, FAS, CD44 and integrinβ1 was analysed by flow cytometry (BD FACS Canto II). Flow cytometry data was analysed via either FlowJo (FlowJo LLC, Ashland, USA) version 10 or WinMdi version 2.9 (The Scripps Research Institute, La Jolla, USA)

Table 2.7 Primary and secondary antibodies used in Flow cytometry analysis

Primary antibody or lectin	Secondary antibody
B 27.29	FITC anti mouse antibody
Biotinylated PNA	FITC conjugated avidin
Biotinylated VVA	FITC conjugated avidin
Mouse anti E-cadherin	FITC anti mouse antibody
Mouse anti Fas receptor	FITC anti mouse antibody

Mouse anti CD44	FITC anti mouse antibody
Mouse anti integrin beta 1	FITC anti mouse antibody

2.12 Epithelial cell- endothelial adhesion

HUVEC cells were released from the T25 flask using trypsin as above and suspended at $1x10^5$ cells/ml with EGM culture medium. A 13-mm sterile coverslip was inserted in each well of 24-well plats. 200 µl of HUVEC cell at $1x\ 10^5$ cell/ml was then added to each well of the 24 well plate and a HUVEC cell monolayer was grown on the glass coverslip (~ 3 days). The 24 well plate was incubated at 37^0 C at 5% CO_2 for 24 hours. The cells were deemed suitable for adhesion assay at 100% confluence.

ACA19+, ACA19-, ATD, HCA1.7+, HCA1.7- and HTD cells were maintained in DMEM containing fetal calf serum (FCS) before being detached from the T25 flask using 1 ml NECDS at 37^0 C for 5 minutes. Cells were washed twice with serum-free DMEM followed by centrifugation at 300 RCF for 5 minutes between washes, as mentioned above. The cells were then re-suspended in 1 ml serum-free DMEM and 5 µl/ml of fluorescent dye, Calcein-AM, was added and the cells followed by an incubation at 37^0 C for 30 minutes. Once the ACA19+ cells were fluorescent labelled, they were re-suspended at $1\ x\ 10^5$ cells/ml.

The 24 well plates, containing glass coverslips with a monolayer of HUVEC cells were washed carefully with PBS and 0.5 µl/ml of fluorescent dye, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiL) was added for 30 minute at 37^0 C. HUVEC cells were again washed twice with PBS and 300 µl of the incubated ACA19+

cells with galectin-3 (2 µg/ml), control (BSA 2 µg/ml) or 214 D (2 µg/ml) were added to corresponding well on the 24 well plate containing the glass coverslip with confluent HUVEC monolayer. The 24 well plates were then incubated at 37^0 C for 2 hours.

To finish, the medium was removed from the 24 well plates and the plates were carefully washed with PBS. The cells were also fixed with 2% paraformaldehyde for 10 minutes. The glass coverslip was carefully taken out from the wells and mounted with mounting medium for fluorescence (HI200 with DAPI from Vector labs). The cover slips were covered with a new 22mm cover slide. The slides were blinded with tape and fluorescence-labelled cells remaining on the endothelial monolayer were counted in 10 random fields of view under fluorescence microscopy (Olympus B51 fluorescence microscope).

2.13 Immunofluorescence

Sterile 13 mm coverslips were inserted into each well of a 24 well plate before cells to be assessed were added at a concentration of 1 x 10^5 cells/ml. The cells were grown to about 60% confluence before media was changed to serum free media 16 hours prior to treatment. Following treatment (details of which are given in individual result chapters) the cells were washed with either PBS or TBS before being fixed with 4% paraformaldehyde for 10 minutes at room temperature. Following fixation, the cells were washed twice with either PBS or TBS before being blocked in 5% goat serum for 30 minutes at room temperature. This was followed by addition of primary antibodies in 1% goat serum (details and concentration given in individual

result chapters) and the cells were incubated at room temperature for 2 hour. The cells were washed twice before an appropriate FITC or Alexa fluor conjugated secondary antibody was added and the cells were incubated in dark at room temperature for a further 60 minutes. The cells were washed twice before mounting using DAPI containing fluorescent mounting media. The slides were analysed via either AQM 6 (Olympus B51 fluorescence microscope) or with 3i confocal microscope (Marianas SDC, 3i Imaging) and Slidebook 6 Reader version 6.0.4 (Intelligent-imaging).

2.14 Caspase 8 activity assay

Like the caspase 3/7. The Caspase 8-Glo® Buffer was thawed and equilibrated prior to use. The Caspase-Glo® substrate was equilibrated to room temperature prior to use. All of the Caspase-Glo® Buffer was transferred into the amber bottle containing Caspase-Glo® substrate to reconstitute the lyophilized enzyme/substrate mixture.

All the caspase 8 assays in this study was done at either 0 or 2-hour time-points. The cells were plated at a concentration of 2 X 10^5 cells/ml in a flat white (block) transparent 96 well plate. For 0-time point, assessment was done on cells under adhesion condition and for 2-hour time points the cells were plated in a poly-HEMA coated plate. FAs ligand treatment included introduction of 100 ng/ml Fas-L for 0 and 2 hr before measuring the cellular caspase-8 activity using caspase-8 Glo assay.

Prior to the addition to cells, Caspase-Glo® Reagent was mixed gently vortexing, swirling or by inverting the contents to obtain a homogenous solution. One hundred

µl of this solution was added to cells in media in a 1:1 ratio. The control wells were added with medium only to obtain a value for background luminescence. Contents were mixed for 2 minutes on an orbital shaker to lyse the cells. The plate was allowed to incubate at room temperature for 30 minutes to equilibrate. A luminescence reading was taken after 30 minutes using Tecan infinite 200.

Application:	Tecan i-control, 1.3.3.0
Device:	infinite 200
System:	MED17-86467C
Plate:	Corning 96 flat bottom transparent polystyrol
Mode:	Luminescence
Attenuation:	none
Integration time:	500 ms
Settle time:	0ms

2.15 C1GT knockdown

Plasmids containing shRNA for C1GT and Puromycin resistance (Vector-pLKO.1-Clone Number TRCN0000289384) or control vector (MISSION® TRC2 pLKO.5-puro Empty Vector Control Plasmid DNA SHC201) were purchased from Sigma-Aldrich. Prior to any stable transfection, a kill curve assay was performed.

2.15.1 Killing curve

Cells to be transfected (SW620, HCT116 MUC1 full and HCT116 MUC1 neo) were plated in a 96 well plate at a concentration of $1x10^5$ cells/ml. The cells were allowed to grow till 70-80% confluent before the media was changed to 200 µl/well complete media (10% FCS, 2mM glutamine, penicillin 100 U/ml, streptomycin 100 µg/ml) containing a range of different puromycin concentrations from 0 µg/ml to 15 µg/ml. The cells were observed at 6 hours interval for up to 3 days after which the minimum puromycin concentration required to kill all the cells of a particular cell type was recorded. This concentration was used later in the selection phase of the stable transfection

2.15.2 shRNA transfection

HCT116 MUC1 full, HCT116 MUC1 neo and SW620 cells were seeded in corresponding complete media (McCoy's 5A for HCT116 MUC1 full and HCT116 MUC1 neo, DMEM for SW620) until the cells were 60-70% confluent. ShRNA for C1GT or control shRNA (100 ng) was pre-mixed in a 1:4 ratio with Metafectene transfection reagent in serum-free and antibiotic-free McCoy's 5A media (100 µl) for 30 min before addition to the cells in antibiotic-free and serum-containing medium (100 µl) in a single 96-well plate. The cells were then cultured at 37^0C and 5% CO2 for 6 hours.

After 6 hours culture the culture media was removed and replaced with complete media containing puromycin concentration pre-determined using the kill curve assays. The cells were incubated at 37^0C and 5% CO_2 for a further 72 hours. The surviving cells were released by trypsin, were re-suspended in a very low cell density and seeded into new 96 well plates. Wells containing single cells were identified

under microscope and were allowed to proliferate before being selected and analysed for successful shRNA transfection.

2.15.3 Transfection Validation

C1GT is an essential transferase that encodes for Core 1 structure following 0-glycosylation. Absence of C1GT leads to a reduction of core 1 structure as well as increased availability of sialyl-Tn and Tn cellular glycans. Using this justification, we tested for MUC1 expression (using B27.29 and CT2 to detect extracellular and intracellular domain of MUC1), Core 1 expression (Using PNA that binds to core 1 structure on MUC1) and Tn expression (using VVA that recognises the Tn antigen) via both immunoblotting and flow cytometry analysis.

shRNA transfection was considered successful for C1GT knockdown when Core 1 expression (PNA binding) was reduced and Tn expression was increased.

2.16 EGFR activation assays

The cells to be assessed were seeded at a concentration of $1x10^5$ cells/ml in a 24 well plate under complete media, till 50-60% confluent. At this point the medium was changed to serum-free for 16 hours. The cells were washed with sterile TBS before addition of different treatment (details of treatment and durations are explained in corresponding result chapter). The cells were then incubated at 37^0C and 5% CO_2 for varying times after which they were washed again in ice cold TBS before being immediately lysed in 2x SDS-sample buffer on ice. The lysates were collected in 1.5 ml eppendorfs and stored at -80^0C prior to immunoblot analysis. In immunoblots

detecting either pEGFR or pERK1/2, the wash buffer and blocking buffer were made in TBS to reduce non-specific interactions.

2.17 EGFR crosslinking

A detailed protocol for EGFR crosslinking using BS3 cross-linker is published by Turk *et al*. (434). We followed the general principles of this protocol and adapted it for our assay as follows:

The cells were seeded in a T25 flask at a concentration of 2x 10^5 cells/ml and were grown till 50-60% confluent, at which point the complete medium was removed and the cells were serum-starved overnight (16 hours). The following day the cells were washed twice with Ca^{2+} and MG^{2+} free PBS and were treated with serum-free media containing the treatments (details of treatments are given in corresponding chapters). Following treatment, the cells were incubated in 37^0C and 5% $C0_2$ for 10 minutes after which they were washed with ice-cold Ca^{2+} and Mg^{2+} free PBS and incubated with 3mM BS3 cross-linker in Ca^{2+} and Mg^{2+} free PBS on ice for 20 minutes. Excess BS3 was quenched with 250 mM glycine in PBS for 5 minutes at 4^0 C , as per manufacturer's instruction. The cells were washed three times with ice cold PBS, lysed in SDS-sample buffer and analysed by immunoblotting with antibodies against EGFR.

2.18 Immunoprecipitation

Sub-confluent cells were incubated in serum-free medium containing 0.5mg/ml BSA overnight. The cells were with TBS and incubated with EGF (20ng/ml), EGF (20ng/ml) and galectin-3 (2μg/ml), galectin-3 (2 μg/ml) or 20 ng/ml BSA (control) in serum free media for 10 min at 37^0 C. The cells were washed with ice cold PBS, scraped and collected in a 1 ml PBS containing 1% TritonX-100 and protease inhibitors (Calbiochem). The cells were lysed on ice for 30 min followed by centrifugation at 10,000g at 4^oC for 15 minutes. The supernatants were collected and pre-cleared by adding 20 µl of the protein A/G beads and incubating at 4^0C for 30 minutes with gentle agitation. One ml lysates (protein concentration 2mg/ml) were incubated with B27.29 (1 μg/ml), anti-EGFR (DB81) (2 µg/ml) or isotype-matched IgG (rat anti-mouse IgG1 for B27.29 and Armenian hamster anti rabbit for anti-EGFR, produced against a synthetic hapten) at 4^0C with continuous agitation for 16 hours. Thirty µl of protein A/G plus agarose beads were added for 4 hr and the beads were washed five times at 4^0C with ice cold PBS. Proteins were eluted from the beads by boiling in SDS-sample buffer for 10 minutes before application to SDS-PAGE and subsequent immunoblotting.

2.19 Statistics

Data is presented as mean or percentage of control ± SEM or ±SD (stated in individual figures). For the experiments repeated in at least triplicates, a one-way ANOVA with either Dunnett or Bonferroni post-hoc test was used to establish statistical

significance where appropriate. A Fisher exact test was used to assess statistical differences in MUC1 polarization when treated with MUC1 polarizing agents (antibody 214D and galectin-3) and control Bovine serum albumin (BSA). A two-way ANOVA with Sidak post-hoc test was used to assess statistical difference in EGFR activation when tested with ligand EGF, EGF in combination with galectin-3 or control (galectin-3). For all statistical analysis $P<0.05$ was considered significant.

3 Investigation into the impact of different domains of MUC1 on cellular resistance to anoikis

3.1 Hypothesis

Different domains of MUC1 may contribute differently to MUC1-mediated resistance to anoikis.

3.2 Aims

To investigate the contributions of MUC1 extracellular and intracellular domains on MUC1-mediated resistance to anoikis.

3.3 Introduction

Anoikis is apoptosis induced in cells following inadequate or inappropriate cell-cell, cell-matrix contact (126, 435). This fundamental process ensures tissue homeostasis and prevents displaced epithelial/endothelial cells from seeding to inappropriate sites. Absence or resistance to anoikis is one of the first steps in eventual tumour metastasis. Metastasising tumour cells are able to survive independently in suspension and are able to 'seed' at secondary tumour sites leading to tumour metastatic spread (436).

Many cell surface and intracellular proteins are involved in regulating anoikis. It is generally accepted that following loss of contact with extra-cellular matrix, anoikis signalling starts from the cell surface through activation of cell surface anoikis-initiating molecules such as integrins, cadherins and death receptors. Loss of the integrin-mediated cell-basement matrix contact (131), loss of the E-cadherin-mediated cell–cell contact (437, 438) or ligation of the cell surface death receptors with their ligands (131, 439) have been shown to induce conformational changes or oligomerization of these cell surface anoikis-initiating molecules. This triggers a series of events leading to activation of either the caspase-8-mediated extrinsic apoptotic signalling pathway or the mitochondrion-mediated intrinsic apoptotic signalling pathway leading to eventual accumulation of executioner caspases, caspase 3 and 7 (135).

MUC1 is a large transmembrane mucin protein that is physiologically expressed exclusively on the apical side of normal epithelial cells and some other cell types. MUC1 consists of a large extracellular domain, a transmembrane region and a short

cytoplasmic tail. The MUC1 extracellular domain contains a variable number of tandem repeats (VNTR) that are heavily glycosylated (up to 50% of the MUC1 molecular weight) with complex O-linked mucin-type glycans (440) and flanked by a unique N-terminal domain and an SEA domain. The cytoplasmic tail of MUC1 contains 72 amino acids and harbours several phosphorylation sites and is able to interact with various growth factor receptors and intracellular signalling proteins (32, 116, 441).

MUC1 is overexpressed by up to at least 10-fold in many epithelial cancer cells (442) and this overexpression of MUC1 is closely associated with high metastatic potential and poor prognosis in many cancer types (61, 443). In epithelial cancer cells, the overexpressed MUC1 loses its apical membrane polarization and becomes expressed over the entire cell surface (431). Overexpression of MUC1 in epithelial cancer cells has been shown in our previous study to lead to increased resistance of the cells to anoikis (433). A model for this effect was proposed in which MUC1 forms a 'protective barrier' on the cell surface that prevents activation of anoikis-initiating molecules in response to loss of cell-matrix contacts (25)

This part of the study aims to determine the influence of MUC1 extracellular and intracellular domains on MUC1-associated cellular resistance to anoikis

3.4 Methods

Immunoblotting

The immunoblotting protocol has been described in detail previously. Here antibody B27.29 (1µg/ml) was used to detect MUC1 extracellular matrix, biotinylated PNA (2 µg/ml) to detect TF antigen, anti-CT2 (2 µg/ml) to detect MUC1 intracellular domain, and actin (1 µg/ml) to ensure equal loading.

Caspase-3-7 activity assay

Ninety-six well plates were washed with sterile PBS and coated with 10 mg/ml poly-HEMA. The cells were released by NECDS from culture flasks, washed with PBS and re-suspended to $5x10^4$ cells/ml with serum-free DMEM containing 0.5 mg/ml BSA and seeded at 1 x 10^4 cells/well and cultured for 24 hours at 37^0C. Anoikis was assessed by using Caspase- Glo® 3/7 Assay kit according to manufacturer's instructions.

Apoptosis Array

Proteome Profiler™ Human Apoptosis Array Kit (ARY009) was purchased from R&D systems. HBl100 breast epithelial cells transfected with either the full length MUC1 (HCA1.7+), no MUC1 (HCA1.7-) or MUC1 Δ CT (HDT), or HCT116 cells transfected with either full length MUC1 (HCT116 MUC1 F3), intracellular domain deleted MUC1 (HCT116 MUC1 Δ CT, extracellular domain deleted MUC1 (HCT116 MUC1 ΔTR) or MUC1 negative control (HCT116 MUC1 neo) were grown in serum-free media while suspended in a poly-HEMA coated 6 well plate for 24 hours at 37^0C. The suspended cells were collected, lysed and protein concentration was measured using BCA

protein assay kit. Four hundred µg/ml of lysate from each cell line was added to an apoptosis array and apoptosis proteins were analysed and quantified as per manufacturer's instructions

Adhesion assay

HUVEC cells were cultured in 96-well plates at 1 X 10^5 cells/ml till a monolayer formed (~48 hours). Subconfluent ACA19+, ACA19-, ATD, HCA1.7+, HCA1.7- and HTD cells were labelled with Calcein-Am at a concentration of 5 µl Calcein-AM in 1 ml containing $1x10^6$ cells for 30 min at 37^0 C. The cells were then washed twice with PBS and mixed with either BSA (2 µg/ml) , recombinant galectin-3 (2 µl/ml) or antibody 214-D (50 µg/ml) in serum-free media before being added to the 96-well plate containing HUVEC monolayer. The plate was incubated at 37^0C with 5% CO_2 for 1 hour before being washed twice with PBS. A final fluorescence reading was taken using a TECAN plate reader.

3.5 Results

3.5.1 Validation of MUC1 status and cell morphology in MUC1-transfected cells

The cells used in this study were the A375 Melanoma cells, HBL100 human breast epithelial cells and HCT116 colon cancer cells that were stably transfected with either the full length, control MUC1 neo, MUC1 ΔCT or MUC1 ΔTR. Prior to any further investigation to assess the effect of these transfection, the cells were validated to confirm the correct MUC1 status.

Expression of full length MUC1 can be validated by detection of both MUC 1 extracellular domain with B27.29 anti-MUC1 antibody and intracellular domain by anti-MUC1 CT2 antibody in all HCT116 MUC1 full (figure 3.1 A), HCA 1.7+ (figure 3.1 B) and ACA19+ cells (figure 3.1C). No MUC1 expression was detected in Control MUC1 negative HCT116 MUC1 neo HCA1.7- and ACA19- cells by either B27.29 or CT2 antibodies in all three cell types. As expected, ΔCT cells could only be detected by B27.29 and not CT2 (HCT116 MUC1 ΔCT , HTD and ATD) whereas ΔTR cells could only be detected by CT2 and not B27.29 antibodies (HCT116 MUC1 ΔTR).

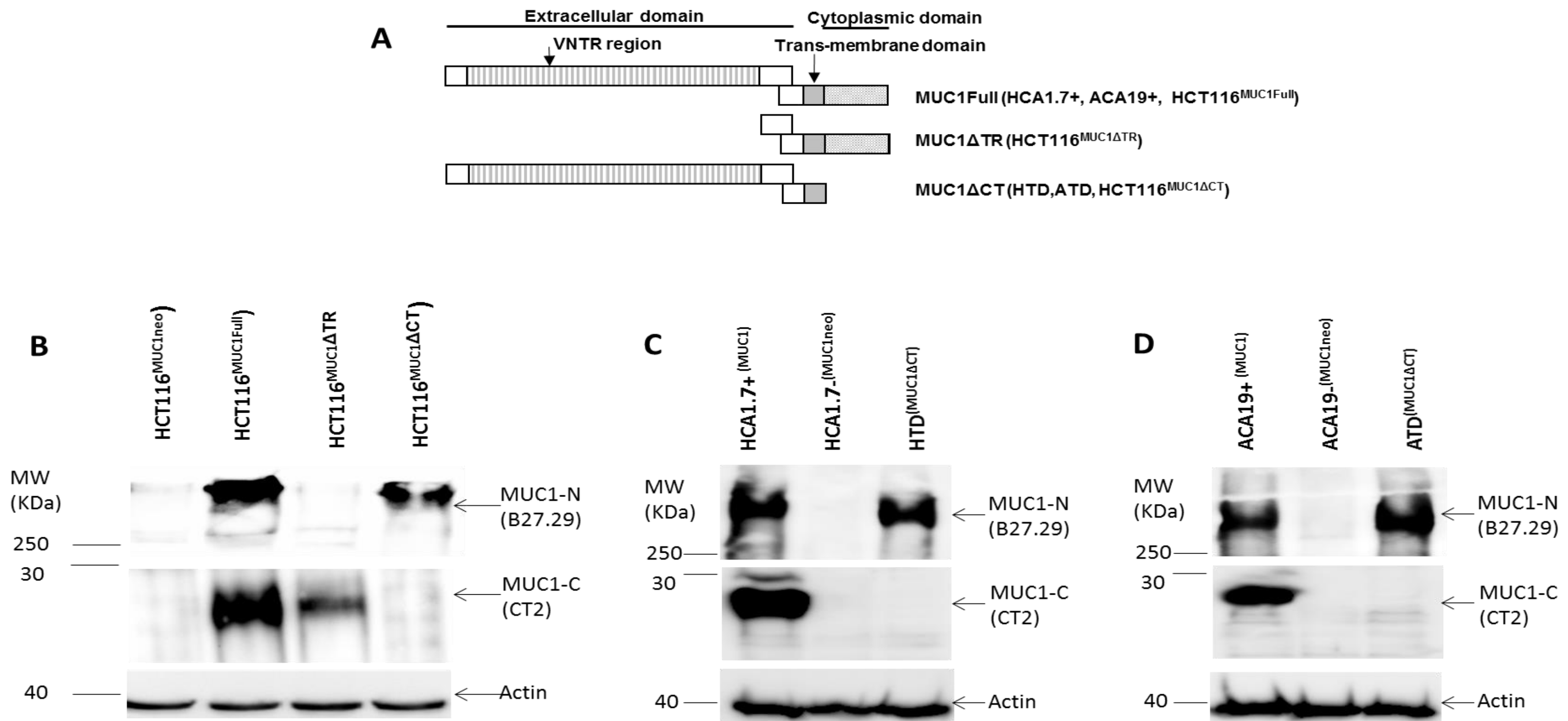


Figure 3.1: Immunoblot analysis of MUC1 transfected cells. HCT116 (panel B), HBL100 (panel C) and A375 (panel D) cells were stably transfected with either full length, ΔTR, ΔCT or control variant. A schematic diagram of transfection is shown (panel A). Cells transfected with full length MUC1 could be detected with both antibodies B27.29 and CT2, which targets the extracellular and the intracellular domains of MUC1 respectively. ΔCT cells could be detected by only B27.29 and not CT2, whereas ΔTR cells could be detected only by antibody CT2 and not B27.29. MUC1 neo cells were negative for both anti-MUC1 antibodies.

The morphology of these cells was also checked (figure 3.2). Although morphologically very similar, cells transfected with full length or only the extracellular domain of MUC1 tended to be more spindle shaped, whereas those transfected with control vector tended to be rounder. It was also observed that cells transfected with full length or either the intracellular or extracellular domain of MUC1 had slower rates of growth compared to control group, supporting the idea that MUC1 expression may have inhibit cell proliferation (64, 67).

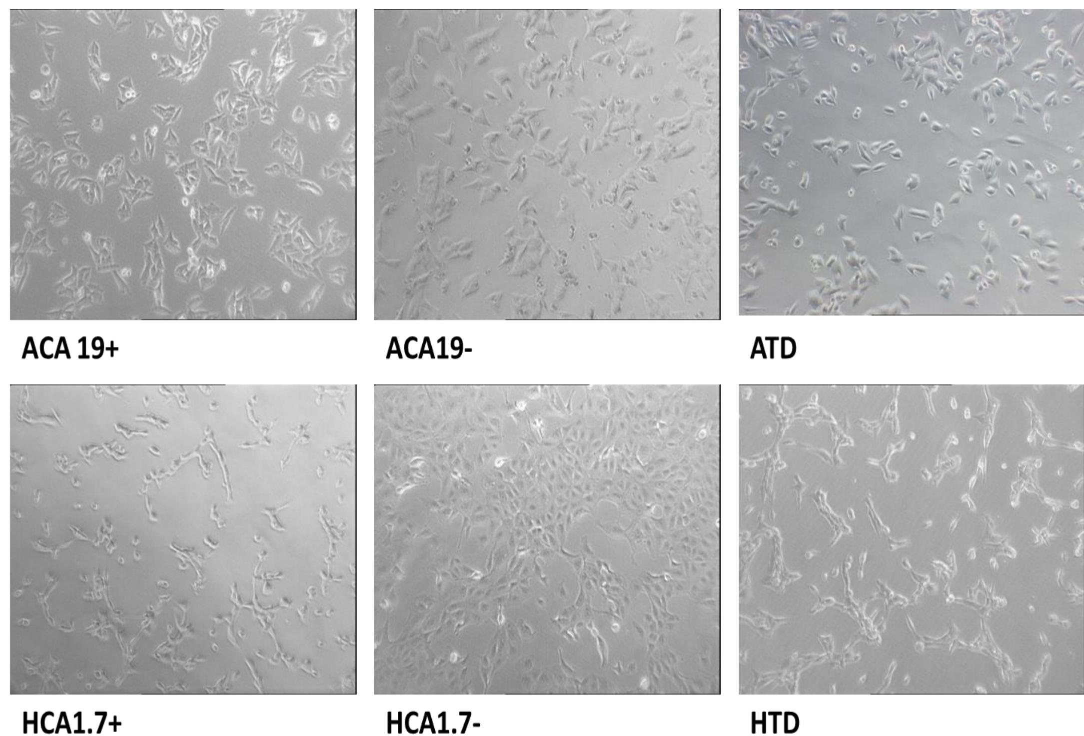


Figure 3.2: Morphology of MUC1-transfected melanoma and Breast epithelial cells Morphology of melanoma (A375) and human breast epithelial (HBL100) cells transfected with either full length MUC1 (ACA19+ and HCA1.7+ respectively), with extracellular domain of MUC1 only (ATD2 and HTD respectively) or control MUC1 neo group (ACA19- and HCA 1.7- respectively) after 2-day culture at 37^0C. Representative images of 10 random field observations from 2 experiments (10 x magnification).

Further validation of MUC1 expression was also done by flow cytometry. Figure 3.3 is a flow cytometry analysis on MUC1 transfected HCT116 cells when tested with antibody B27.29 and appropriate FITC-conjugated secondary antibodies. As

expected, minimal signal is noted for MUC1 neo and MUC1 ΔTR cells with increased signal for MUC1 full and ΔCT variants, indicating the presence of MUC1 extracellular domain in these cells in the MUC1 full and ΔCT variants. MUC1-positive melanoma cell ACA19+, MUC1-negative melanoma cells ACA19-, MUC1 intracellular domain-deleted melanoma cells ADT, MUC1-positive breast epithelial cell HCA1.7+, MUC1-negative breast epithelial cells HCA1.7- and MUC1 intracellular domain-deleted melanoma cells HDT were also tested for TF expression on MUC1 extracellular domain by the TF-binding Peanut agglutinin (PNA). No PNA binding was noticed in MUC1 neo cells but MUC1 full and ΔCT variant all showed strong PNA binding suggesting the presence of TF region on MUC1 extracellular domain. (see figure 3.4)

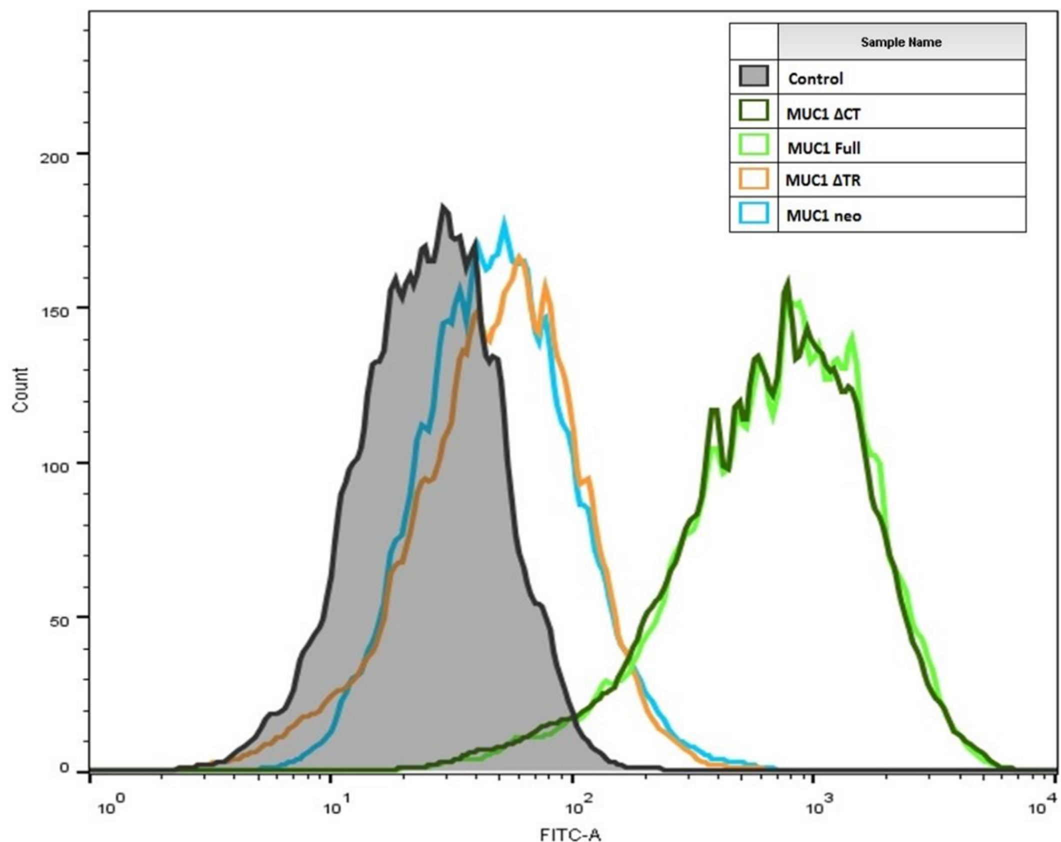


Figure 3.3: Analysis of MUC1 expression by Flow cytometry of the MUC1 transfected HCT116 cells. HCT 116 cells transfected with either the full length (MUC1 Full), intracellular (MUC1 Δ TR), extracellular (MUC1 Δ CT) or control group (MUC1 Neo) were probed with primary antibody B27.29 (to detect MUC1 extracellular

domain) and FITC conjugated secondary antibody followed by analysis with flow cytometry.

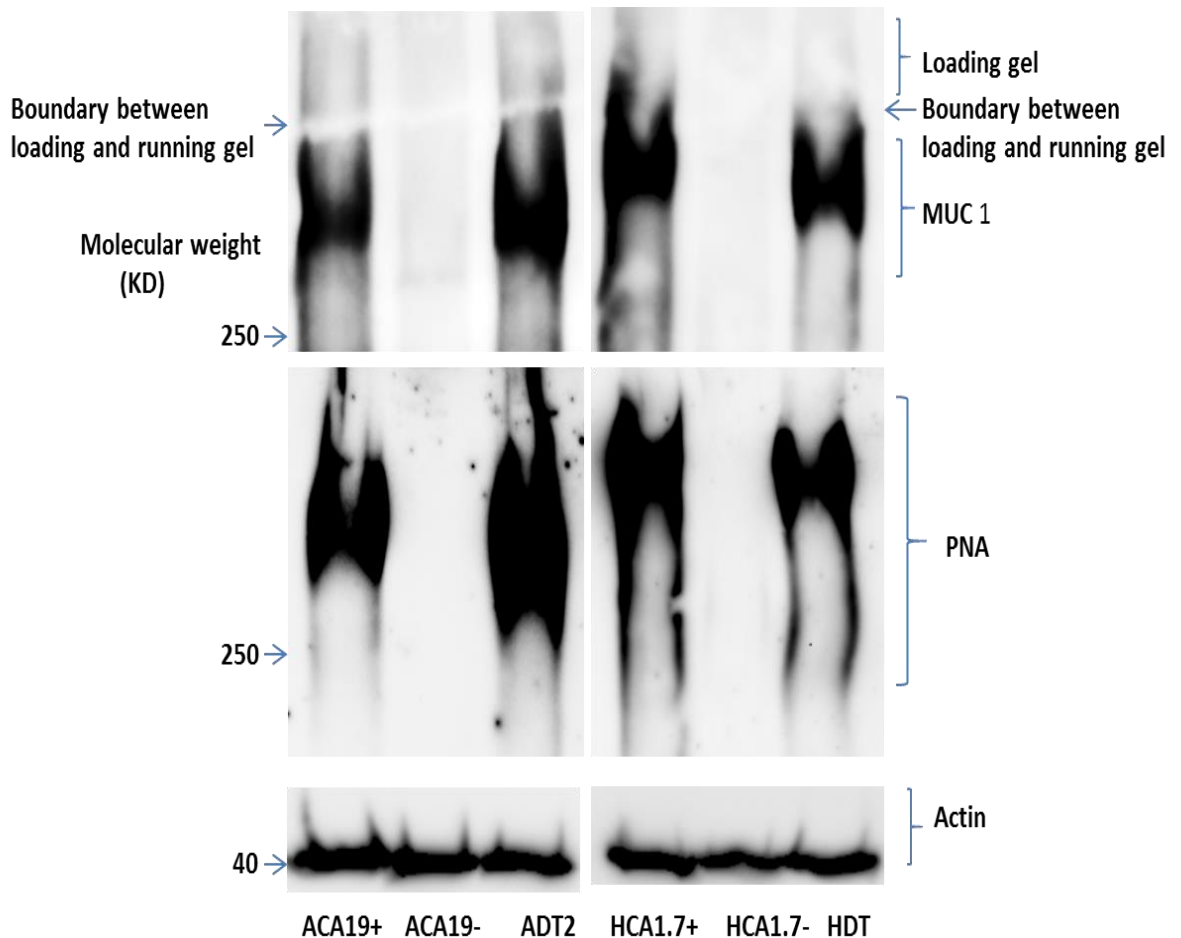


Figure 3.4: MUC1 full and MUC1 ΔCT cells show similar TF expression in Breast epithelial and melanoma cells. Stable Transfected Melanoma cells ACA19+, ACA19-, ADT and stable transfected breast epithelial cells HCA1.7+, HCA1.7-, HDT were immunoblotted using antibodies B27.29 and PNA. PNA shows binding to both MUC1 full and MUC1 ΔCT cells.

3.5.2 MUC1 overexpression leads to resistance to anoikis

Following 24 hours in suspension in poly-HEMA coated plates to mimic anoikis conditions; HCA1.7+ and ACA19+ cells transfected with full length MUC1 showed a marked reduction in caspase 3/7 activity compared to their control variants (Fig 3.5A and Fig 3.5B). These data further confirm our previous report that overexpression of MUC1 leads to resistance to anoikis.(433)

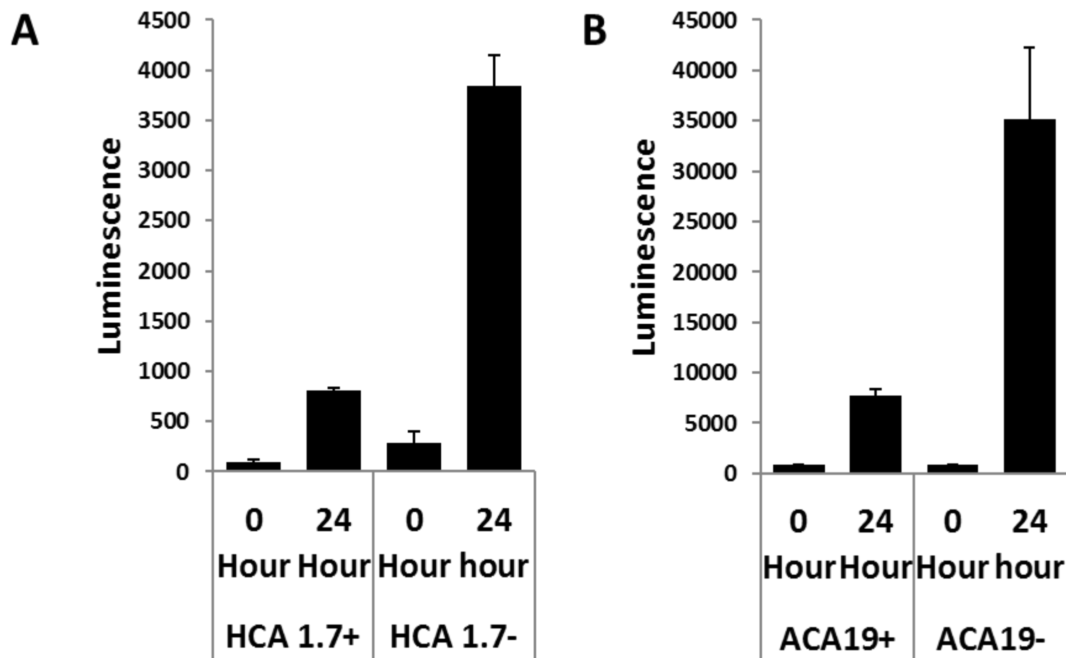


Figure 3.5: MUC1 transfection in human breast epithelial HBL-100 and Melanoma A375 cells inhibits anoikis. Assessment of caspase-3/7 activity of HCA1.7+/- (Figure3.5 A) and ACA19+/- (Figure 3.5 B) cells in response to 24-hour culture in suspension condition shows that MUC1 transfected cells are more resistant to anoikis compared to the MUC1 negative controls. The data are presented as mean +/- SD of triplicate determinations from 2 experiments. N=2, n=3

3.5.3 Both intracellular and extracellular domains of MUC1 contribute to MUC1-mediated anoikis resistance

Having shown that expression of MUC1 leads to resistance to anoikis, we then compared the effect on anoikis of MUC1-mutant cells which express different domains of MUC1. 3 different cell lines transfected with either MUC1 full (ACA19+, HCA1.7+ and HCT116 MUC1 Full), MUC1 ΔCT (ADT, HTD and HCT116 MUC1 ΔCT) and control MUC1 neo cells (ACA19-, HCA1.7- and HCT116 MUC1-neo) were used. For HCT116 cells we also had MUC1 ΔTR variant. Using poly-HEMA coated plates to simulate anoikis conditions, we compared Caspase 3/7 activity in these cells in

response to 24-hour cell suspension culture. Cells expressing the full length MUC1 (HCA1.7+, ACA19+ and HCT116 MUC1 full) were shown more resistant to anoikis compared to MUC1-negative transfects (HCA1.7-, ACA19- and HCT116 neo). This is in accordance with the earlier results (Figure 3.5 and (433)). MUC 1 transfectants without the MUC1 cytoplasmic domain (ATD, HTD and HCT116 Δ CT) showed higher levels of anoikis (~50%) compared to full length MUC1 transfectants (HCA 1.7+ and ACA19+) but significantly less ($p<0.001$) than the MUC1 negative transfect (HCA 1.7- and ACA19- HCT116 MUC1 Neo) (Figure 3.6). When comparing HCT116 Δ TR with HCT116 MUC1 Full and HCT116 MUC1neo, a similar pattern was observed, whereby HCT116 Δ TR had a higher level of anoikis compared to HCT116 MUC1 full but significantly less than HCT116 MUC1neo. Together, these results suggest that both the intracellular and the extracellular domains of MUC1 contribute to the effect of MUC1 on anoikis.

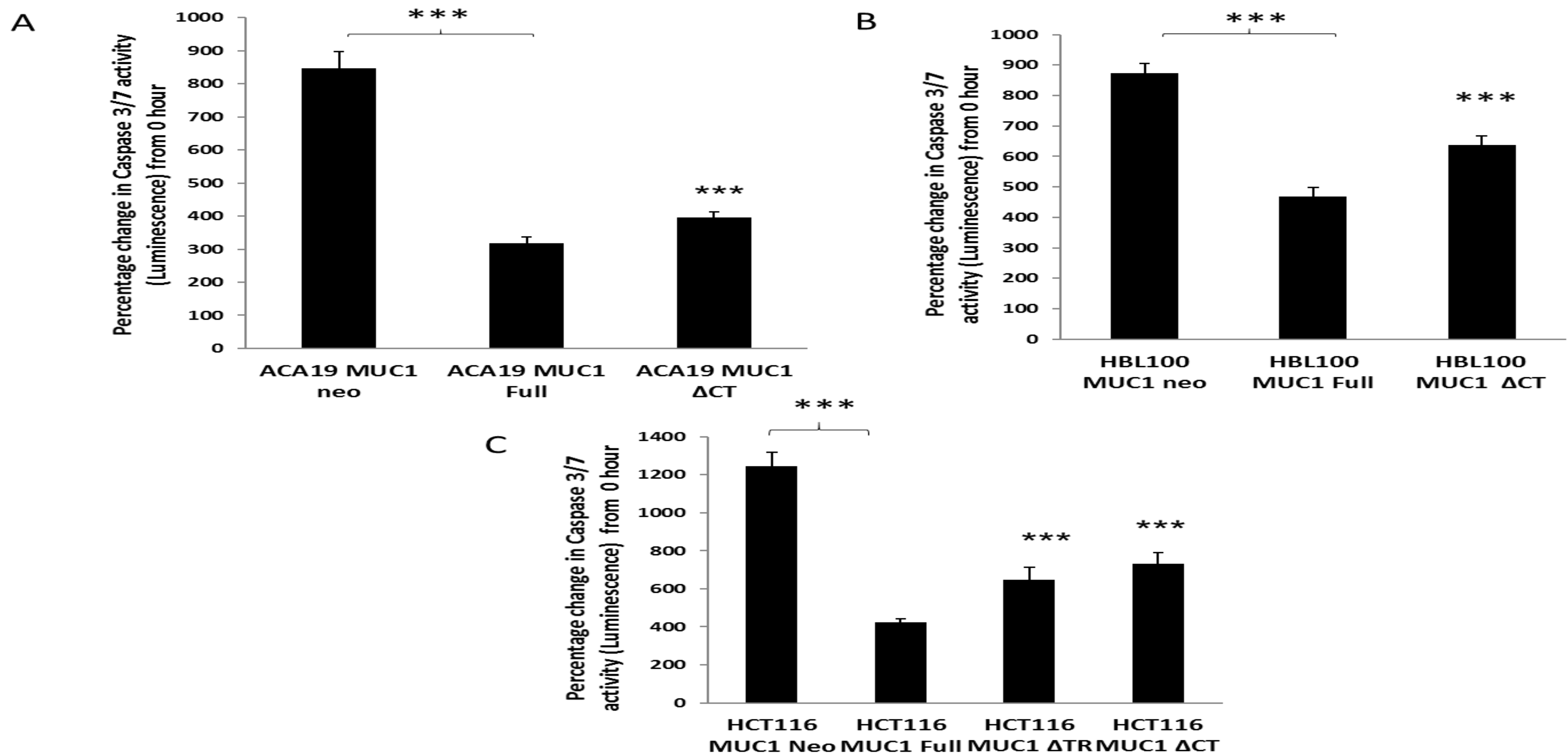


Figure 3.6: Depletion of either MUC1 intracellular (ΔCT) or extracellular (ΔTR) domain leads to a reduction in MUC1 mediated resistance to anoikis. ACA19 MUC1 neo/full/ ΔCT (figure 3.6 A) HBL100 MUC1 neo/full/ ΔCT (figure 3.6 B) and HCT116 MUC1 Neo/Full/ ΔCT and Δ TR (figure 3.6 C) were assed for anoikis resistance. The absence of either of MUC1 extracellular or cytoplasmic domain reduces full MUC1-mediated cell resistance to anoikis when cellular caspase-3/7 activity was assessed. The data are presented as mean +/- SE of triplicate determinations of three experiments. N=3, n=3, one-way ANOVA with Bonferroni, ***p<0.001.

3.5.4 The length of the MUC1 extracellular domain influences the effect of MUC1 on resistance to anoikis

To test whether the length of the MUC1 extracellular domain influences MUC1-mediated resistance to anoikis, we tested anoikis resistance in fibroblasts transfected with MUC1 shRNA consisting of either 0 (parental), 8 15 or 36 variable number of tandem repeat. (Figure 3.7 A) By changing the length of VNTR, the size of extracellular domain of MUC1 can be controlled, with higher numbers of VNTR leading to a longer extracellular domain and increased MUC1 size.

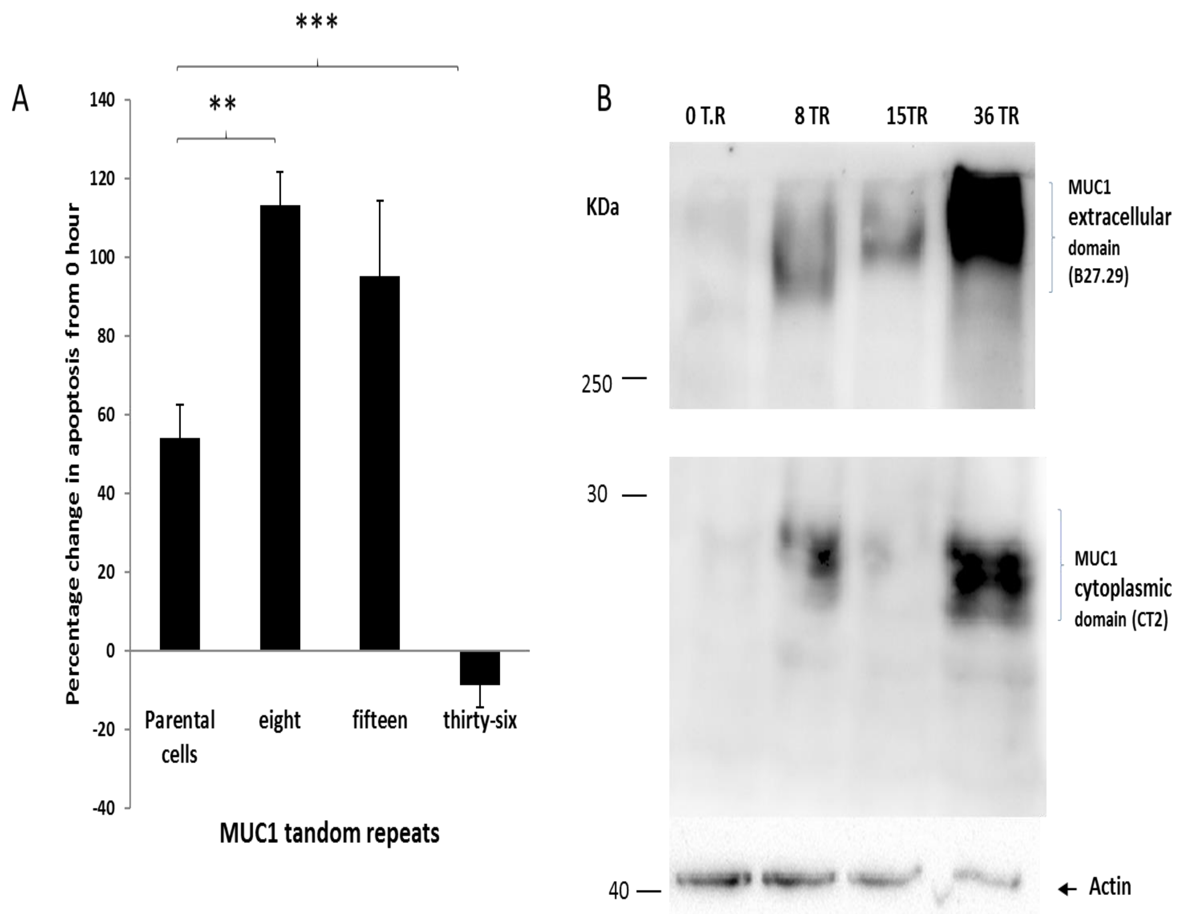


Figure 3.7: Increase in variable number of tandem repeats (VNTR) reduces anoikis in fibroblast cells. A) Human fibroblast cells stably transfected with either 0 (parental), 8, 15 or 36 tandem repeats in MUC1 extracellular domains were assessed for anoikis. b) Immunoblot with antibodies against the MUC1 extracellular domain (b27.29), intracellular domain (CT2) and actin. N=3, n=3, one-way ANOVA with Bonferroni * *p<0.01, ***p<0.001.

Although an increase was noted between parental and 8 or 15 tandem repeats, a marked reduction in anoikis was noted for the cells transfected with 36 tandem repeats in comparison to MUC1 without tandem repeats (0) cells. The effect of the 36-tandem repeat in contrast to 8 and 15 tandem repeats suggests that a large number of VNTR are needed to ensure anoikis resistance in fibroblasts. Further immunoblot analysis of these fibroblasts shows that cells transfected with 36 VNTR MUC1 also had a higher expression of MUC1 compared to 8 and 15 VNTR group (figure 3.7 B), suggesting that the effect on anoikis resistance in 36 VNTR group could be related to the combined effect of increased length as well as increased expression of MUC1 extracellular domain.

3.5.5 Expressions of several apoptotic proteins are altered in MUC1-expressing cells in response to anoikis

Having established that both extracellular and intracellular domains of MUC1 are involved in MUC1-mediated resistance to anoikis, we compared the effects of the presence of different MUC1 domains on expression of key apoptosis proteins in response to anoikis culture using the Proteome Profiler™ Human Apoptosis Array Kit (Ary009) R&D systems) which tests for 35 key apoptosis proteins.

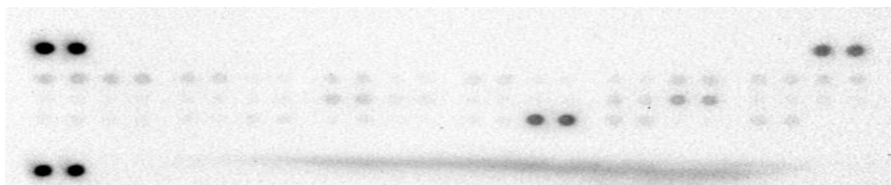
We first tested HBL100 cells transfected with full length MUC1 (HCA1.7+) and compared them with HBL100 neo cells (HCA1.7-). It was noted that expression of several key apoptosis proteins was increased in HCA1.7+ cells compared to HCA 1.7- with several hundred-fold increase noted in pro caspase 3, Cytochrome c and p53

(figure 3.8). Interestingly, a similar pattern as HCA1.7+ was observed in HDT cells. All the key apoptosis proteins noted to have a higher expression in HCA1.7+ also had a higher expression in HTD cells, and in some cases this level of increased expression was almost similar. For example, pro caspase 3 levels were noted to be almost 50-fold and 46-fold higher in HCA 1.7+ and HTD cells, respectively, compared to HCA1.7-. Similarly, P53(s392) level were 148 and 154 folds higher in HCA 1.7+ and HTD cells, respectively, compared to HCA1.7-. However, a few key apoptosis proteins did show a degree of variation. For example, Bcl-X expression level was increased by 503% and 332% in HCA1.7+ and HTD cells, respectively, when compared to HCA1.7- . Similarly, P21 level was increased by 8.7-fold and 3.2-fold in HCA1.7+ and HTD cells, respectively, when compared to HCA1.7- .

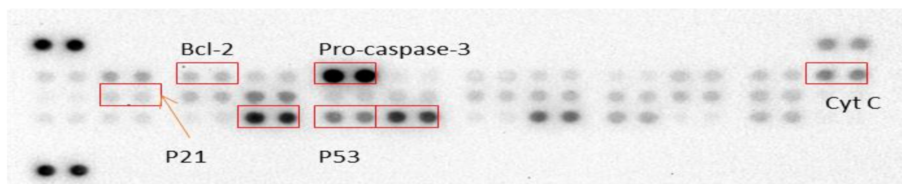
Apoptosis array analysis was also performed in MUC1-transfected HCT116 cells (figure 3.9). In these cells the pattern of expression of key apoptosis proteins was surprisingly different from that seen in MUC1-transfected HBL100 cells. The several-fold increase in key apoptosis proteins as noted in HCA1.7+ and HTD cells when compared with HCA1.7- cells was not noted in HCT116 mutant cells. Instead, the changes in expression level of key apoptosis proteins between these cells were rather modest. The table in figure 3.9 shows several key apoptosis proteins that were noted to be either increased or decreased by 50% in MUC1F3, MUC1 ΔCT or MUC1 ΔTR cells when compared to HCT116 MUC1 Neo. For example, cleaved caspase- 3 levels were decreased by 83.4% in HCT116 MUC1 full (F3) cells, decreased by 67% in HCT116 MUC1 ΔTR but increased by 15.5% in HCT116 MUC1 ΔCT, when compared to HCT116

MUC1 neo. Similarly, P21 level were decreased by 46.5% in F3, increased by 131.8% in ΔTR and decreased by 60.2% in ΔCT cells compared to HCT116 MUC1 neo cells. Thus, expression of MUC1 and MUC1 mutants has a complex effect on expression of several key apoptotic proteins.

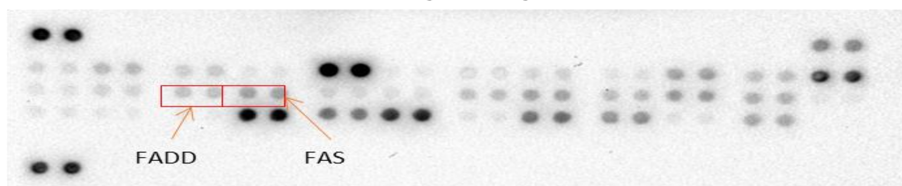
A HBL-100 MUC1 neo (HCA1.7-)



HBL-100 MUC1 full (HCA1.7+)



HBL-100 MUC1 ΔCT (HDT)

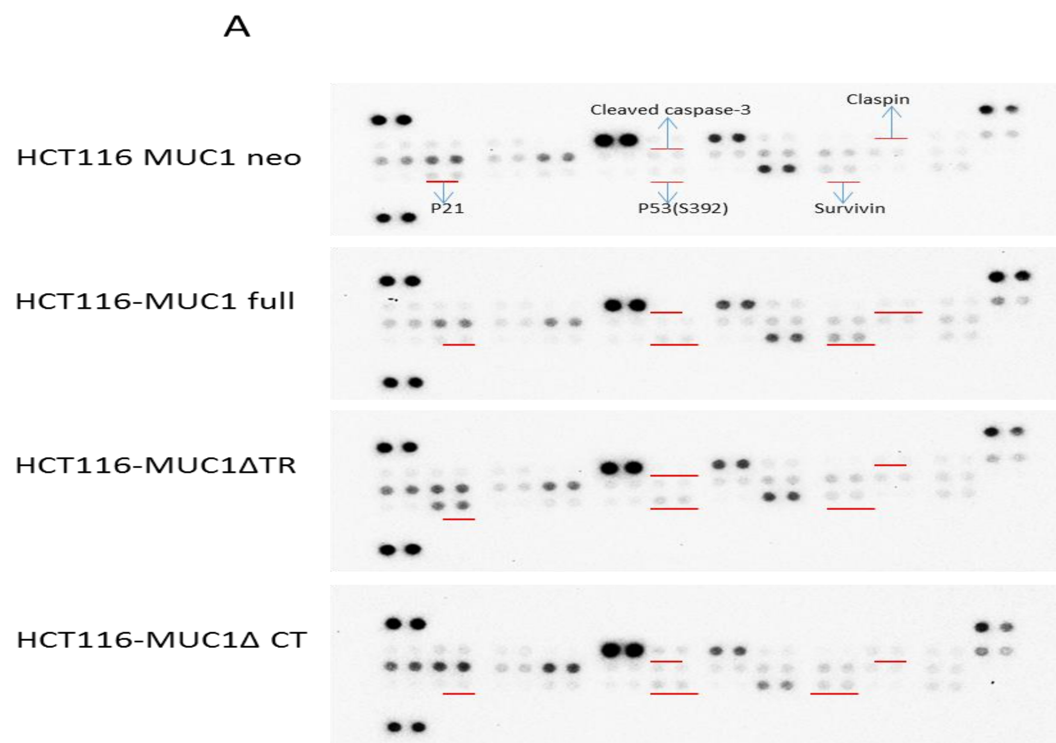


B

Percentage change compared to HCA1.7-

Apoptosis proteins	HCA 1.7+	HDT
Bax	+387 %	+283 %
Bcl-2	+503 %	+332 %
Bcl-x	+7376 %	+ 2797 %
Pro caspase 3	+49 65 %	+4666 %
claspsin	+ 256%	+542%
clusterin	+274 %	+381%
Cytochrome c	+ 995 %	+ 1653 %
FADD	+1507 %	+ 1224%
FAS	+14624 %	+ 9859 %
P21	+ 876 %	+ 321%
P27	+1006 %	+ 446%
P53 (s15)	+6329 %	+ 7146%
P53(s46)	+ 9248%	+10022 %
P53(s392)	+ 14853 %	+ 15444 %
Survivin	+582 %	+831 %
TNF RI/ TNFRSF	+620 %	+ 841 %
XIAP	+411 %	+531 %

Figure 3.8: Apoptosis array on MUC1 transfected HBL100 cells. HBL 100 cells transfected with either no, full length or ΔCT domains of MUC1 were cultured in suspension condition for 24 hours followed by apoptosis array. The apoptosis array and apoptosis proteins were analysed and quantified as per manufacturer's instructions. The table in figure B shows some of the key apoptosis proteins that were noted to be either increased or decreased by 50% or more in HCA1.7+ or HDT cells when compared to HCA1.7-. Representative images from two arrays, N=2.



B Percentage change compared to HCT116 neo

Apoptosis proteins	F3	ΔTR	ΔCT
Cleaved caspase-3	-83.4%	-67.0%	+15.5%
P21	-46.5%	+131.8%	-60.2%
P53 (s392)	+13.3%	+102.0%	+40.0%
Survivin	+49.1%	-6.4%	-14.1%
Bax	-46.0%	-31.2%	+2.0%
cLAP1	-10.4%	-10.1%	-54.2%
Claspin	+110.0%	+78.5%	+128.4%
HO-1/HMOX1/HSP 32	-56.3%	-30.4%	-30.1%
HSP27	-36.2%	-28.4%	-52.0%
HTRA2/0mi	+74.1%	+60.7%	+2.0 %
Livin	+11.1%	-67.6%	-27.3%
P53 (s46)	-28.5%	+50.8%	+2.7%

Figure 3.9: Apoptosis array on MUC1 transfected HCT116 cells. HCT116 cells transfected with either no, full length, ΔCT or ΔTR domains of MUC1 were cultured in suspension condition for 24 hours followed by apoptosis array. The apoptosis array and apoptosis proteins were analysed and quantified as per manufacturer's instructions. The table on the right-hand side shows some of the key apoptosis proteins that were noted to be either increased or decreased by more than 50% in MUC1 full, MUC1 ΔCT or MUC1 ΔTR cells when compared to HCT116 MUC1 Neo. Representative images from two arrays, N=2.

3.5.6 Effect of MUC1 polarization on MUC1 cell surface localization and on cancer cell adhesion to endothelial cells.

The large and abnormally glycosylated MUC1 proteins surround the entire cell surface and acts as a protective barrier against the underlying cell surface molecules. Our previous work has shown that the expression of MUC1 reduces the accessibility to several key anoikis initiating molecules, including integrins, cadherin and FAS (433). Polarization of MUC1, reduces the 'barrier' nature of MUC1 and exposes the underlying anoikis initiating and adhesion molecules.

Galectin-3 is a multi-functional galactoside-binding lectin that is physiologically expressed by many types of human cells. It is found intracellularly, in the circulation and on the cell surface. Intracellular galectin-3 is an apoptosis inhibitor (270) and mRNA splicing promoter (444) whilst cell surface-associated extracellular galectin-3 acts as an adhesion molecule in cell-cell interactions (445, 446) and promotes cancer progression and metastasis (306, 447). The concentration of free circulating galectin-3 is markedly increased in the sera of patients with breast, colorectal, lung (448), head and neck cancers (319) and melanoma.(449) Patients with metastatic disease are seen to have higher concentrations of circulating galectin-3 than those with localized tumours. Recently, we have shown that the transmembrane mucin protein MUC1 is an endogenous ligand of galectin-3 in human colon cancer cells and that the interaction between MUC1 and galectin-3 occurs via binding of galectin-3 to the oncofetal Thomsen-Friedenreich carbohydrate (Galβ1,3GalNAcα-, T or TF) antigen on MUC1.(79) Interaction between cell surface MUC1 and galectin-3 at concentrations similar to those found in the sera of cancer patients increases cancer cell heterotypic

adhesion to endothelium as a result of MUC1 cell surface polarization which leads to exposure of heterotypic cell-cell adhesion molecules that are otherwise concealed by elongated structure of MUC1.(79)

214D is an anti MUC1 antibody that binds to the VNTR region of MUC1 extracellular domain. Given the role of galectin-3 in tumour progression and metastasis, we wanted to assess whether 214D anti-MUC1 antibody, which is known to induce MUC1 cell surface polarization, has a similar effect as galectin-3; as galectin-3 induced MUC1 polarization is associated with cell adhesion.

When binding of the HCT116 MUC1 transfected cells to endothelial cells was tested, the MUC1 neo cells bound to endothelial cells most strongly, when compared to HCT116 MUC1 full and HCT116 ΔCT cells (figure 3.10). This adhesion of MUC1 neo cells to the endothelium was not affected by addition of either galectin-3 or 214D anti-MUC1 antibody. HCT116 MUC1 full and HCT116 ΔCT cells had a lower adhesion to endothelial cells compared to MUC1 neo variant, but the level of adhesion was significantly increased in the presence of either galectin-3 or 214D, suggesting that MUC1 polarisation by galectin-3 or 214D anti-MUC1 antibody increases heterotypic adhesion of MUC1full and MUC1 ΔCT cells (figure 3.10, 3.12). Immunofluorescence analysis of these cells (figure 3.11) showed that presence of galectin-3, but not the truncated galectin-3C, or antibody 214D induced MUC1 cell surface polarization of both MUC1 full and MUC1 ΔCT cells and this effect is independent of the expression/presence of MUC1 intracellular domain.

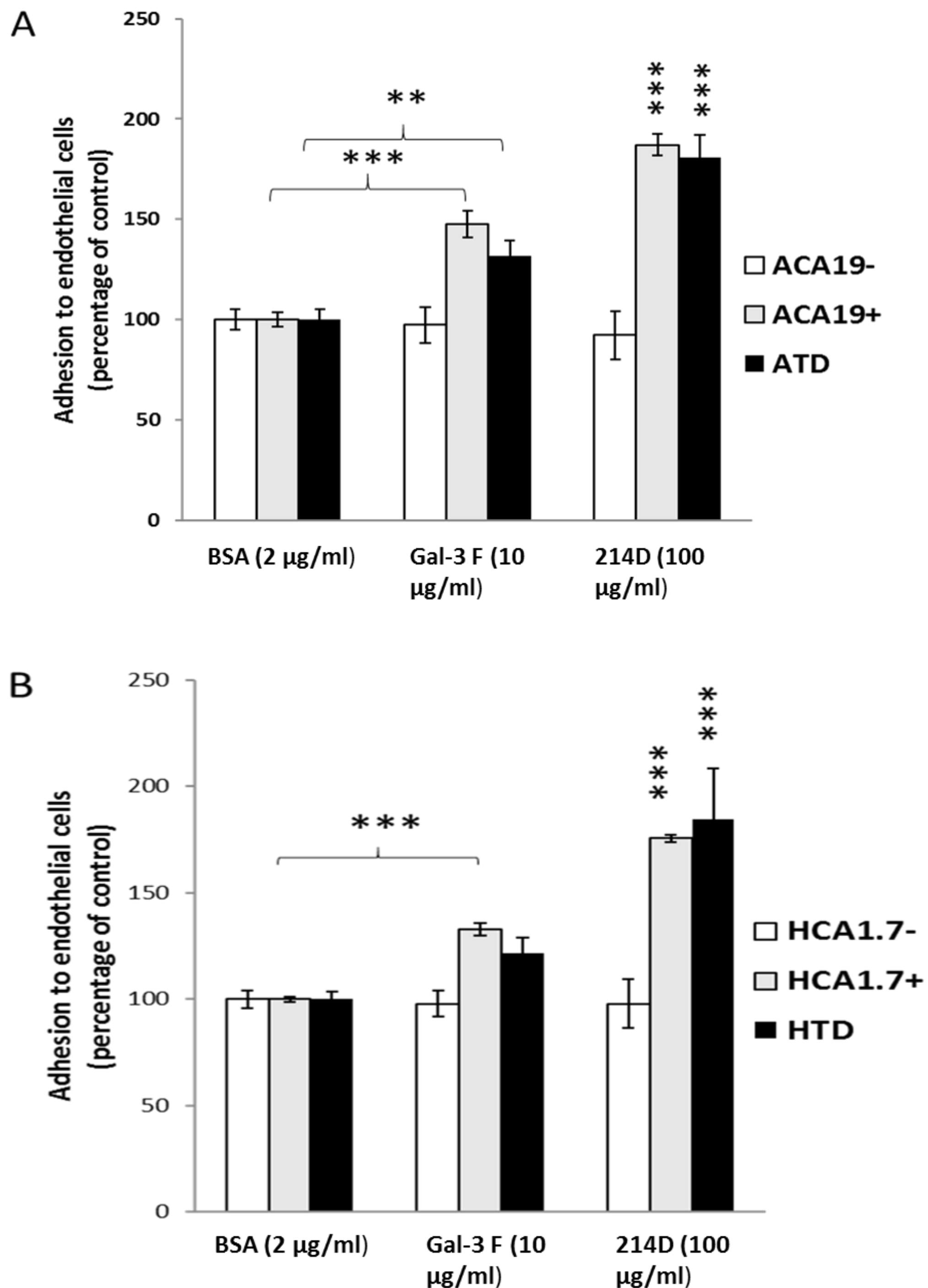


Figure 3.10: exogenous addition of galectin-3 or 214D anti-MUC1 antibody increased adhesion to endothelial cells of both MUC1 full and MUC1 ΔCT cells. ACA19+/ACA19-/ADT (figure 11 A) and HCA1.7+/HCA1.7-/HDT were treated with either control (BSA 2 μg/ml), galectin-3 (2 μg/ml) or 214d (50 μg/ml) before being added to a HUVEC monolayer. Results are shown as percentage to control (BSA binding). Addition of galectin-3 or antibody 214D substantially increased cell adhesion of HCA1.7+, HTD, ACA19+ and ATD cells to the endothelial monolayer. N=3, n=3, ANOVA (Bonferroni), ** = p<0.01, ***= p<0.001.

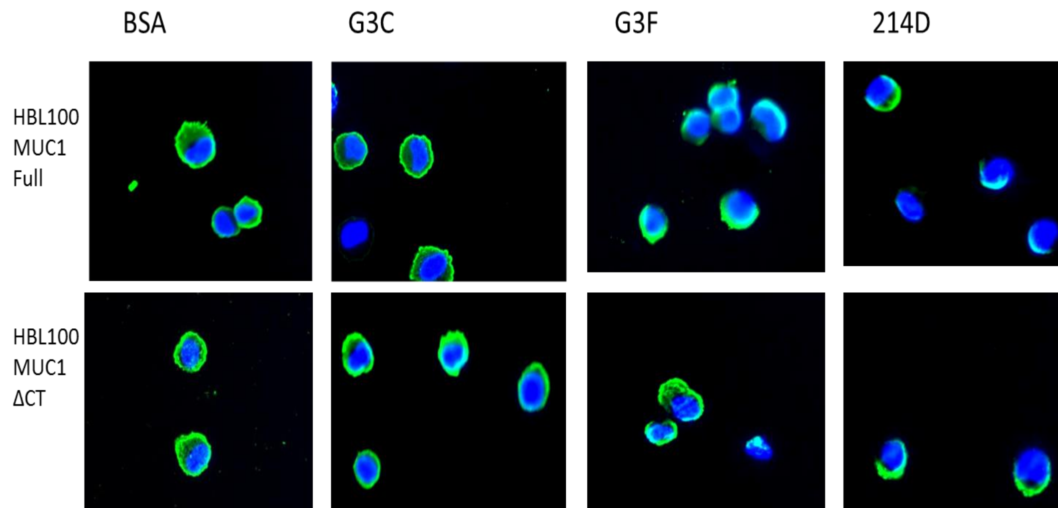


Figure 3.11: Addition of Galectin-3 or antibody 214D induces MUC1 cell surface polarization of MUC1full and MUC1 ΔCT cells. HCA1.7+ and HDT cells were mixed with either control (BSA 2 μg/ml), galectin-3 (2 μg/ml), truncated form of galectin-3 (galectin-3C 2 μg/ml) or 214D (50 μg/ml) before being immunofluorescent labelled by antibody B27.9 and FITC conjugated secondary antibody. Galectin-3, but not galectin-3C, and antibody 214D induces MUC1 cell surface polarisation (as illustrated by discontinuous MUC1 localization on the cell surface) of HCA1.7+ and HTD cells. Representative images of 20 random field observations from 3 experiments (40 x magnification).

Figure 3.12 summarises MUC1 cell surface polarization of the cells in response to galectin-3 or 214D treatment. Following addition of galectin-3, 54% of HCA1.7+ cells and 65.2% of HTD cells showed MUC1 polarisation. Following treatment with anti MUC1 antibody 214D 82% of HCA1.7+ cells and 87% of HTD cells showed MUC1 polarisation.

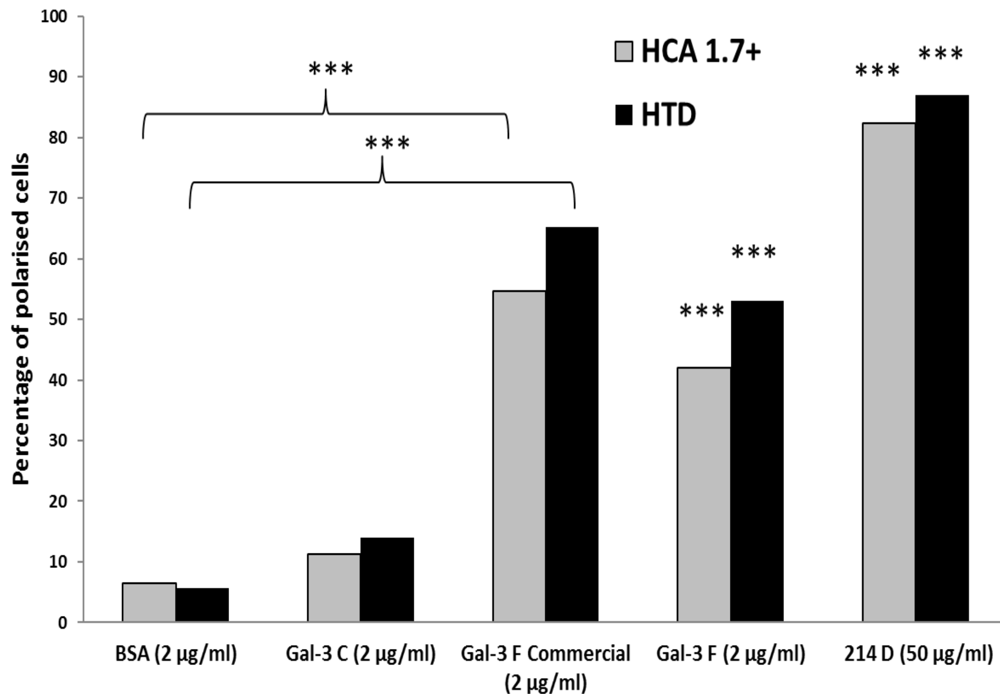


Figure 3.12: Galectin-3 and antibody 214D induces MUC1 cell surface polarization in HCA1.7+ and HTD cells. Following treatment of HCA1.7+ or HTD cells with either control (BSA 2 μg/ml), 2 μg/ml of truncated form of galectin-3 (Gal-3C), 2 μg/ml of commercial galectin-3 (Gal-3F commercial) from R&D systems (1154-GA/CF), 2 μg/ml of galectin-3 produced in our lab (Gal-3 F) or antibody 214D (10 μg/ml) 200 cells in each group shown in the immunofluorescent experiment (figure 3.11) were randomly selected and were quantified for MUC1 cell surface polarization as determined by loss of continuous MUC1 ring on cell surface. Galectin-3 and 214D treated cells showed significant increase in MUC1 polarization when compared to control BSA group. This effect was not seen when cells were treated with Gal-3 C. Fisher exact test, ***= P-value <0.001 vs control (BSA) for the same cells.

3.6 Summary of results

1. Expression of MUC1 by transfection in Human breast epithelial (HBL100), Melanoma (A375) and colon cancer (HCT116) cells is associated with resistance of the cells to anoikis.
2. Both the MUC1 intracellular and extracellular domains are shown to contribute to MUC1 mediated cell resistance to anoikis
3. MUC1 transfected cells showed alteration of several apoptosis-related proteins in response to anoikis culture in comparison to control cells.
4. Exogenous addition of either galectin-3 or 214D anti-MUC1 antibody induces MUC1 cell surface polarization and increases cell adhesion to endothelial cells.

3.7 Discussion

This part of the study shows that expression of MUC1 by transfection in three different types of epithelial cancer cells is associated with increased resistance of the cells to anoikis. This further confirms our earlier work which identified a role of MUC1 expression in anoikis (433). It was found in this study that both the MUC1 extracellular and intracellular domains contribute to MUC1-mediated cell resistance to anoikis, to some degree, independently cell anoikis was reduced in intra- or extra-cellular MUC1 domain depleted cells compared to MUC1 full cells but still significantly lower than the MUC1 neo cells.

The role of MUC1 in tumour progression is well documented (23, 37, 450, 451). It has been shown that the MUC1 intracellular domain, with a potential 22 phosphorylation sites, has an ability to facilitate the phosphorylation of key downward signalling proteins, such as ERK,(452) AKT(453) and PI3K(81). A study published by Kumar *et al* (454) shows that MUC1 extracellular domains are found in nuclear speckles and associate with spliceosomes. It was found in this study, that depletion of the MUC1 extracellular domain abolishes ~61% of the MUC1-mediated cell resistance to anoikis (Figure 3.6), suggesting a predominant role of the MUC1 extracellular domain in MUC1-mediated cell resistance to anoikis. It was also found that MUC1 transfection without the extracellular domain still causes anoikis inhibition of the cells, albeit much less efficiently. This indicates that the MUC1 cytoplasmic domain also makes significant contribution to the MUC1-mediated cell resistance to anoikis through different mechanisms.

The role of the MUC1 extracellular domain in MUC1-mediated anoikis became more evident when we assessed anoikis in fibroblasts stably transfected with different numbers of MUC1 VNTR. (Figure 3.7) A significant increase in anoikis resistance was noted between transfects with lower numbers of VNTR than with cells with 36 VNTR. It is found that the cells transfected with 8 and 15 MUC1 VNTR did not add any ability to the cells to resist anoikis (albeit even slightly higher anoikis) in comparison to MUC1 neo cells. However, transfection of the cells with higher (n=36) MUC1 VNTR showed significantly increased resistance to anoikis. It is noted the expression of MUC1 in the 8 and 15 VNTR transfected cells is much lower than the 36 VNTR transfected cells. It is possible therefore that the lack of effect of the 8 and 15 VNTR transfected cells on anoikis as compared to 36 VNTR transfected cells is due to insufficient expression of MUC1 by these cells. It is also possible that 36 MUC1 VNTR reflects a threshold in fibroblast cells, of the MUC1 extracellular domain required for its effect on anoikis. It is also possible that fibroblast cells behave differently from epithelial cells where MUC1 is usually expressed. Further investigation in this area will be needed.

Initiation of anoikis starts from the cell surface with various triggers followed by common downstream apoptosis signalling. Cell surface proteins such as integrins and cadherins detect cell-matrix and cell-cell contact respectively. Disruption in these contacts leads to integrin- and cadherin- mediated activation of apoptotic activators Bid and Bim. Bid and Bim promote the assembly of oligomers within the outer mitochondrial membrane (OMM) Bim also interacts with Bcl-XL, neutralizing its pro

survival effect. Formation of oligomers in OMM leads to the release of cytochrome C from mitochondria. Cytochrome C binds to apoptotic protease activating factor (APAF1) leading to eventual cleavage of pro-caspase 9 into activated caspase 9. Activated caspase 9 then activates the caspase pathway leading to eventual cleavage of executioner caspase-3 and cellular proteolysis. Fas receptor, also found on the cell surface, once activated by its ligand leads to formation of Fas-associated death domain and eventual cleavage of caspase 3 via extrinsic apoptotic pathway, as detailed previously.

We had previously reported that expression of MUC1 leads to a reduced accessibility to underlying anoikis initiating molecules including FAS, integrins and cadherins (433). We have also shown that MUC1 expression reduces the activation of the extrinsic pathway following addition of recombinant FAS-ligand. The apoptosis array in this study paints a complex picture of expression of key apoptotic proteins in MUC1 transfected cells. Following 24 hours in anoikis conditions, the ratio of pro-caspase-3 to cleaved caspase 3 is increased in cells transfected with full length MUC1 compared to MUC1 neo transfected cells. Also, the level of survivin is increased in cells with transfected with full length MUC1 compared to their MUC1 neo counterparts. However, further work is needed to understand signalling transduction of MUC1-mediated cell resistance to anoikis. This will enhance our understanding of anoikis and may also help development of new therapeutic agents for cancer treatment.

Previous studies have shown that exogenous introduction of galectin-3 induces MUC1 cell surface polarization in epithelial cancer cells (25, 337, 455). MUC1

polarisation induced by galectin-3 leads to MUC1 cell surface polarization and exposure of underlying cell surface adhesion molecules. This subsequently leads to an increase of tumour cell homotypic aggregation and heterotypic adhesion in tumour cell metastatic spread. 214D anti-MUC1 antibody was generated against VNTR region of MUC1 and has been reported to induce MUC1 polarization. It is found in this study that indeed introduction of the 214D anti-MUC1 antibody induces MUC1 cell surface polarization of MUC1-full and MUC1ΔCT cells. This effect of 214D is found, like galectin-3, to also increase cancer cell adhesion to endothelial cells. This discovery further supports a role of MUC1 cell surface localization in cell-cell interaction in cancer progression.

4 Investigating the effect of MUC1 O-glycosylation on MUC1-mediated resistance to anoikis

4.1 Hypothesis

The heavy *O*-glycosylation of MUC1 on its extracellular domain may contribute to MUC1-mediated cell resistance to anoikis.

4.2 Aims

To assess the influence of *O*-glycosylation of the MUC1 extracellular domain on MUC1-mediated resistance to anoikis

4.3 Introduction

The presence of a variable number of tandem repeat regions (VNTR) is a defining feature of all mucins and the MUC1 external domain is no exception (33). The number of these tandem repeats and their amino acid sequences varies, but they are generally rich in serine, threonine and proline amino acids which act as a building block for initiating *O*-linked glycosylation (456, 457). The length and size of MUC1 extracellular domain, determined by the number of VNTR and the amount of *0*-glycosylation on the VNTR region, ensures that MUC1 extracellular domain extends far outward from cell surface and is heavily glycosylated. The MUC1 *0*-glycosylation is critical for its function, as it confers specific molecular feature that modulates ligand-receptor interactions and cell-extracellular matrix (ECM) interactions (458, 459). As the number and sequence of the tandem repeats is highly variable, mucins present a wide array of potential glycosylation patterns.

The process by which mucin type *O*-linked glycosylation occurs is well characterized, though we know relatively little about its regulation (43-46). The initiating step involves the addition of *N*-acetylgalactosamine (GalNAc) to serine or threonine residues present in the mucin backbone to form the Tn-epitope, a step that is catalysed by a large family of polypeptide GalNAc-transferases (GalNAc-Ts).(43, 47) These structures can then be further extended to form Core 1, 2, 3, or 4 structures based on the identity of the carbohydrate and linkage (460). Core 1 structures are formed by addition of galactose (Gal) in a β1-3 linkage to GalNAc, which is catalyzed by a single enzyme, Core 1 Gal-transferase (C1GT) (48). Core 1 structures can then be extended for Core 2 structures by addition of *N*-acetylglucosamine (GlcNAc) in a β1-6 linkage to the existing GalNAc of the Core 1 structure by Core 2 GlcNAc transferases

(C2GnTs) (49-51). As an alternative to Core 1, Core 3 structures can be generated through addition of GlcNAc in a β1-3 linkage to the Tn epitope (52). Like Core 1 structures, Core 3 structures may be extended or act as the scaffold for Core 4 structure generation through addition of another GlcNAc in a β1-6 linkage (51). While other core structures do exist, Core 1, 2, 3, and 4 structures comprise the primary glycan structures observed in humans.

Table 4.1 Structure of O-glycan cores

O-Glycans	Structure
Core	
Tn antigen	GalNAcαSer/Thr
Sialyl-Tn antigen	Siaα2-6GalNAcαSer/Thr
Core 1 or T antigen	Galβ1-3GalNAcαSer/Thr
Core 2	GlcNAcβ1-6(Galβ1-3)GalNAcαSer/Thr
Core 3	GlcNAcβ1-3GalNAcαSer/Thr
Core 4	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcαSer/Thr
Core 5	GalNAcα1-3GalNAcαSer/Thr
Core 6	GlcNAcβ1-6GalNAcαSer/Thr
Core 7	GalNAcα1-6GalNAcαSer/Thr
Core 8	Galα1-3GalNAcαSer/Thr

Many tumours exhibit aberrant *O*-glycans. Alterations in the glycobiology of tumours occur principally through two mechanisms: neo-synthesis and incomplete synthesis (85). The expression of truncated Core 1 based structures, such as T, Tn, or sialyl-Tn

(STn), are observed in a majority of human carcinomas. These structures are typically absent in healthy tissues (86-88). In many instances, expression of these truncated structures is driven by alterations to the expression of enzymes involved in the glycosylation process. Recent studies have also found that the localization of GalNAc-Ts is a critical factor in the generation of *O*-glycan structures (461, 462). Relocation of GalNAc-Ts from the Golgi to the endoplasmic reticulum (ER) results in changes to the compartmentalization of the initiation machinery and the normal *O*-glycosylation process.

Phenotypically, altered expression of mucin-type glycoproteins bearing aberrant *O*-glycans is associated with increased aggressiveness and metastatic behaviour in a variety of cancers (87, 92, 98). These effects result in part from changes to binding properties of secreted and cell surface proteins that modulate interactions between tumour cells and binding partners in the extracellular environment (e.g., selectins and integrins) (98) and from effects on other ligand-receptor interactions that alter signal transduction in affected cells. Re-expression of enzymes involved in the extension of the carbohydrate chain, such as Cosmc or Core 3 synthase, results in a decrease in these aggressive properties in pancreatic cancer cells by influencing these interactions. (99)

Recent work done in our lab (433) and others (463) has revealed that overexpression of MUC1 on the cell surface protects the cell from anoikis. The effect was noted in breast epithelial cells, melanoma cells and colon cancer cells transfected with full length MUC1. A substantial amount of evidence exists that MUC1 intracellular domain, with its 22 potential phosphorylation sites, are able to relay signals to key

anoikis resistance proteins, by increasing the activation of AKT (81, 453), PI3K (81) and ERK (452). Our recent work (433), part of which is presented in chapter 3, demonstrated that both the intracellular as well as extracellular domain of MUC1 have a role in MUC1 mediated resistance to anoikis; with a bigger influence coming from the MUC1 extracellular domain. This has led us to hypothesize that overexpression of MUC1, as seen in the majority of metastasising epithelial cancers, leads to the formation of a MUC1 'protective shield' around the cell which reduces the access/activation of anoikis-initiating molecules found on the cell surface. Indeed, our previous work, comparing the MUC1-transfected breast epithelial HBL100 cell with its control variant showed that over-expression of MUC1 on the cell surface leads to reduced access/activation of integrin β, CD44, E-cadherin and Fas-receptor (433).

The discovery of the impact of MUC1 extracellular domain on anoikis and the nature of MUC1 extracellular domain heavy O-glycosylation led us to speculate that the glycosylation and length (size) of the MUC1 extracellular domain may have an influence on MUC1-mediated cell resistance to anoikis.

4.4 Methods

Generation of core-1 galactosyl transferase knockdown MUC1 mutants and control variants

ShRNA plasmid against C1GT (SHCLND-NM_020156-C1GALT1) or control vector were purchased from Sigma Aldrich (Gillingham, UK). 0.2 µg of the plasmid in antibiotics and serum-free media was premixed with to 0.6 or 0.8 µg of Metafectene

transfection reagent and added to approximately 60% confluent HCT116 MUC1 full (HCT116 MUC1-F3) or HCT116 MUC1-neo cells in 100 µl antibiotics-free and serum-containing media in a 96-well plate for 6 hours at 37^0C with 5% CO_2. The culture medium was replaced with serum-containing medium for 24 h before the cells were cultured in normal growth medium containing 1 µg/ml puromycin for 3 days at 37 °C. Surviving cells following the puromycin treatment were proliferated in serum-containing media with 0.5 µg/ml puromycin. The cells were released, heavily diluted in media containing puromycin and plated in 96-well plates to ensure single cell clones. At this point the cells were named based on the transfection they had and the well number from a 96 well plates where a single cell colony was confirmed, for example F3 sh-C1GT (B7) corresponds to single cell colony originating in well number B7 in a 96 well plate following ΔC1GT transfection of HCT116 MUC1 positive cells. Single-cell clones were then proliferated and analysed for C1GT knockdown. Selected C1GT colonies were initially analysed via slot blot based on a reduction of PNA binding and an increase in VVA binding. Selected colonies were then further analysed by immunoblotting and flow-cytometry. C1GT knockdown was confirmed based on reduced side of MUC1 extracellular domain (tested with antibody B27.27), reduced PNA binding and increased VVA binding compared to control vectors. Control colonies were selected based on similar PNA and VVA binding to non-transfected HCT116 MUC1 full cells. A schematic diagram of stable transfection knockdown and colony selection is illustrated in figure 4.1.

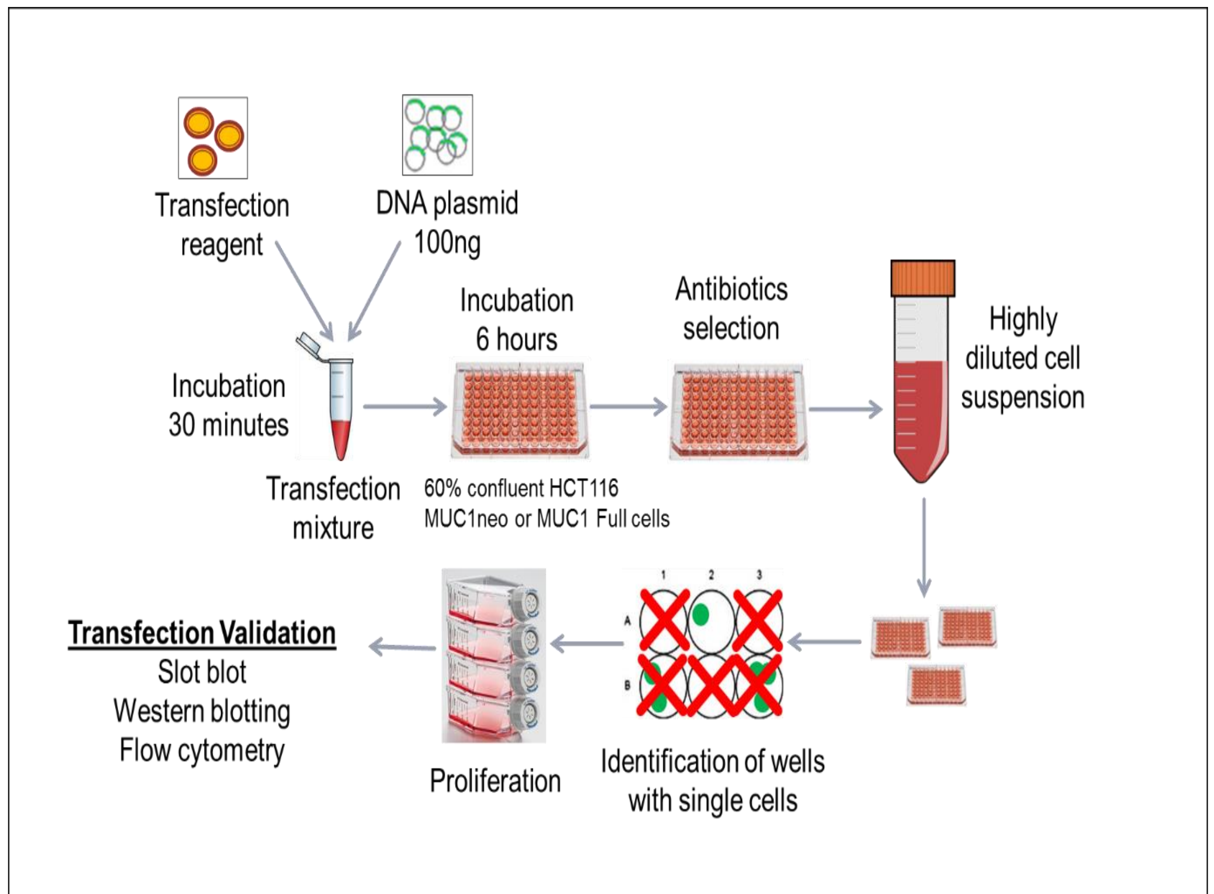


Figure 4.1: General plan for shRNA transfection and validation of C1GT knockdown in HCT116 MUC1 full and HCT116 MUC1 neo cells. 200 ng of plasmid was incubated with transfection reagent Metafectene in 1:4 ratios before being added to sub-confluent HCT116 MUC1 full cells for 6 hours. Following incubation, the cells were subjected to antibiotic selection using 0.5 µg/ml of puromycin in McCoy's 5A complete medium. Surviving cells were highly diluted to clones originating from a single cell. Single clones were selected, proliferated and shRNA knockdown was validated using immunoblot and flow cytometry analyses Following transfection, successful C1GT knockdown colonies were initially identified via slot blot and were further validated via immunoblotting and flow cytometry.

Assessment of cell anoikis

The condition required to promote anoikis was created by culturing the cells in suspension as described previously. Cells undergoing apoptosis (anoikis) were assessed by both Caspase- Glo® 3/7 assay and using an Annexin-V/PI apoptosis detection kit followed by flow cytometry.

Analysis of the expression of cell surface adhesion molecules

The cells in this analysis were released from culture flasks with NECDS and fixed immediately 2% paraformaldehyde for 15 min at room temperature. After washing with PBS, the cells were incubated with 5% goat serum in PBS for 30 min. The cells were re-suspended to 5×10^5 cells/ml with 1% goat serum in PBS and incubated with antibodies (1 µg/ml) against, E-cadherin, CD44, integrinβ_1, Fas-L or control IgG and lectins (1 µg/ml) against peanut agglutinin (PNA), Vicia Villosa Lectin (VVA) and Griffonia Simplicifolia Lectin II (GSL-II) for 1 h at room temperature. After three washes with PBS, FITC-conjugated secondary antibody (1 : 500 in 1% goat serum in PBS) was applied for 1 h. The cells were washed three times with PBS before flow cytometry analysis.

Effect of exogenous Fas-L on caspase-8 activation under anoikis conditions

HCT116 MUC1 full and null cells with validated C1GT knockdown and corresponding control were released with NECDS and were diluted to 2×10^5/ml in serum-free McCoy's 5A. One hundred μl/well of these cells was transferred to a 96 well plates triple-coated with 10 mg/ml poly-HEMA with or without introduction of 100 ng/ml Fas-L for 0 and 2 h followed by assessments of the cellular caspase-8 activity using a Caspase- Glo® 8 Assay kit.

4.5 Results

4.5.1 C1GT knockdown leads to reduced TF expression and increased Tn expression

Suppression of C1GT knockdown using siRNA has previously been shown to lead to a reduction of TF and reciprocal increase of Tn, sialyl-Tn and core 3 glycans in human colon cancer cells (54). In this study we used a reduction in TF binding as an initial confirmation of successful C1GT knockdown. Further confirmation of C1GT knockdown was validated by monitoring the size of MUC1 molecular weight in SDS-PAGE and expressions of Tn and core 3 structures by slot-blotting and flow cytometry.

The control (empty vector) and C1GT shRNA vectors used in this study contains resistance to the antibiotic puromycin. Following C1GT shRNA transfection of the MUC1+ (HCT116 MUC1-F3) and MUC1 – (HCT116 MUC1-neo) transfected HCT116 cells, single cell colonies were selected, proliferated and tested for the expressions of TF (via PNA), Tn (via VVA) and core 3 structures (GSL-II) via slot blot (figure 4.2). A number of colonies (e.g. B7,D5,E7, C6 and F11) showed successful C1GT knockdown. Control transfected colonies (e.g. F2 and C2) showed a similar TF and Tn expression to non-transfected cells

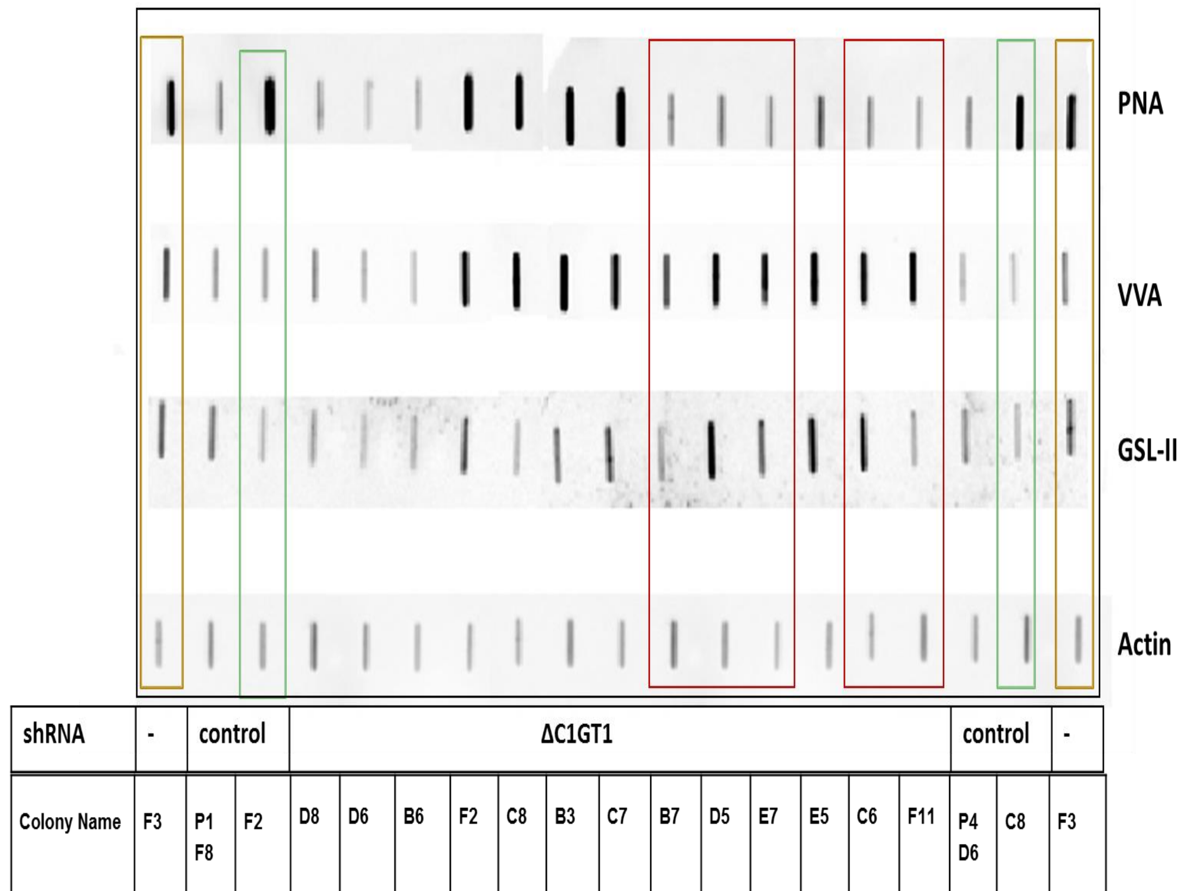


Figure 4.2: Slot blot analysis for C1GT and control shRNA transfected HCT116 MUC1 full cells. 4 selected colonies from control ShRNA transection and 13 colonies from C1GT transfected cells, along with non-transfected HCT116 MUC1 full (F3) were tested for binding to PNA, VVA, and GSL-II. Actin was used as a measure of control of equal loading. C1GT knockdown colonies B7, D5, E7, C6 and F11 were selected on the basis of reduced expression of TF (detected by low PNA binding) and higher expression of TN (detected by VVA) and equally higher expression of core 3 structure (Detected by GSL-II) compared to F3 cells. Based on these criteria two potential transfection control colonies (F2 and C8) were selected along with 5 potential C1GT knockdown colonies (B7, D5, E7, C6 and F11) for further evaluation.

These selected colonies were then analysed by immuno/lectin blotting with antibodies against MUC1 (B27.29 and CT2) or TF-binding peanut agglutinin. Figure 4.3 shows that all the selected shRNA C1GT transfected cells showed on average ~ 30% reduction in size of the MUC1 extracellular domain, compared to control

transfectants and non-transfected cells (as detected by B27.29 antibody against the MUC1 extracellular domain), suggesting reduction of MUC1 glycosylation following C1GT knockdown. Confirmation of successful C1GT knockdown was further validated by a reduction in PNA binding in these cells compared to both the control transfect and the non-transfected cells. When adjusted for actin, expression and movement of MUC1 intracellular domain in SDS-PAGE (as shown by anti-cytoplasmic tail MUC1 antibody CT2) were not expected to be altered in response to the shRNA C1GT, as the MUC-1 *O*-Glycosylation occurs in the extracellular VNTR region. However, in some selected Δ C1GT colonies, when adjusted for actin, a reduction in CT2 expression is noted in F3-C1GT (C6) and F3-C1GT (F11), indicating that the transfection process might have influenced overall MUC1 expression by these cells. Based on the data from these immunoblots, F3-sh-con (C8) and F3-sh-con (F2) were chosen as control colonies due to similar expression of MUC1 extracellular domain, PNA binding and MUC1 intracellular domain as non-transfected HCT116 MUC1 full cells. F3-sh-C1GT (B7) and F3-sh-C1GT (E7) were chosen as ideal C1GT knockdown colonies.

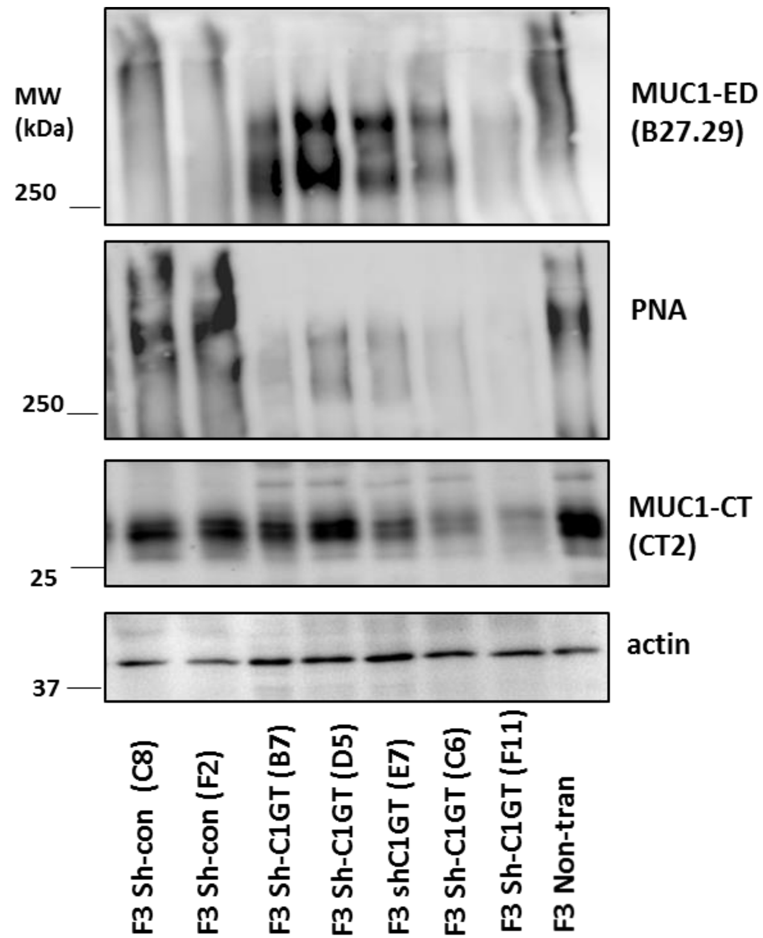


Figure 4.3: Immunoblot analysis for C1GT and control shRNA transfected HCT116 MUC1 full cells. two transfected colonies from control F3 sh-con (C8) and F3 sh-Con (F2) and 5 transfected colonies from C1GT knockdown F3 sh-C1GT (B7) F3 sh-C1GT (D5), F3 sh-C1GT (E7), F3 sh-C1GT (C6) and F3 sh-C1GT (F11) were immunoblotted and probed for MUC1 extracellular domain expression (B27.29), MUC1 intracellular domain (via CT2) and Actin and lectin blotted for core 1 structure (PNA binding),.

HCT116 MUC1-negative cells were also transfected with shRNA for stable C1GT knockdown and with control vector. (figure 4.4) Figure 4 A shows that when adjusted for actin, there is a reduction in PNA binding in C1GT transfects compared to control or non-transfected HCT116 MUC1-negative cells. When immunoblotted for PNA on a 10% polyacrylamide gel, multiple sites are identified in C1GT knockdown colonies

(highlighted by black arrows on figure 4.4B) where there is reduction in PNA binding compared to control and non-transfected cells.

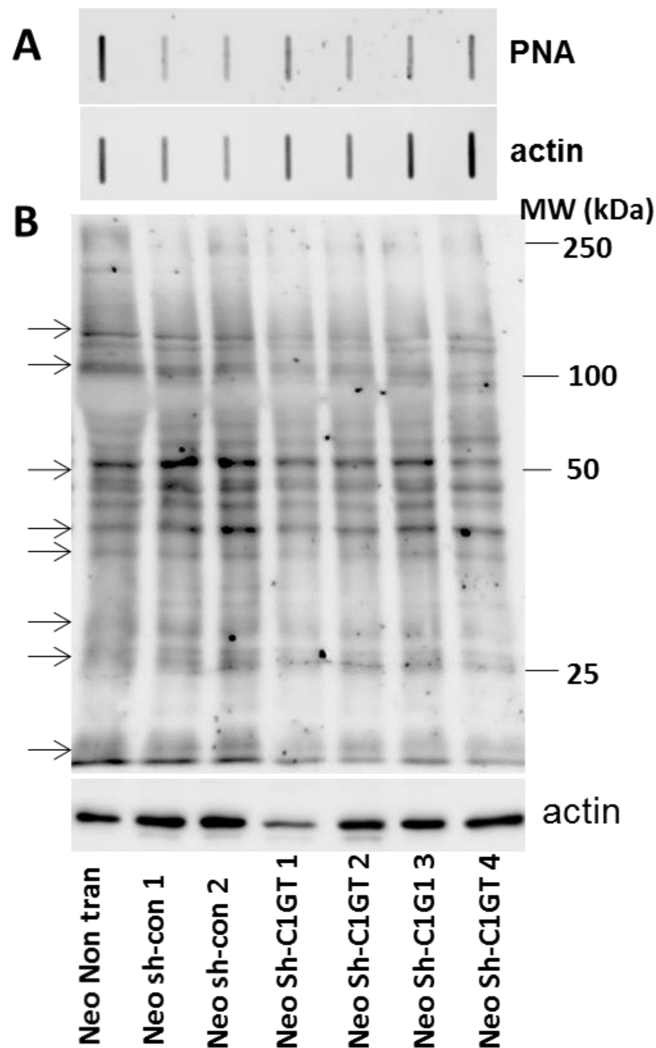


Figure 4.4: Immunoblot analysis for C1GT and control shRNA transfected HCT116 MUC1 neo cells. HCT116 MUC1 neo cells were transfected with control shRNA and ShRNA for C1GT knockdown. The colonies were initially assessed for PNA binding on slot blot. When adjusted for actin the colonies transfected with C1GT knockdown showed a reduction in PNA binding compared to control and non-transfected variant. A lectin blot for PNA binding in these cells was assessed using 10% polyacrylamide gels. Although there are some non-specific bands there are multiple areas (highlighted by the arrow) which shows a reduction of TF expression compared to control variant. The percentage knockdown level was calculated by doing a densitometry analysis on the entire lane and normalizing it to actin. Based on these analysis Neo sh-Con 1 and Neo sh-Con 2 were selected as control transfectants along with Neo sh-C1GT 3 and Neo sh-C1GT 4 as C1GT knockdown colonies for further analysis.

Metastasising colon cancer cells, SW620, were also transfected with shRNA for stable C1GT knockdown and control shRNA vector (figure 4.5). Following immunoblotting with B27.29and CT2 and lectin blotting with PNA and VVA, colonies SW620 sh-con was selected as control colony and SW620 sh-C1GT was selected as being C1GT knockdown cells.

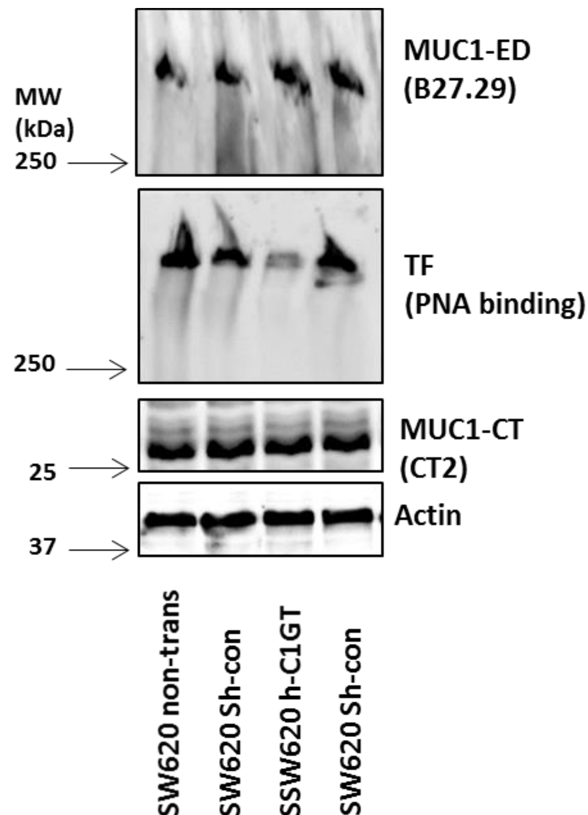


Figure 4.5: Immunoblot analysis for Δ C1GT and control shRNA transfected SW620 cells MUC1 expressing SW620 cells were transfected with either the control or ΔC1GT shRNA. Colonies were selected and immunoblotted for B27.29 and CT2 to detect MUC1 extracellular and intracellular domain respectively. PNA was used to detect Core 1 structure. Control transfected SW620 sh-con showed a similar level of PNA as non-transfected SW620 non-trans, whereas ΔC1GT transfected SW620 sh-C1GT showed a marked reduction in PNA binding.

Flow cytometry analysis for PNA and VVA binding in HCT116 MUC1 + C1GT knockdown and control variants shows that there is significant reduction in PNA

binding in both C1GT knockdown F3-sh-C1GT (B7) and F3-sh-C1GT (E7) cells compared to control F3-sh-con (C8) and F3-sh-con (F2) (p <0.001 following a one-way ANOVA with Dunnett's test) (figure 4.6). Such a reduction was not noted between HCT116 MUC1 neo C1GT knockdown (Neo sh-C1GT 3 and neo sh-C1GT 4) and control variants (Neo sh-Con 1 and Neo-sh Con 2) suggesting that C1GT knockdown in MUC1+ cells leads to reduction in core1 structure (figure 4.7). MUC1 expressing SW620 cells also showed a similar reduction in PNA binding in SW620 sh-C1GT when compared to the control transfected SW620 sh-Con (figure 4.8)

Tn expression, as detected by VVA, was noted to be increased in C1GT knockdown transfectants compared to control transfectants in both HCT116 MUC1+ and SW620 cells (p<0.001, one-way ANOVA with Dunnett's test). This effect was not noted in HCT116 MUC1 neo transfected cells suggesting that C1GT knockdown in MUC1+ cells leads to a reduction in core 1 structure (TF) and a corresponding increase in Tn structure.

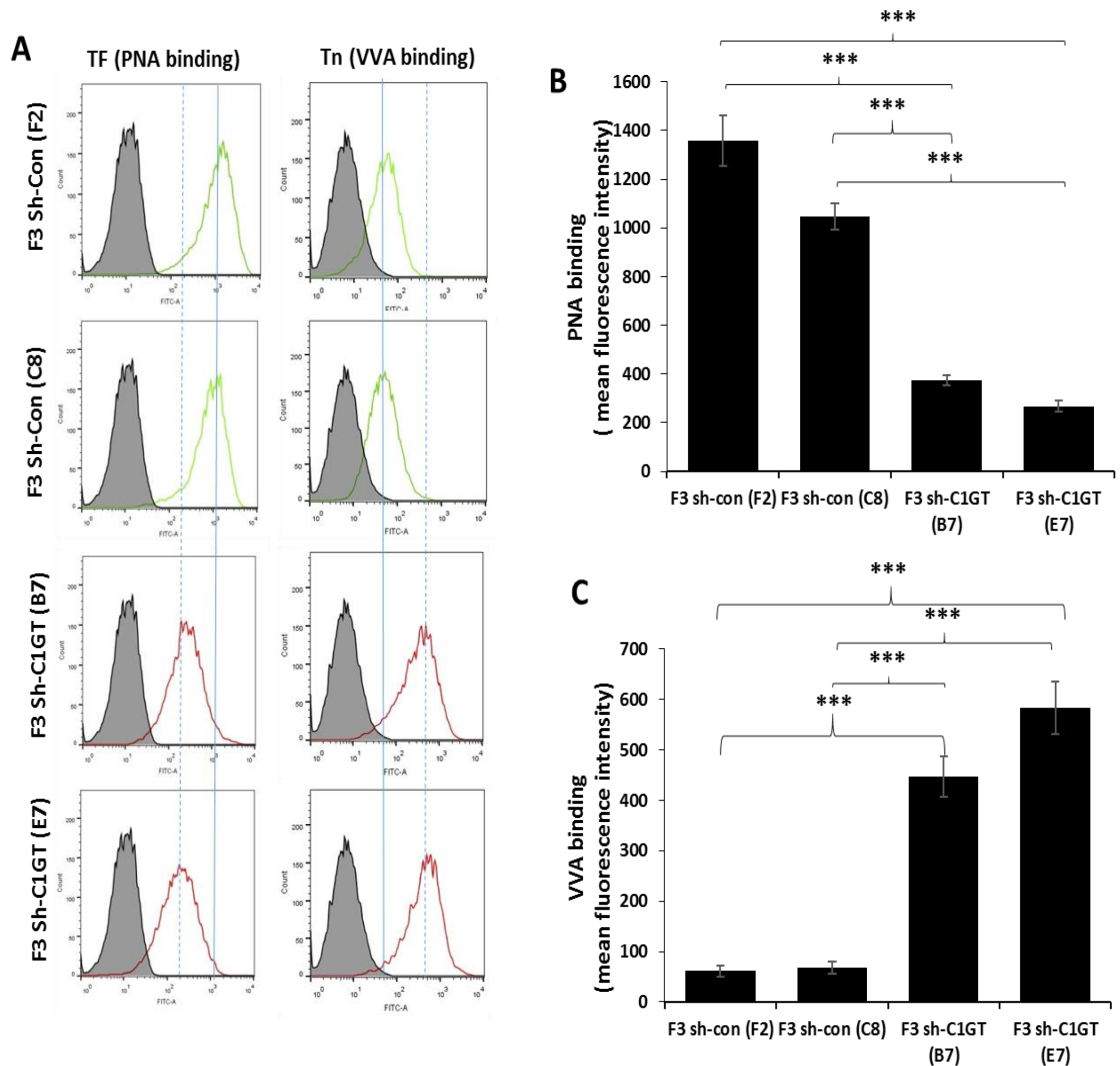


Figure 4.6: C1GT knockdown leads to a reduction in PNA binding and an increase in VVA binding in HCT116 MUC1 full cells. Control shRNA transfected cells F3 sh-Con (F2) and F3 sh-Con (C8) were assessed for PNA and VVA binding along with C1GT knockdown coloniesF3 sh-C1GT (B7) and F3 sh-C1GT (E7). F3 sh-C1GT (B7) and F3 sh-C1GT (E7) showed a statistically significant reduction in PNA binding (p value <0.001) when compared with control variant F3 sh-Con (F2) and F3 sh-Con (C8). VVA binding on the other hand was significantly increased in C1GT knockdown cells compared to control suggesting that C1GT knockdown leads to a reduction in TF and a subsequent increase in Tn N=2, n=2, ANOVA with Dunnett's test, ***= P<0.001

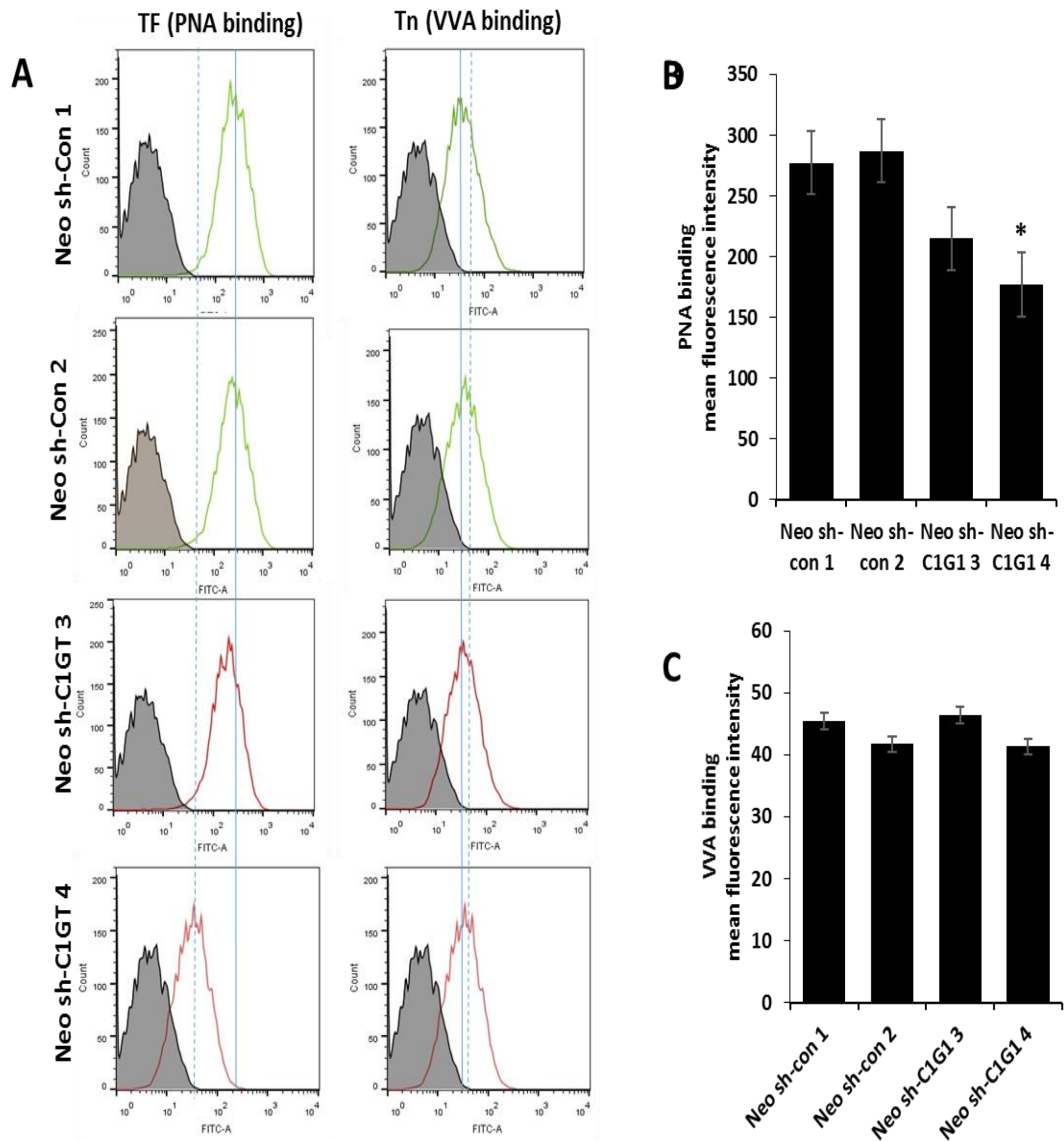


Figure 4.7: C1GT knockdown leads to no significant difference in PNA and VVA binding in HCT116 MUC1 neo cells. HCT116 MUC1 neo cells transfected with C1GT knockdown showed a slightly reduced PNA binding compared to control variants and this effect was statistically significant in Neo sh-C1GT4.when compared to control transfected Neo sh-Con 1 and Neo sh-Con 2 when mean fluorescence intensity was analysed. No difference was noted in VVA binding between C1GT knockdown cells and control. N=2, n=2, ANOVA with Dunnett's test, *= P<0.05

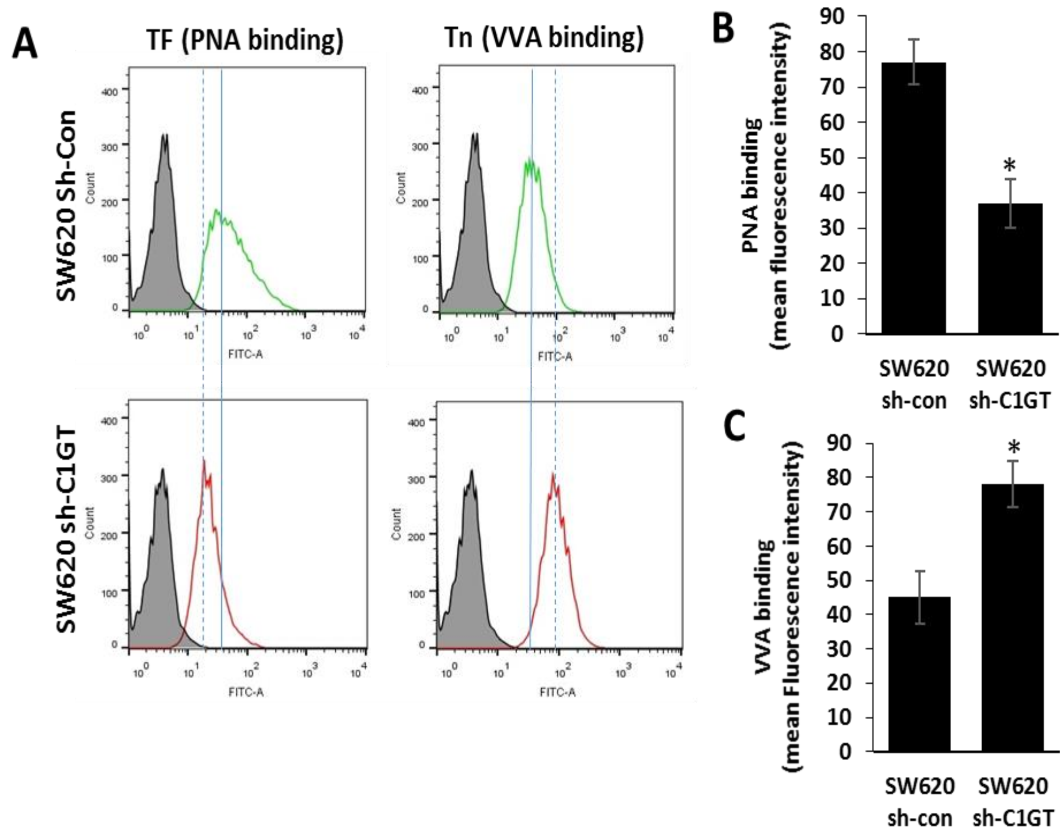


Figure 4.8: C1GT knockdown in MUC1 expressing SW620 leads reduced PNA binding. SW620 cells, which normally overexpress MUC1, were transfected with control and C1GT knockdown ShRNA and were assessed for PNA and VVA binding. C1GT knockdown cells SW620 sh-C1GT showed a significant reduction of for PNA binding. VVA expression for C1GT knockdown cells was higher compared to control SW620 sh-Con, N=2, n=2, ANOVA with Dunnett's test, *=$p<0.05$

4.5.2 Reduction in *O*-glycosylation by C1GT knockdown makes cells more susceptible to anoikis

HCT116 MUC1 full cells transfected with/without C1GT shRNA were cultured in suspension in a poly-HEMA coated plate and were tested for anoikis using caspase 3/7 Glo®assay. Readings were taken at 0 and 24 hours and percentage change in the

recorded luminescence level from 0 hour was analysed. HCT116 MUC1 neo cells and HCT116 MUC1 full cells were also tested and analysed as a means of internal control within the assay (figure 4.9).

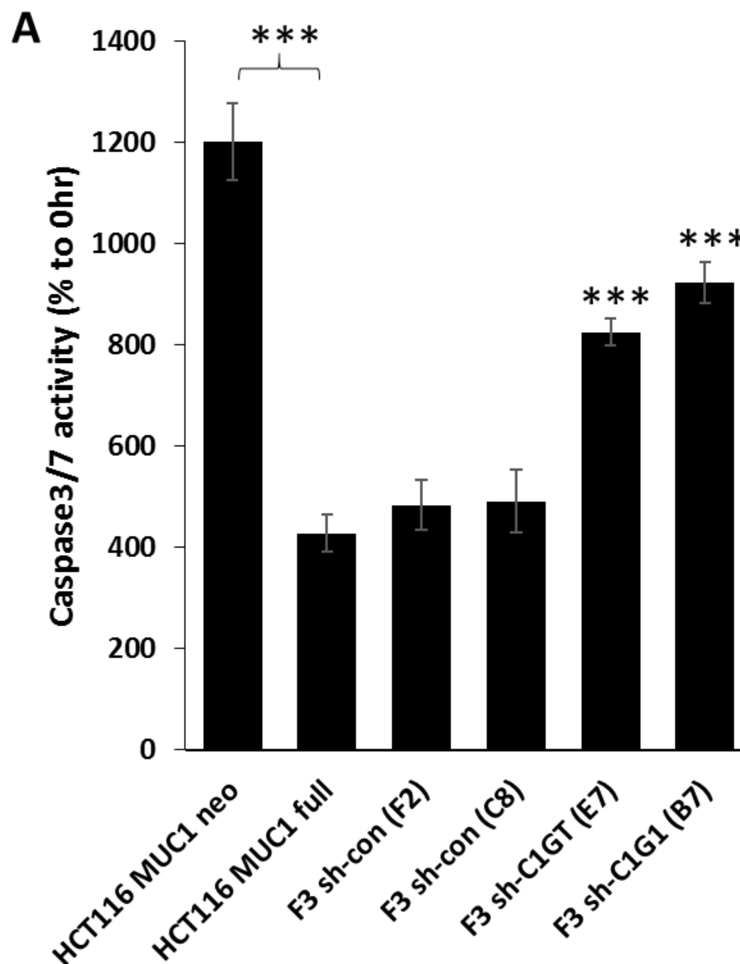


Figure 4.9: C1GT knockdown leads to increased anoikis in HCT116 MUC1 full cells. HCT116 MUC1 full, HCT116 MUC1 neo, HCT116 MUC1 control transfected cells (F3 sh-Con (F2) and F3 sh-Con (C8)) and HCT116 MUC1 full Δ C1GT cells (F3 sh-C1GT (E7) and F3 sh-C1GT (B7)) were cultured in suspension condition. A caspase 3/7 glo assay was conducted at 0 hour and 24 hours. When percentage change in luminescence from 0 hour is analysed, HCT116 MUC1 neo cells show a roughly 3-fold increase in anoikis compared to HCT116 MUC 1 full cells (P<0.001 ANOVA with Dunnett's test). Control transfected F3 sh-Con (F2) and F3 sh-Con (C8) cells show a similar level of luminescence as non-transfected HCT116 MUC1 full cells. C1GT knockdown cells F3 sh-C1GT (E7) and F3 sh-C1GT (B7) show a roughly 2-fold increase in anoikis compared to control transfectant (F3 sh-Con (F2) and F3 sh-Con (C8)) and non-transfected HCT116 MUC1 full cells (***= p<0.001 ANOVA with Dunnett correction) (N=3, n=3)

HCT116 MUC1 full cells showed a marked reduction in anoikis compared to HCT116 MUC1 neo cells as noted previously (chapter 3). HCT116 MUC1 full cells transfected with control shRNA showed a similar level of anoikis at 24 hours as non-transfected HCT116 MUC1 cells, suggesting that the transfection process does not have much effect on anoikis. HCT116 MUC1 full cells transfected with C1GT shRNA showed a marked increase in anoikis compared with both the control transfectant and non-transfected HCT116 MUC1 full (P <0.001, ANOVA with Bonferroni correction) but still lower than that in HCT116 MUC1 neo cells (Fig 4.9). This reduction in anoikis in C1GT knockdown transfectant could be due to the anoikis prevention properties of MUC1 intracellular region as noted previously (chapter 3, Figure 3.6). HCT116 MUC1 full Δ C1GT cells showed a similar level of anoikis as HCT 116 Δ TR (chapter 3, Fig 3.6 C), suggesting that *O*-glycosylation of the MUC1 extracellular domain is involved in MUC1-mediated resistance to anoikis.

HCT116 MUC1 neo cells stably transfected with control shRNA or C1GT shRNA showed a different pattern of anoikis (figure 4.10). Although a slight increase in caspase 3/7 activity was noted in C1GT knockdown transfected cells compared to control variant following suspended culture, it was not statistically significant. This suggests that expression of MUC1 plays a significant role in resistance to anoikis and controlling the size and glycosylation properties of the MUC1 extracellular domain makes a significant difference in the ability to resist anoikis.

MUC1+ SW620 cells transfected ShRNA for C1GT knockdown and control variant showed a similar pattern of anoikis following 24 hours suspension culture as that noted for HCT116 MUC1 full cells but to a lesser extent (figure 4.11)

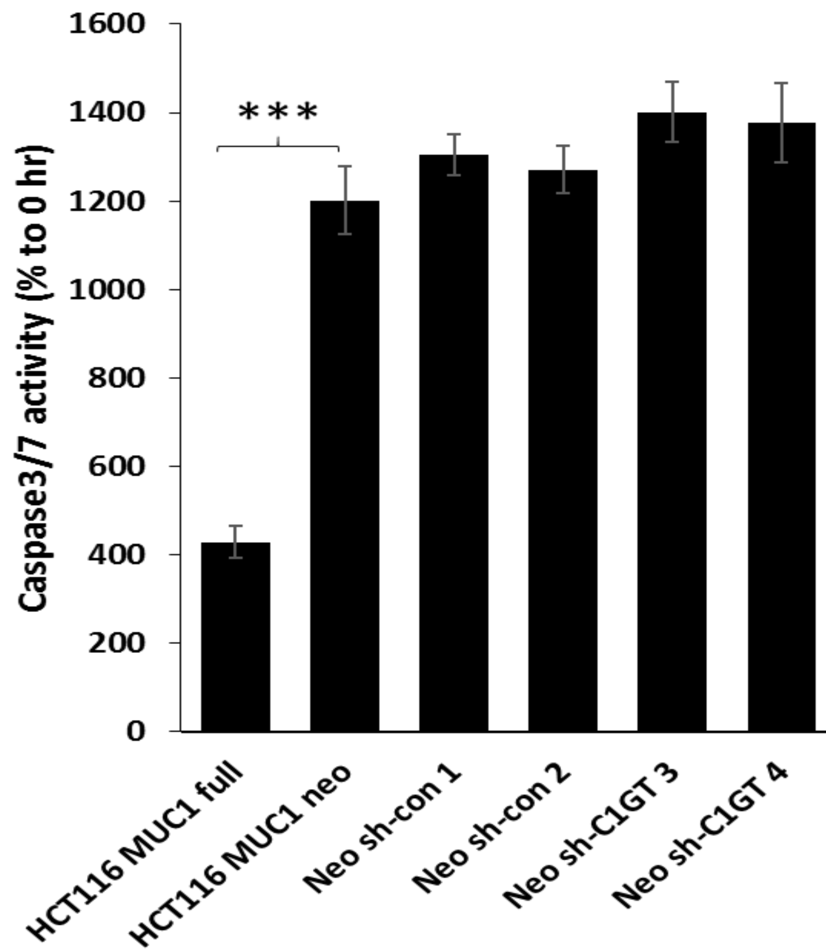


Figure 4.10: C1GT Knockdown in HCT116 MUC1 neo has no significant effect on resistance to anoikis. HCT116 MUC1 neo cells transfected with C1GT knockdown (Neo sh-C1GT 3 and Neo sh-CC1GT 4) had a slightly higher percentage change compared to control transfects (Neo sh-con 1 and Neo sh-con 2) but the difference was not statistically significant. Non-transfected HCT116 MUC1 full and HCT116 MUC1 neo were compared to test the validity of the assay and as per previous results (Figure 4.9), showed statistically significant difference. (N= 3, n=3) ANOVA with Dunnett's test, ***= P<0.001.

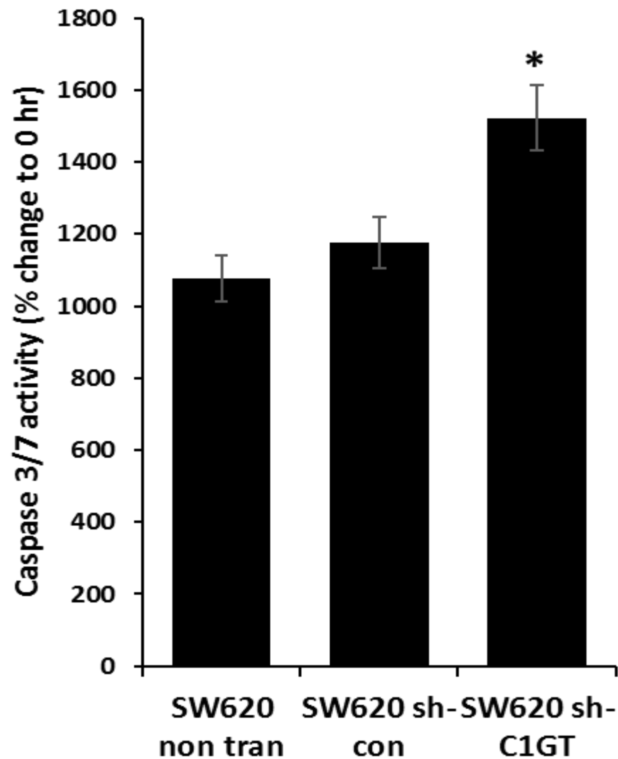


Figure 4.11: C1GT knockdown in MUC1 expressing SW620 cells leads to an increase in anoikis. MUC1 expressing SW620 cells were transfected with control shRNA (SW620 sh-con) showed a similar level anoikis at 24 hours as non-transfected SW620 cells (SW620 non tran). CIGT knockdown SW620 cell (SW620 sh-C1GT) showed a statistically significant increase in anoikis at 24 hours when compared to control SW620 non tran. N=3, n=3, ANOVA with Dunnett correction, *= P<0.05

We also tested the level of anoikis in C1GT knockdown transfectant cell lines and control variant using the annexin V assay with PI. Annexins are a family of calcium-dependent phospholipid-binding proteins that preferentially bind phosphatidylserine (PS). Under physiological conditions, PS is predominantly located in the inner leaflet of the plasma membrane. Following initiation of apoptosis, PS loses its asymmetric distribution across the phospholipid bilayer and is translocated to the extracellular membrane leaflet, marking cells as targets of phagocytosis. Once on the outer surface of the membrane, PS can be detected by fluorescently labelled

Annexin V in a calcium-dependent manner. Propidium iodide (PI), a ready-to-use and red-fluorescence dye which binds to DNA. PI cannot cross the membrane of live cells, making it useful to differentiate necrotic, apoptotic and healthy cells. Combined together an annexin V/PI assay detects live cells at the bottom left (annexin V negative, PI negative), early apoptotic cells at the bottom right (Annexin-V-positive and PI-negative) and late apoptotic cells at the top right (Annexin-V-positive and PI-positive).

When comparing early and late apoptosis at 24 hour from 0 hour, there was 37.9% increase in HCT116 MUC1 neo cells compared to 8.6% increase noted in HCT116 MUC1 full cells (figure 4.12). Our previous data using HBL100 MUC1 Full (HCA1.7+) and HBL100 MUC1 neo had shown a similar level of increase of early and late apoptosis in MUC1 neo cells compared to MUC1 positive cells (433).

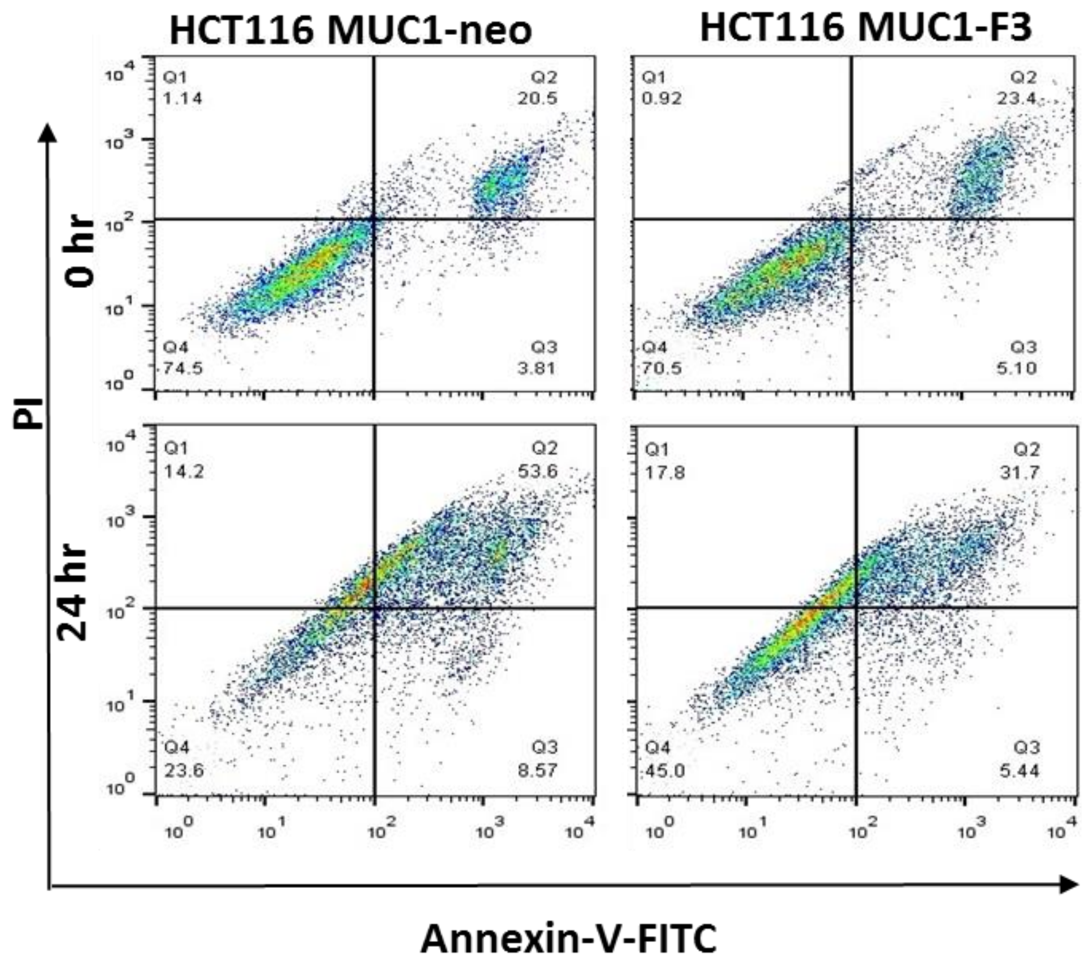


Figure 4.12: Expression of MUC1 reduces anoikis in HCT116 cells. HCT116 cells transfected with MUC1 (HCT116 MUC1 full) and control variant (HCT116 MUC1 neo) were assessed for Annexin V binding at 0 and 24 hour following suspension in poly-HEMA coated plates. At 24 hours MUC1 neo cells showed a higher level of early and late apoptosis than MUC1 full cells.

When we compared HCT116 MUC1 full ΔC1GT cells and control variant using the annexing V assay, we found that control variant C8 had a slightly higher level of early and late apoptosis following 24 hours in suspension culture (42.1%) compared to non-transfected HCT116 MUC1 full cells (37.1%) (figure 4.13). The two selected HCT116 MUC1 Full cells with C1GT knockdown, F3 sh-C1GT (B7), showed a much higher level of early and late apoptosis following 24 hours under suspension

condition (53.9%) compared to the control variant F3 sh-con (C8) (42.1%). When we compared the percentage change in early and late apoptosis from 0 hour, we found that there was a 42.0% increase in early and late apoptosis for F3 sh-C1GT (B7), compared to a 25.3 % increase noted in control variant F3 sh-con (C8).

We then compared selected HCT116 MUC1 neo Δ C1GT cells and its control variant (figure 4.14). Annexin V/PI analysis looking at early and late apoptosis between HCT116 MUC1 neo, Neo sh-con 1 and Neo sh-C1GT cells were very similar. Early and late apoptosis noted at 24hr following suspended culture were 62.3% for control transfected Neo sh-con 1, compared to 62.2 % for non-transfected HCT116 MUC1 neo cells. When compared with C1GT transfected cells, early and late apoptosis at 24 following suspended culture were 64.9% for neo sh-C1GT 3.

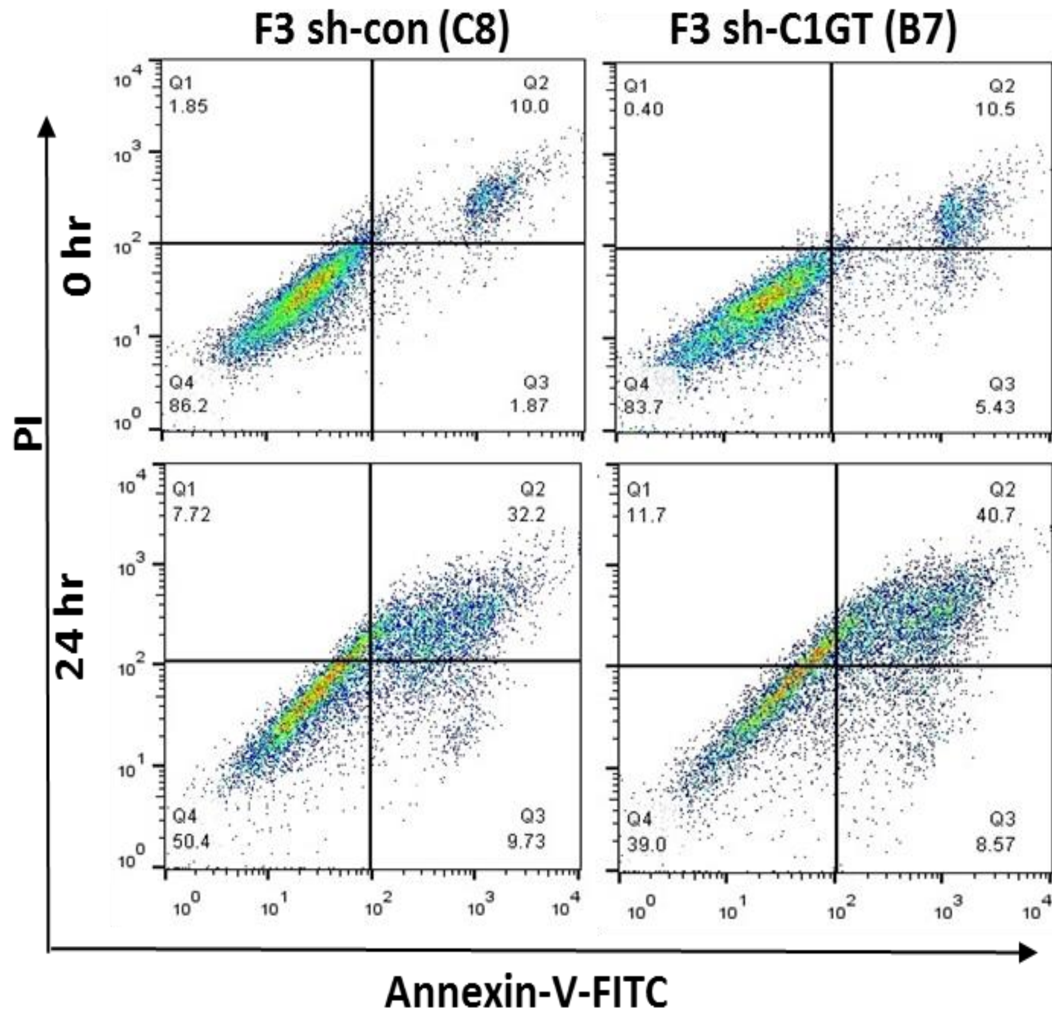


Figure 4.13: C1GT knockdown in HCT116 MUC1 full cells makes cells more susceptible to anoikis. HCT116 MUC1 full cells transfected with control shRNA (F3 sh-con (C8)) and C1GT knockdown (F3 sh-C1GT (B7) were assessed for Annexin V binding at 0 and 24 hour following suspension in poly-HEMA coated plates. F3 sh-con (C8) had a similar level of late (32%) and early apoptosis (9.73%) at 24 hours as the non-transfected HCT116 MUC1 noted earlier. C1GT knockdown variant F3 sh-C1GT (B7) showed a higher level of early (noted in Q3) and late apoptosis (noted in Q2) compared to both the control F3 sh-con (C8) and non-transfected HCT116 MUC1 full noted earlier, However the level of anoikis noted at 24 hours in F3 sh-C1GT (B7) was still lower than that noted for HCT116 MUC1 neo cells (figure 4.12).

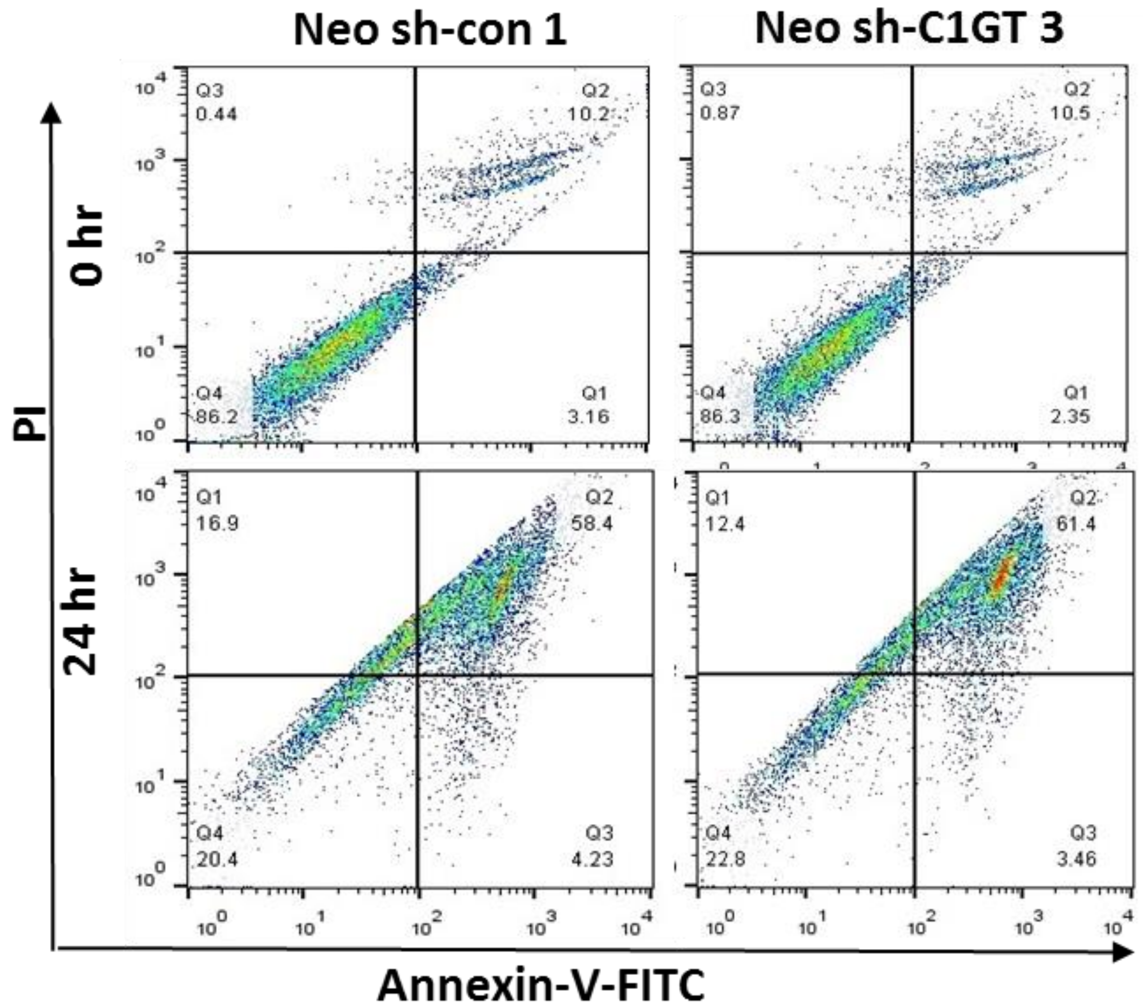


Figure 4.14: C1GT knockdown in HCT116 MUC1 neo cells has no significant effect on anoikis. HCT116 MUC1 neo cells transfected with control shRNA (Neo sh-con 1) and C1GT knockdown (Neo sh-C1GT 3) were assessed for Annexin V binding at 0 and 24 hour following suspension in poly-HEMA coated plates. Like the effect noted in HCT116 MUC1 full cells, the early(Q3) and late apoptosis (Q4) levels were similar to no transfected HCT116 MUC1 neo cells (figure 4.12). Unlike the effect noted in HCT116 MUC1 full cells, however, the C1GT knockdown cells (Neo sh-C1GT 3) did not show much change in anoikis level compared to control transfectant or the non-transfected HCT116 MUC1 neo cells

MUC1+ SW620 cells transfected ShRNA for C1GT knockdown and control variant showed a similar pattern of anoikis following 24 hours suspended culture as that of HCT116 MUC1 full cells but to a lesser extent (figure 4.15). Like the effect for HCT116 cells, the control transfectant SW620 sh-con cells showed a similar level of early and late apoptosis at 24 hours following suspension as the non-transfected SW620 cells (10.8% and 9.6%, respectively). This compares to an early and late apoptosis level of 14.2% as noted in C1GT knockdown cells SW620 sh-C1GT, 12.7% of which was late apoptotic cells. The lower Annexin V level in SW620 transfectants compared to HCT116 MUC1 cells could be due to the fact that SW620 cells are more robust compared to HCT116 cells (as they have a higher tolerance to puromycin compared to HCT116 MUC1-F3 cells, 8 µg/ml compared to 0.5 µg/ml), have a different level of MUC1 expression and potentially different glycosylation state following C1GT knockdown.

Collectively, these data show that the size of the MUC1 extracellular domain and its glycosylation state have a role in MUC1-mediated cell resistance to anoikis.

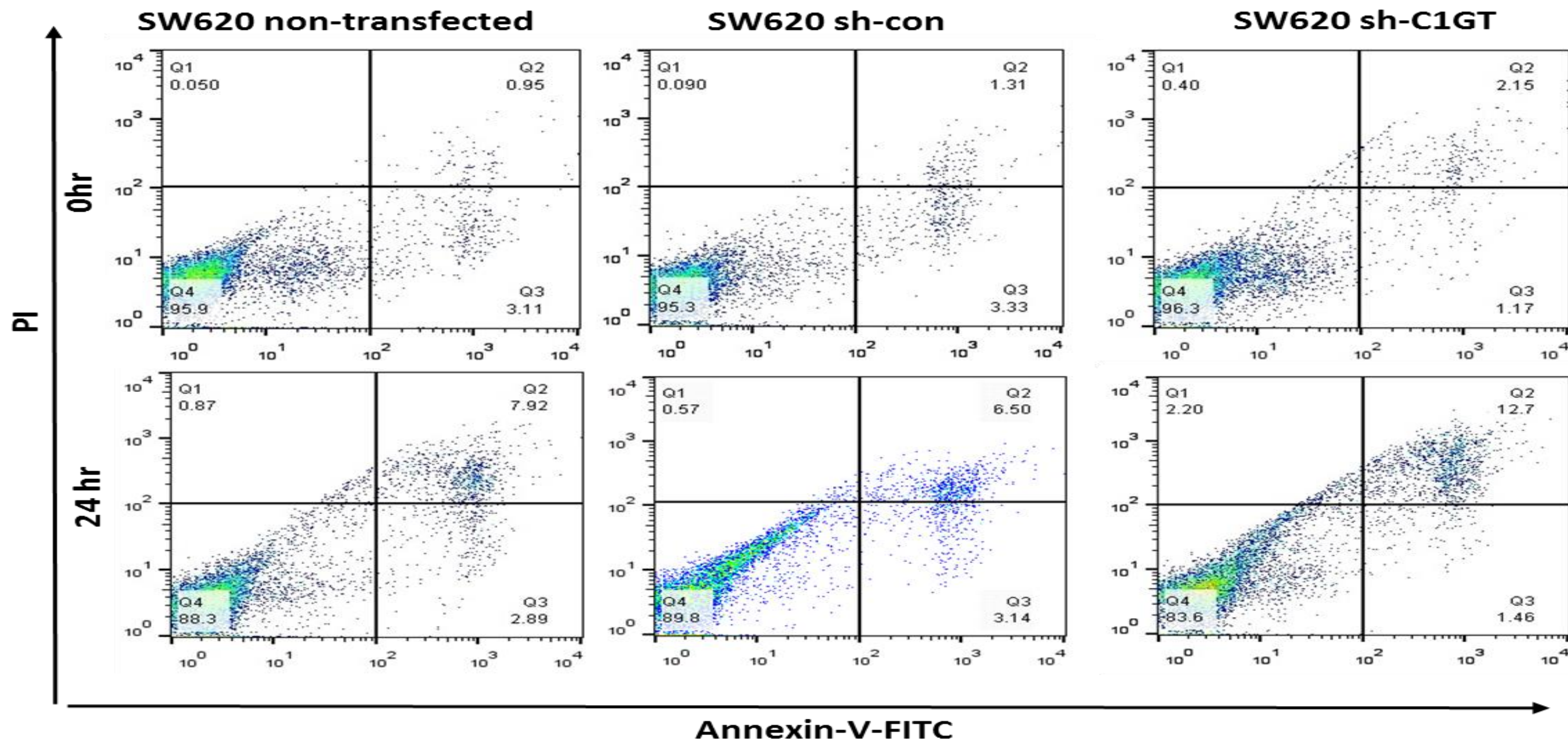


Figure 4.15: C1GT knockdown in MUC1 expressing SW620 cells leads to increase of anoikis. MUC1 expressing SW620 cells were transfected with control and C1GT Knockdown shRNA and were tested for annexin v with PI analysis at 0 and 24 hours along with non-transfected Sw620 cells. Control transfected SW620 cells SW620 sh-con, showed a similar level of early and late apoptosis as non transfected SW620 ells. C1GT transfected SW620, SW620 sh-C1GT however showed a higher level of apoptosis with particular increase noted in late apoptosis at 24 hours (Q2), 12.7 % compared to 6.5 % noted in control transfected SW620 sh-con.

4.5.3 C1GT Knockdown leads to increased caspase 8 activity following activation with Fas-L ligand (Fas-L)

We had previously reported that HBL100 breast epithelial cells transfected with full length MUC1 show a reduced response of caspase 8 activation in response to Fas-L when compared to MUC1 neo transfectant (433). In this study, we found a similar pattern when we compared HCT116 MUC1 full with HCT116 MUC1 neo cells. (figure 4.16).

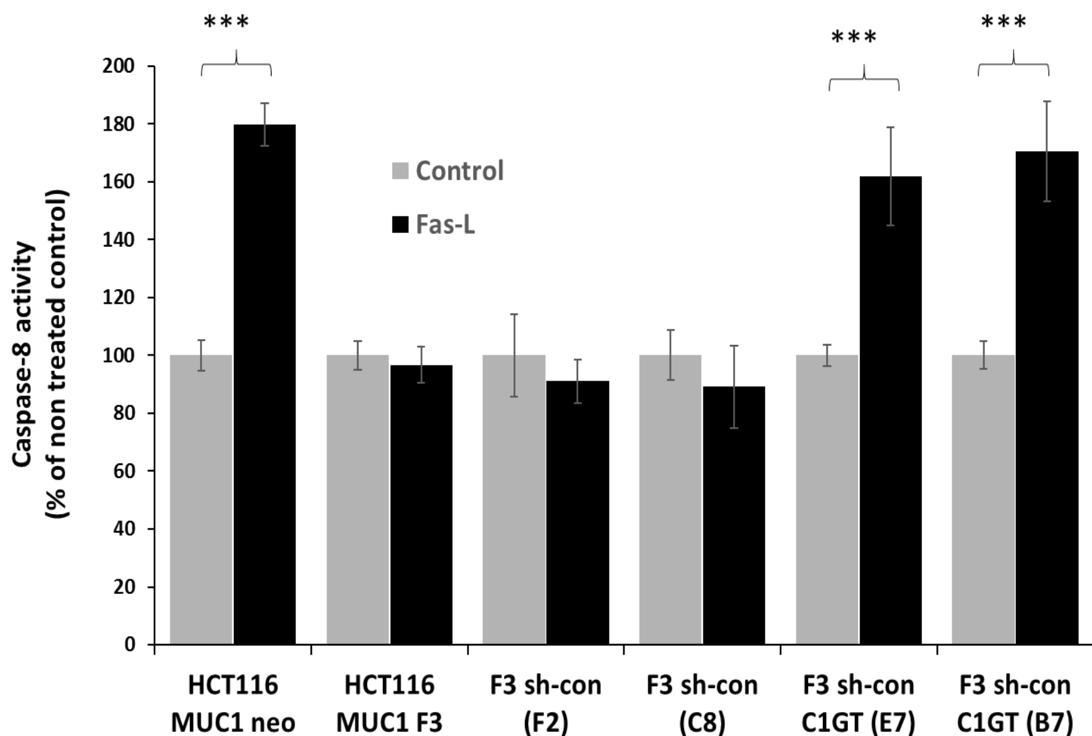


Figure 4.16: C1GT knockdown in HCT116 MUC1 full cells leads to an increase in fas-L induced caspase 8 activation. HCT116 MUC1 full (HCT116 MUC1 F3) and HCT116 MUC1 neo along with HCT116 MUC1 full cells transfected with either control or ΔC1GT shRNA were tested for caspase 8 activation following simulation with either the fas-L or control (PBS). HCT116 MUC1 neo cells showed an 80% increase in caspase 8 activity following simulation by fas-L, whereas no statistically significant difference was noted in HCT116 MUC1 full and control shRNA transfected F3 sh-con (F2) and F3 sh-con (C8). ΔC1GT transfected F3 sh-C1GT (E7) and F3 sh-C1GT (B7) showed a 60% and 65% increase, respectively, in caspase 8 activity following simulation with fas-L. N=3, n=3, ANOVA with Dunnett's test, ***= $P<0.001$.

HCT116 MUC1 neo cells had on average 80% greater caspase 8 activity following 2-hour simulation with Fas-L compared to control variants which were not simulated. In comparison, there was a slight reduction in caspase 8 activity in HCT116 MUC1 full cells simulated with Fas-L when compared to control. This suggests that expression of MUC1 expression is associated with reduced activity of Fas-L on induction of caspase-8 activation in anoikis.

When we assessed HCT116 MUC1 full cells transfected with control shRNA, both F3-sh-con (F2) and F3 sh-con (C8) (figure 4.16) showed a similar pattern of caspase 8 activation as non-transfected HCT116 MUC1 full cells. Interestingly the C1GT knockdown variants, F3- sh-C1GT (E7) and F3 sh-C1GT (B7), both had a much higher caspase 8 activation when simulated with Fas-L compared to non-simulated control. A similar pattern of expression is also noted in SW620 cells (figure 4.17), with control transfectant SW620 sh-con showing a similar and non-significant caspase 8 activation when simulated with fas-L or control. SW620 Cells transfected with shRNA for C1GT knockdown however shows a marked increase in cell caspase 8 activity when treated with Fas-L compared to control.

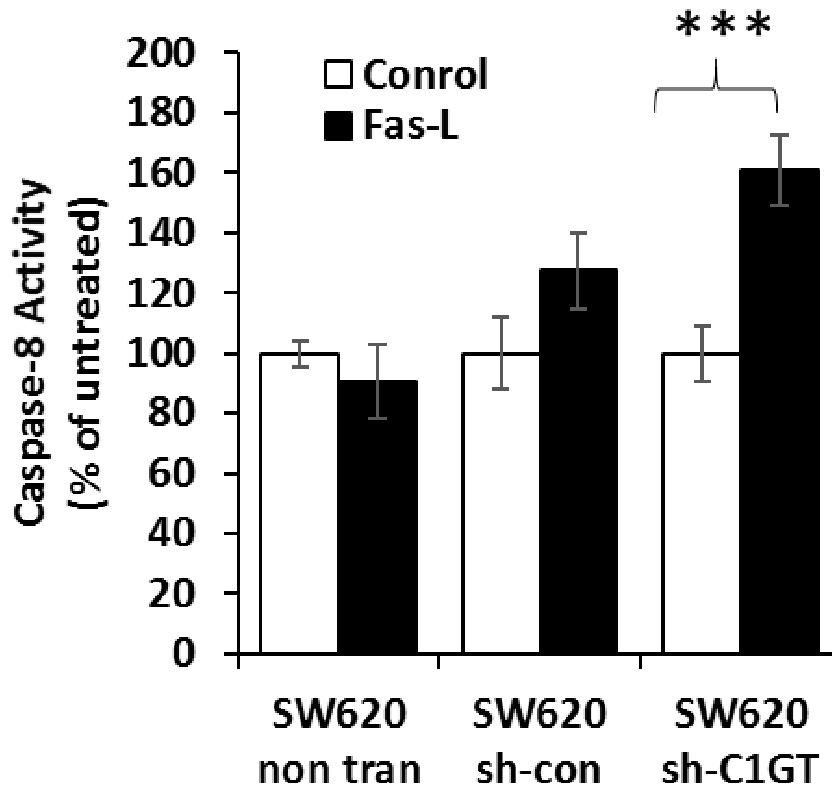


Figure 4.17: C1GT knockdown in MUC1 expressing SW620 cells leads to an increase in Fas-L mediated caspase 8 activation. MUC1 expressing SW620 cells transfected with either control or Δ C1GT shRNA were tested for caspase 8 activation following simulation with either the Fas-L or control (PBS). A caspase 8-Glo® assay was performed at 0 hours and 2 hours following cells suspension culture. The percentage change in cellular caspase 8 activity compared to non-treated control was analysed. Non-transfected SW620 were also used as an internal control. Like the effect noted for HCT116 cells, no significant difference in caspase-8 activation was noted for non-transfected or control transfected, SW620 sh-con cells when simulated with either the Fas-ligand or control. SW620 sh-C1GT however, showed a 56% increase in caspase 8 activity following simulation with Fas-ligand.

Collectively these data suggest that MUC1 expression and its glycosylation state has an effect on anoikis initiated by Fas-L.

4.5.4 C1GT knockdown leads to increased accessibility to cell surface anoikis-initiating molecules

Our previous work has shown that expression of MUC1 reduces the accessibility of antibodies to key cell surface anoikis initiating molecules integrin beta 1, CD44, E-cadherin and Fas (433). We therefore assessed whether reduction of MUC1 *O*-glycosylation affected antibody accessibility to these cell surface anoikis initiating molecules.

Using HCT116 cells transfected with C1GT knockdown, F3 sh-C1GT (E7) and F3 sh-C1GT (B7), and comparing them with control shRNA transfectant F3 sh-Con (F2) and F3 sh-Con (C8) we found that C1GT knockdown cells had an increased accessibility of antibodies to integrin beta 1 ($p < 0.001$), E-cadherin ($p < 0.001$) and also Fas-L cell surface binding ($p < 0.001$) when compared with control variant. (figure 4.18, 4.19) Although there seemed a slight increase in accessibility of antibodies to CD44 in ΔC1GT cells, it was not statistically significant. SW620 cells transfected with C1GT knockdown, SW620 sh-C1GT and control transfectant SW620 sh-con also showed a similar pattern of cell surface anoikis-initiating molecule antibody accessibility as noted for HCT116 cells above. (figure 4.20, 4.21). SW620 sh-C1GT cells showed a marked increase of antibody accessibility to cell surface integrin beta ($p < 0.001$), e-cadherin ($p < 0.001$) and Fas-L binding ($p < 0.001$) when compared to control (SW620 sh-con) non-significant rise in cell surface accessibility to CD44.

Collectively, these results show that reduction of MUC1 glycosylation leads to an increase of the accessibility of antibodies against cell surface adhesion molecules.

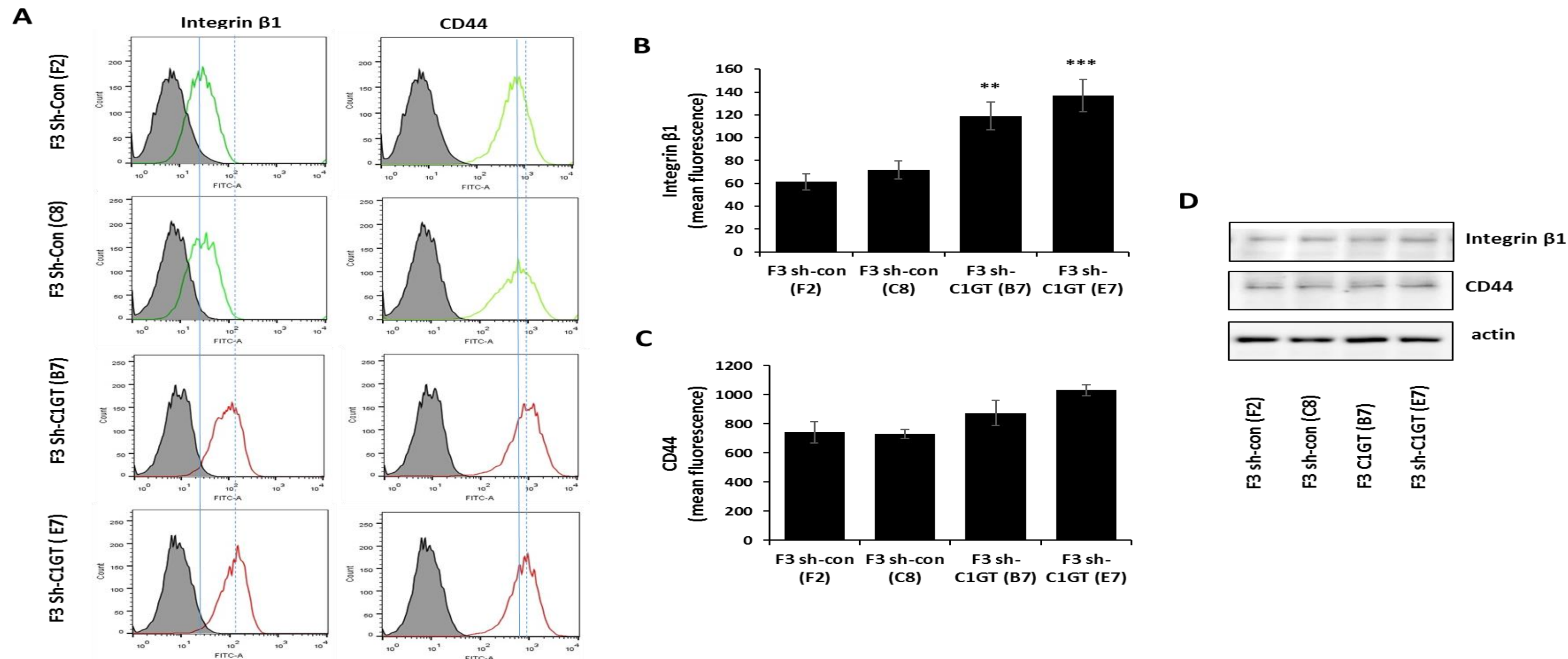


Figure 4.18: C1GT knockdown in HCT116 MUC1 full cells leads to an increase of anti-integrin beta antibody accessibility. HCT116 MUC1 full cells transfected with either the control or Δ C1GT shRNA were tested for cell surface accessibility of antibodies to Integrin β1 and CD44. The cells were released with NECDS and were targeted with either anti integrin β1 or anti CD44 followed by appropriate fluorescently labelled secondary antibody before being analysed by flow-cytometry. Both ΔC1GT transfected F3 sh-C1GT (B7) and F3 sh-C1GT (E7) showed a statistically significant increase in integrin β1 antibody accessibility compared to control shRNA transfected F3 sh-con (F2) and F3 sh-con (C8). Anti-CD44 antibody accessibility between these cells was found to be non-significant. The expression level of integrin β1 and CD44/antibody binding were also compared between transfectants and were found to be similar. N=2, n=2, ANOVA with Dunnett's test, ***= $P<0.001$

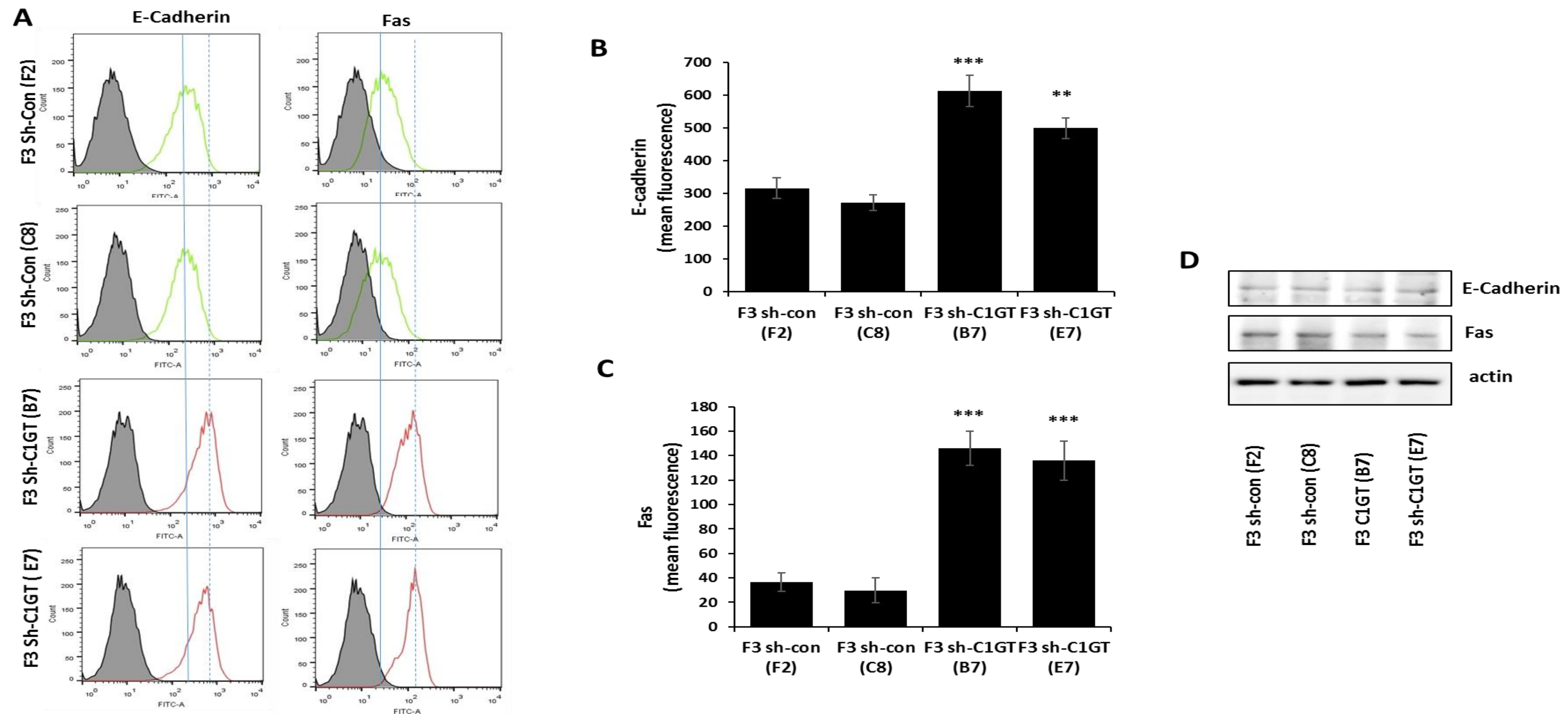


Figure 4.19: C1GT knockdown in HCT116 MUC1 full cells leads to increased accessibility of antibodies to E-cadherin and Fas. HCT116 MUC1 full cells transfected with either the control or Δ C1GT shRNA were tested for cell surface accessibility anti e-cadherin antibody and fas-L binding, using flow-cytometry analysis. Both Δ C1GT transfected F3 sh-C1GT (B7) and F3 sh-C1GT (E7) showed a statistically significant increase in anti-e-cadherin and Fas-L accessibility when compared to control shRNA transfected F3 sh-con (F2) and F3 sh-con (C8). The expression level of e-cadherin and fas were also compared between transfectants and were found to be similar. N=2, n=2, ANOVA with Dunnett's test, ***= P<0.001

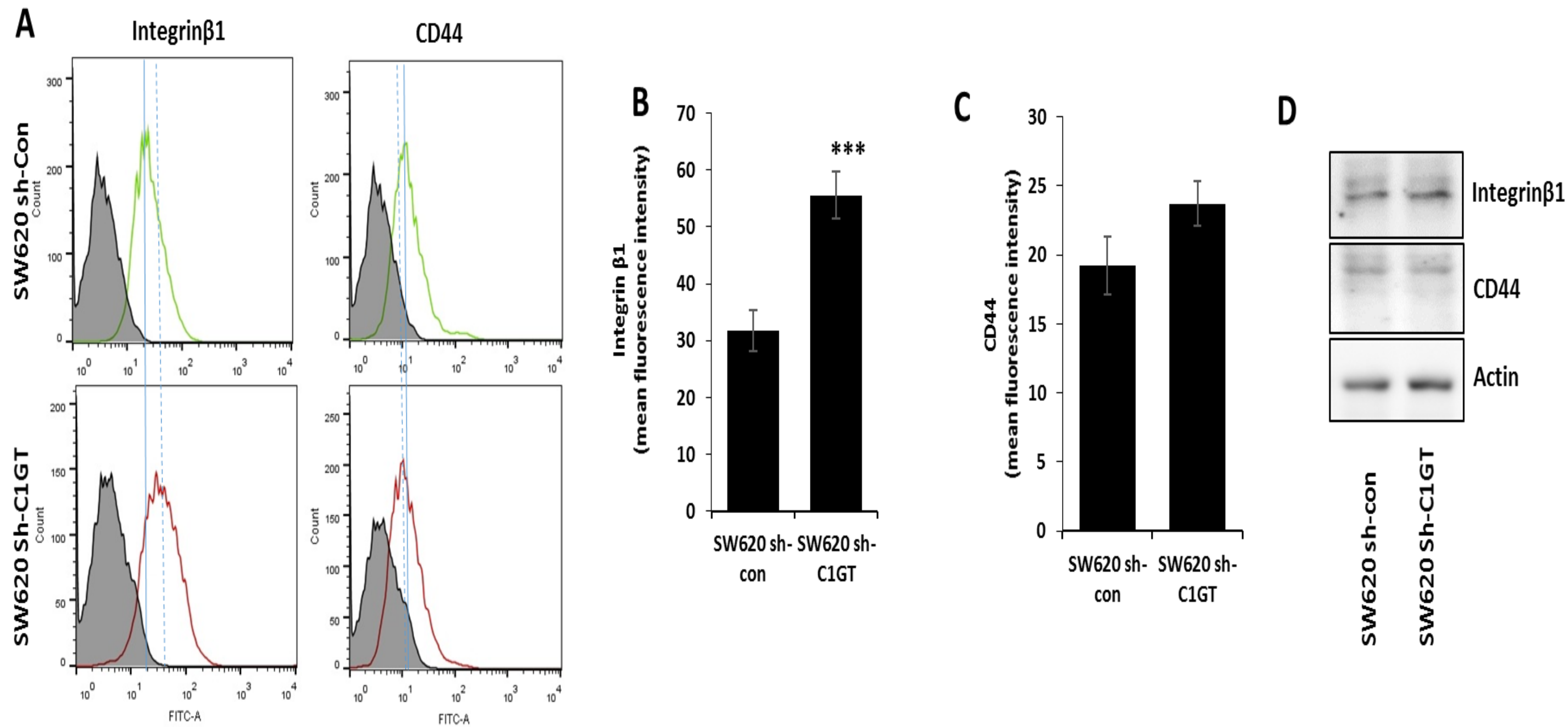


Figure 4.20: C1GT knockdown in MUC1 expressing SW620 cells leads to increased accessibility of anti-integrin beta antibody. MUC1 expressing SW620 cells transfected with either the control or Δ C1GT shRNA were tested for cell surface binding of antibodies to Integrin β1and CD44 and analysed by flow-cytometry. Δ C1GT transfected SW620 sh-C1GT showed a statistically significant increase in anti-integrin β1 accessibility compared to control shRNA transfected SW620 sh-con. Anti- CD44 accessibility between these cells was found to be non-significant. The expression level of integrin β1 and CD44 were also compared between transfectants and were found to be similar. N=2, n=2, ANOVA with Dunnett's test, ***= P<0.001

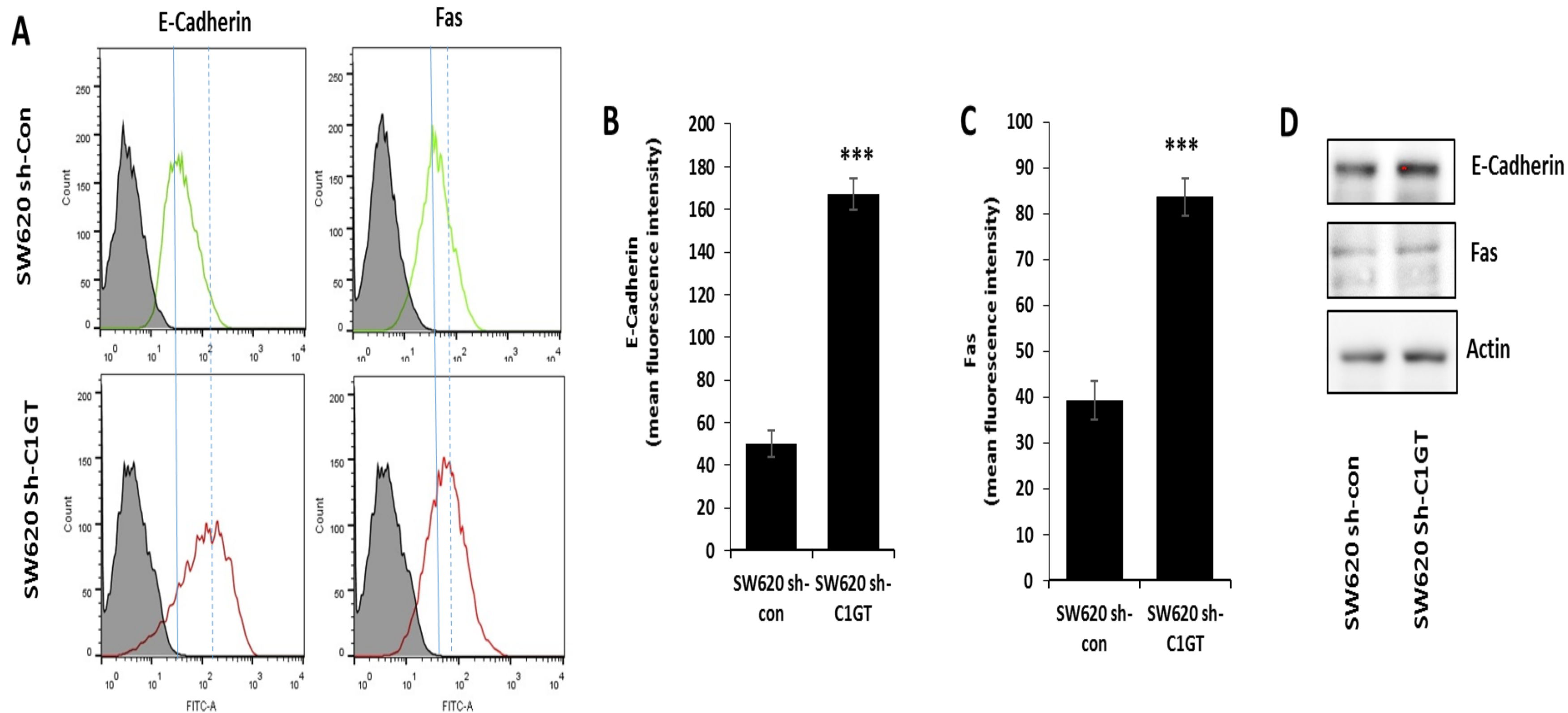


Figure 4.21: C1GT knockdown in MUC1 expressing SW620 cells leads to increased accessibility of antibodies to E-cadherin antibody and Fas-L. MUC1 expressing SW620 cells transfected with either the control or Δ C1GT shRNA were tested for cell surface binding of antibodies to E-cadherin and Fas-L and analysed using flow-cytometry. ΔC1GT transfected SW620 sh-C1GT showed a statistically significant increase both E-cadherin and Fas-ligand accessibility compared to control shRNA transfectedSW620 sh-con. The expression level of E-cadherin and Fas were also compared between transfectants and were found to be similar. N=2, n=2, ANOVA with Dunnett's test, ***= P<0.001

4.6 Summary of results

1) C1GT knockdown in HCT116 MUC1 full cells and SW620 cells leads to a reduction in TF expression and an increase in Tn expression, an effect not seen in MUC1 neo cells of same cell type

2) C1GT knockdown in MUC1 expressing cells leads to a significant increase in anoikis in response to suspended culture

3) C1GT knockdown in MUC1-expressing cells leads to increased caspase 8 activity in response to treatment with Fas-L.

4) C1GT knockdown in MUC1-expressing cells increased accessibility of antibodies to cell surface integrin β1, E-cadherin and Fas receptor but not to CD44 in both HCT116 MUC1 full cells and MUC1-expressing SW620 cells

4.7 Discussion

The results from this study show that suppression of C1GT, the glycosyl transferase that controls the biosynthesis of the Core 1 structure of mucin type *O*-linked glycans, is accompanied by increased expression of sialyl-Tn (as detected by VVA) and core 3 structure (as detected by GSL-II binding). This supports our previous work which showed that there is a competitive modification of the GalNAc residue of GalNAcα-Ser/Thr between C1GT, C3GnT and ST6GalNAc-T in the biosynthesis of complex *O*-linked mucin type glycans (54). It was found in this study, that reducing the size of MUC1 extracellular domain, by reducing its glycosylation through suppression of C1GT, leads to an increase in cell anoikis of both HCT116 and SW620 cells. Flow cytometry analysis of cell surface proteins showed that suppression of C1GT leads to an increase of the accessibility of antibodies to cell surface integrin β, E-cadherin and Fas, but not CD44. It also increased Fas-L cell-surface binding and induction of caspase-8 in subsequent anoikis culture. This highlights the significance of the size of MUC1 extracellular domain in MUC1-mediated cell resistance to anoikis and is in keeping with the 'protective shield' effect of MUC1 over-expression, as we reported.

Suppression of the C1GT was seen in this study to result in marked increase of cellular Tn expression, as detected by VVA (figure 6 and 8). This indicates that the ultimate formation of cellular TF, Tn, sialyl-Tn and Core 3 glycans are controlled not solely by the activity of the competitive glycosyltransferases. The concentrations of nucleotide sugar-donor and the rate of substrate transport throughout the Golgi have been shown previously to contribute to the expressions of specific glycans. The relative positioning of the glycosyltransferases within the Golgi is also reported to be an

important determinant. Work by Kellokompu and colleagues (94, 95) and by Campbell *et al* (96) has shown that Golgi derangement occurs in epithelial cancers and can be mimicked by agents that block normal Golgi acidification, in both cases leading to increased formation of oncofoetal carbohydrate antigens. Furthermore, the expression and action of ER-localized molecular chaperones can also play a role in the expression of the oncofoetal glycans by controlling the folding and hence the activity of the relevant glycosyltransferases (48). Thus, the overall cellular expression of Tn, sialy-Tn, TF and Core 3 structures are the consequence of a range of complex factors that include competition between the relevant glycosyltransferases, the spatial arrangement of the glycosyltransferases within the Golgi, the availability of nucleotide sugar-donors in the Golgi apparatus and actions of relevant molecular chaperones.

O-linked glycosylation, one of the most abundant post-translational modifications observed within the cell, plays crucial roles in creating and modifying the structure and function of the molecules. MUC1 is overexpressed and abnormally glycosylated in many epithelial cancers. Stable knockdown of C1GlT is shown to lead to increased anoikis in response to suspended culture. We had previously proposed that due to its massive size, overexpression of MUC1 on the surface of epithelial cancer cells, provides a protective microenvironment that prevents activation of anoikis-initiating molecules in response to loss of cell-matrix contact (433). The increased accessibility/activation of antibodies to cell surface molecules Integrin β, e-cadherin and Fas-L following C1GT knockdown indicates that the 'protective environment' of MUC1 is severely compromised after reduction of its *O*-glycosylation.

In a mouse model of breast cancer, C1GT knockdown has been shown to decrease the incidence of tumour development.(84) While, presumably, loss of C1GT should favour formation of truncated glycans and tumor progression, loss of Core 1 glycans may favour formation of Core 3 or 4 structures that correlate with less aggressive tumours (99, 464, 465). This model also disrupted MUC1 expression and impacted downstream effectors, including extracellular signal-regulated kinase (ERK), RAC-alpha serine/threonine-protein kinase (AKT), and phosphoinositide 3-kinase (PI3K) activation (84). This may also account for the observed decrease in tumour incidence. Conversely, overexpression of C1GT in breast cancer cells increased association between MUC1 and β-catenin by promoting the shedding of the MUC1 extracellular domain, which was correlated with increased migratory and invasive behaviour (466). Overexpression of C1GT may also potentiate increased formation of TF structures, as well as potential extension to form sialyl-Lewis moieties associated with metastasis. Interestingly, overexpression of MUC1 in human breast cancer lines as well as murine lines results in decreased expression of the extension enzymes core 2 β1,6-*N*-acetylglucosaminyl transferase 1 (C2GnT1) and ST3 β-galactosidase α-2,3-sialyl transferase 1(ST3Gal1) suggesting that MUC1 can potentiate expression of truncated glycans in a feed forward manner (467).

Expression of specific glycans can play a critical role in the metastatic spread of tumour cells by allowing lectin interactions. Expression of the carbohydrate structures sialyl-Lewis^{X} and sialyl-Lewis^{A} on MUC1 enable the binding of MUC1 to both E-selectin and intercellular adhesion molecule (ICAM)-1 (467). Likewise, glycosylated forms of MUC16 have been shown to bind both E- and L-selectin (468).

As interactions with selectins on endothelial cells and other cell types are critical for extravasation of immune cells from vasculature and subsequent trafficking through tissues, these interactions are proposed to similarly affect extravasation, invasion, and metastasis of tumour cells (105).

5 Investigation of the impact of MUC1 expression and MUC1-galectin-3 interaction on EGFR activation in epithelial cancer cells

5.1 Hypothesis

MUC1 expression and its interaction with galectin-3 influences EGFR activation of epithelial cells in response to EGF

5.2 Aims

1. To assess the influence of the MUC1 intra- and extra-cellular domains on EGF-induced EGFR activation in epithelial cancer cells
2. To access the impact of MUC-1- galectin-3 interaction on EGF-induced EGFR activation.

5.3 Introduction

Amongst the hallmarks of metastasising epithelial tumour cells are the pathological changes in MUC1 (440). In tumour cells, MUC is overexpressed, heavily glycosylated and is without apical polarization. This leads to a MUC1 'protective barrier' around the cell (433). Given the length of MUC1 (up to 200 nm in some cells) and heavy glycosylation; combined with overexpression and loss of apical polarization; the 'protective barrier' leads to reduced ligand accessibility to underlying cell surface molecules (433). It has been hypothesised that this is perhaps the reason why tumour cells have a low immunological profile and are more resistant to pharmacological treatment aimed at cell surface receptors (469).

MUC1 is known to interact with a variety of cellular proteins, through both its intracellular (470) and extracellular domain (79), and has been shown to influence cell signalling essential in cell proliferation (471), adhesion (62, 472) and immunomodulation (473). MUC1 has been shown to interact with ERBB2/Her/Neu, beta catenin (110), growth factor receptor-bound protein-2 (GRB2), son of sevenless homolog 1 (SOS) (75) and has been thought to have a significant effect on cell growth and proliferation through these interactions. The glycosylation profile of MUC1 is particularly important as it influences lectin binding affinity on the MUC1 extracellular domain and influences cell surface ligand accessibility to underlying molecules.

A key protein which has been reported to be associated with MUC1 is Epidermal growth factor receptor (EGFR) (339, 430, 474). EGFR is a member of the ErbB family of receptor tyrosine kinases that includes EGFR/ ErbB1 (Her1), ErbB2 (Her2/c-Neu), ErbB3 (Her3) and ErbB4 (Her4) (219). EGFR is involved in regulation of multiple cellular process including proliferation and survival and its activity is directly linked with tumorigenesis and metastasis (219). EGFR normally exists in an inactive conformation. Binding to its extracellular domain by ligands such as EGF induces EGFR conformation change and enables its interaction with another member of ErbB family proteins to form homo- or hetero-dimers (219). This leads to activation of EGFR tyrosine kinase domain and autophosphorylation of specific tyrosine residues at its cytoplasmic domain. These phosphorylated residues then serve as binding sites for proteins containing Src homology and phosphotyrosine binding domains, leading to activation of downstream signalling pathways such as the Ras/extracellular signal regulated kinase (ERK) pathway, the phosphatidylinositol 3-kinase (PI3) pathway, the Janus kinase/Signal transducer and activator of transcription (JAK/ STAT) pathway (219), crucial in cell proliferation, migration and survival.

In physiological conditions, EGFR activation is tightly regulated by its expression and the availability of binding ligands to ensure that cell proliferation matches tissue requirements for homeostasis. In tumours however EGFR activation is often increased due to either increased EGFR expression, EGFR mutation or increased availability of the EGFR ligand produced by the same cells that express the ErbB receptors or by surrounding cells (413),(414). Aberrant expression of EGFR by

tumours typically also confers a more aggressive phenotype and is an indicator of poor prognosis in several types of epithelial cancer (475-477). Not surprisingly, EGFR is currently a principal target for therapeutic intervention in cancer.

Galectin-3 is a β-galactoside-binding protein expressed by many types of human cells and particularly by epithelial and immune cells. Galectin-3 is distributed in the cytoplasm, nuclei, cell surface, extracellular space and in circulation. Overexpression of galectin-3 commonly occurs in most types of epithelial cancers such as colorectal, breast, lung, prostate, pancreatic, head and neck cancer and melanoma (306). The level of circulating galectin-3 is also markedly elevated (up to 30-fold) in cancer patients and particularly in those with metastasis (478). Overexpression of galectin-3 by cancer cells is increasingly shown to influence cancer cell-cell and cancer-microenvironment communication and contributes to cancer development, progression and metastasis as a result of galectin-3 interaction with an array of galactose-terminated glycans carried by glycoproteins and glycolipids on the cell surface as well as in the extracellular matrix (479).

Recently studies from our group (79) as well as others (480, 481) have revealed that galectin-3 is a natural ligand of MUC1 in epithelial cancer cells. The interaction between galectin-3 and MUC1, via binding of galectin-3 to the oncofetal Thomsen-Friedenreich (T or TF) carbohydrate antigen on MUC1 (79), induces MUC1 cell surface polarization and exposure of the underlying smaller cell surface molecules. This leads to increased cancer cell homotypic aggregation (25) and cancer cell heterotypic cell

adhesion to vascular endothelium (337), two important steps in the cancer metastasis cascade. As MUC1 is also associated with EGFR in epithelial cancer cells, the effect of galectin-3 on MUC1 cell surface localization led us to examine the impact of galectin-3-MUC1 on EGFR activity in epithelial cells.

5.4 Methods

Immunoblotting

Cellular proteins (cell lysate or immunoprecipitates) separated by SDS-PAGE were electro-transferred to 0.2 µm nitrocellulose membrane. The membranes were first incubated with specific primary antibodies [anti-p-EGFR (SC-23420), EGFR (SC-03), anti- pERK (SC-7383) and ERK (SC-94) at a concentration of 1:500. Antibodies against MUC1 (B27.29 and CT2) or actin at a concentration of 1:5000 were applied for 16 hr at 4^0C. The blots were washed 3 times with 0.05% Tween-20 in TBS before incubated with peroxidase-conjugated secondary antibody (1: 3000) for 1 hour. After 6 washes with 0.05% Tween-20 in TBS, the protein bands were developed using chemiluminescence Super Signal kit and visualized with Molecular Imager® Gel Doc™ XR System (Biorad). The density of the protein bands was quantified using Imagelab version 3.0.1.

EGFR activation

Sub-confluent cells were incubated in serum-free medium containing 0.5 mg/ml BSA overnight. The cells were washed with PBS before incubation with EGF (20ng/ml), EGF (20ng/ml) and galectin-3 (2 µg/ml), galectin-3 (2 µg/ml), galectin-3C (2µg/ml) or

BSA (2 μg/ml) (control) in the absence or presence of EGFR inhibitor lapatinib (2mM) for various time at 37^0C and 5% CO_2. The cells were washed immediately with ice cold TBS before lysed with SDS- sample buffer and analysed by immunoblotting.

5.5 Results

5.5.1 MUC1 extra- and intra-cellular domains both contribute to EGFR activation

Interaction between MUC1 and EGFR has been shown to influence EGFR activity in breast cancer (338), endometrial cancer (339), non-small cell lung cancer cells (482) by unknown mechanisms. In this study, we first assessed the influence of MUC1 expression on EGFR activation in human breast epithelial and colon cancer cells and then tested the influence of MUC1 intra- and extra-cellular domains to the effect.

Following simulation by EGF, EGFR activation was significantly increased in MUC1 transfected HCT116 MUC1-full and HCA1.7+ cells when compared to MUC1 negatively transfected controls HCT116 Muc1-neo and HCA1.7- cells. The HCT116 MUC1 full cells and HCA1.7+ cell react differently when subjected to 20 ng/ml of EGF with the highest EGFR phosphorylation being noted at 10 minutes for HCA1.7+ cells and 60 minutes for HCA1.7 MUC1 full cells. The EGFR phosphorylation almost reaches baseline level after 120 minutes of 20 ng/ml EGF simulation (figure 5.1 and 5.2). Comparing EGFR phosphorylation with EGFR level in MUC1 positive and negative transfected cells at different timepoints following EGF stimulation, EGFR is noted to be rapidly phosphorylated in full length MUC1-expressing cells HCT116 MUC1 full and HCA1.7+ but almost no EGFR phosphorylation is detected in control MUC1-

negative cells HCT116 MUC1 neo and HCA1.7- (Fig 5.1 and 5.2). In comparison to MUC1- negative cells, a 26-fold and 19-fold increase of EGFR phosphorylation were observed at 10 min and 60 min, respectively, for HCT116 MUC1 full and HCT1.7+ cells (Fig 5.1B and 5.2B).

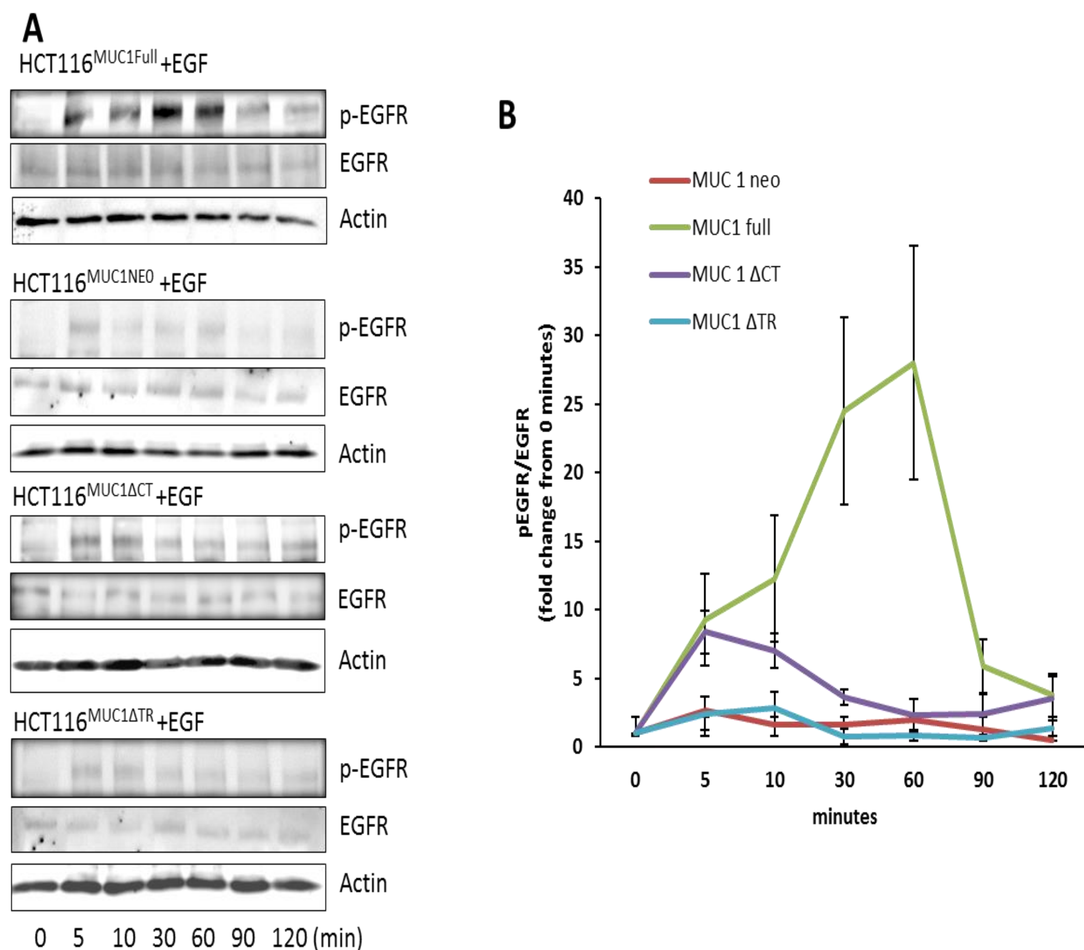


Figure 5.1: Both MUC1 extra and intra cellular domains influence EGF induced EGFR activation in colon cancer epithelial cells. MUC1 transfectants of human colon cancer (HCT116 cells) cells were treated with 20 ng/ml EGF for various times before EGFR phosphorylation was analysed by immunoblotting. The blots were also probed with anti-EGFR antibody to detect EGFR level and anti-actin antibody for protein loading. Densitometry scanning of the bands from three independent experiments is shown in B as fold-changes (mean ± SD) of p-EGFR/EGFR ratio. The cells transfected with full-length MUC1 showed rapid EGFR phosphorylation while MUC1 negative cells showed little response. Depletion of MUC1 extracellular domain had a bigger effect on MUC1 mediated EGFR phosphorylation compared to MUC1 intracellular domain deletion. Representative blots are shown in A. (N=

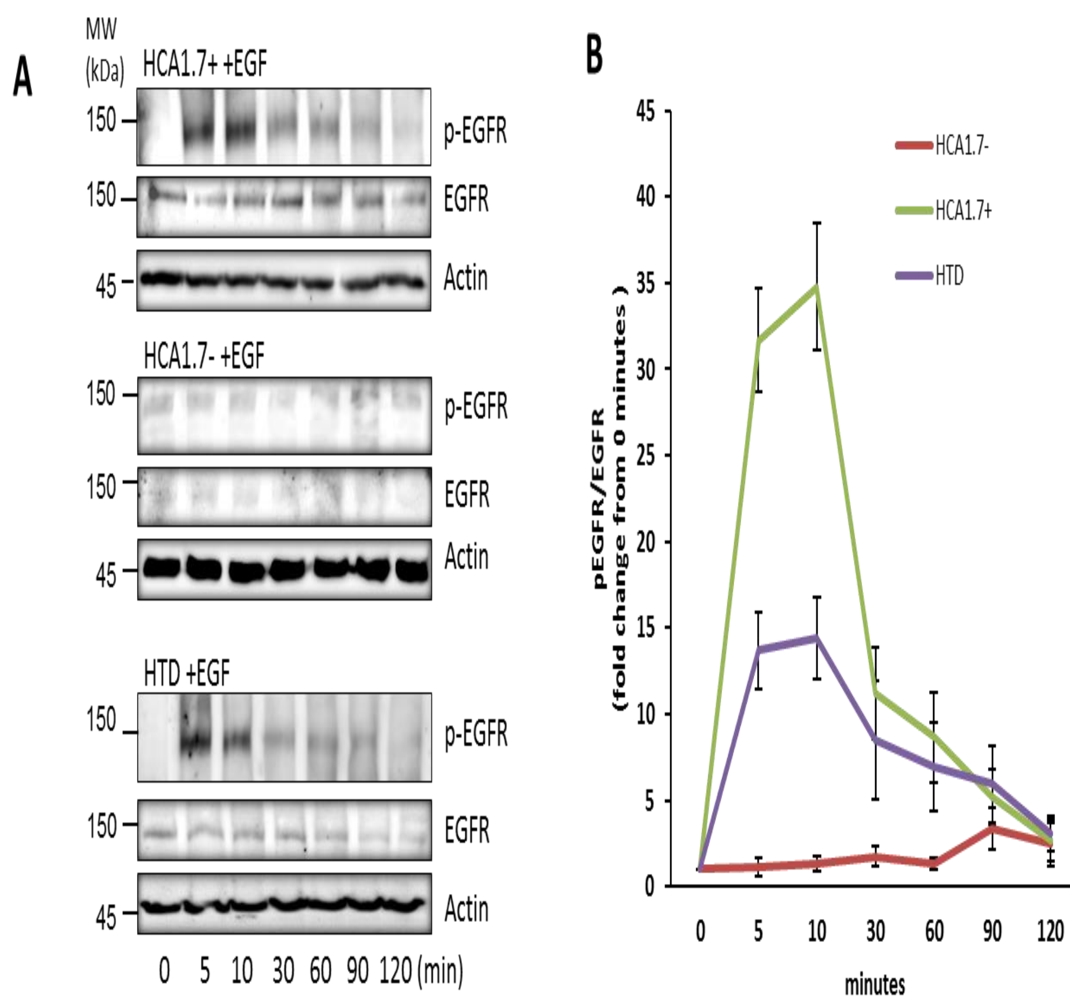


Figure 5.2: The extracellular domain of MUC1 has a significant influence on EGF induced EGFR activation in human breast epithelial cells. MUC1 transfectants of Breast epithelial cells (HBL100) were treated with 20 ng/ml EGF for various times before EGFR phosphorylation was analysed by immunoblotting. The blots were also probed with anti EGFR antibody to detect EGFR level and anti-actin antibody for protein loading. Densitometry scanning of the bands from three independent experiments is shown in B and is shown as fold-changes (mean ± SD) of p-EGFR/EGFR ratio. Like the HCT16 cells, HBL100 cells transfected with full-length MUC1 showed rapid EGFR phosphorylation while MUC1 negative cells showed little response. Depletion of MUC1 intracellular domain had a moderate effect on EGFR phosphorylation, with levels at early time points multiple folds higher than the MUC1 negative variants; suggesting that following simulation by EGF, MUC1 extracellular domain has a bigger role in MUC1 mediated EGFR activation than initially thought. Representative blots are shown in A. (N=2)

Depletion of the MUC1 extracellular domain almost completely inhibited EGFR activation in HCT116 MUC1 ΔTR (Fig 5.1A and 5.1B) with MUC1 ΔTR cells showing similar level of EGFR activation as HCT116 MUC1 neo cells. Depletion of the MUC1 intracellular domain resulted in less but still substantial EGFR phosphorylation in HTD and HCT116 MUC1 ΔCT. To get an indication of the level of contribution from different domains of MUC1, at 10-minute time points following the addition of 20 ng/ml EGF, EGFR activation was recorded to be 12-, 7-, 2.8- and 1.64-fold higher in HCT116 MUC1 full, HCT116 MUC1 ΔCT, HCT116 MUC1 ΔTR and HCT116 MUC1 neo cells, respectively, compared to 0-time point. Although we did not have a MUC1 ΔTR variant for HBL100 transfected cells, we did notice similar intermediate level of EGFR activation for MUC1ΔCT variant when compared to MUC1 full and MUC1 neo variant as noted for HCT116 MUC1 transfected cells. At 10-minute time point following the addition of 20 ng/ml EGF, there was a 34.7-, 14.4- and 1.34- folds increase in EGFR activation in HCA1.7+, HTD and HCA1.7- cells respectively when compared to 0-time point (figure 5.2).

Collectively, these results suggest that expression of MUC1 is critical to EGF-induced EGFR activation and both the MUC1 intra- and extra-cellular domains contribute to the MUC1-associated increase of EGFR activity with predominant influence coming from the MUC1 extracellular domain. We had previously shown that galectin-3 is able to polarise MUC1 on the cell surface in both MUC1 full and MUC1 ΔCT variant cells (chapter 3). It has been proposed by ourselves (25) and by others (483) that polarization of MUC1 on the cell surface leads to exposure of underlying cell surface

molecules. Since MUC1 expression affects EGF-induced EGFR activation, we were interested in what effect, if any, galectin-3 mediated MUC1 polarization has on EGFR activation in our MUC1 transfected cells.

We initially began by testing the effect of EGF on mutant MUC1 transfected cells. Once again, our results showed that the expression of MUC1 is associated with increased activation of EGFR compared to MUC1 neo transfected variant (figure 5.3 compared to 5.4, 5.6. compared to 5.7) and this effect is lower in MUC1 ΔCT cells (figure 5.3 compared to 5.5). There is evidence in literature which shows galectin-3 interacting with EGFR (474, 484, 485). There have also been speculations that perhaps galectin-3 may act as a ligand for EGFR, leading to its activation. We also tested this theory across our MUC1 transfects. Addition of 2 µg/ml galectin-3 alone had no effect on EGFR activation on any of MUC1 full transfected HCT116MUC1 full and HCA 1.7+ cells. The same was also noted for HCT116MUC1 neo, HCA1.7- and HDT cells, suggesting that galectin-3 on its own is not able to activate EGFR.

When galectin-3 was added in conjunction with EGF, galectin-3 presence caused substantially further (53 fold at 5 min) increase of EGFR activation to the MUC1-positive HCA1.7+ cells (Figure 5.3B) but had no effect to the MUC1-negative HCA1.7- cells (Figure 5.5 B). Similar results were observed with colon cancer HCT116 cells. When 20ng/ml EGF and 2 µg/ml galectin-3 were both introduced, a 30-fold further increase of EGFR activation was observed in the MUC1-positive HCT116 MUC1 full than that treated with EGF alone (Figure 5.6 A and B). MUC1 ΔCT transfected HTD

cells also showed similar effect. In comparison to HTD cells treated with EGF alone, the presence of galectin-3 along with EGF showed to cause 30-fold more increase of EGFR activation in response to EGF in the MUC1-cytoplamic domain depleted HTD cells (Figure 5.5 A and B).

Comparing the activation of EGFR receptor in MUC1 full and ΔCT cells following simulation by either EGF alone or EGF in conjunction with galectin-3, galectin-3 addition with EGF seems to not only enhance but also prolong EGFR activation. The EGFR phosphorylation was mostly higher at later time points, in cells treated with both EGF and galectin-3, compared to cells treated with EGF alone. For example, the EGFR phosphorylation in HCA1.7+ cells treated with both galectin-3 and EGF at 90 minute is 13.1 fold higher than 0 hour, which is higher than the 10.4 fold change noted for the same cells when treated with EGF alone at 60 minutes (figure 5.3 B). Similarly, for HTD cells EGFR phosphorylation for cells treated with both galectin-3 and EGF at 90 minute is 14.7 fold higher than 0 hour, which compares to 15.3 % fold change noted for the same cells when treated with EGF alone at 10 minutes (figure 5.5 B).

Collectively these data show that galectin-3 on its own is not able to activate EGFR activation but enhances and prolongs EGFR activation induced by EGF.

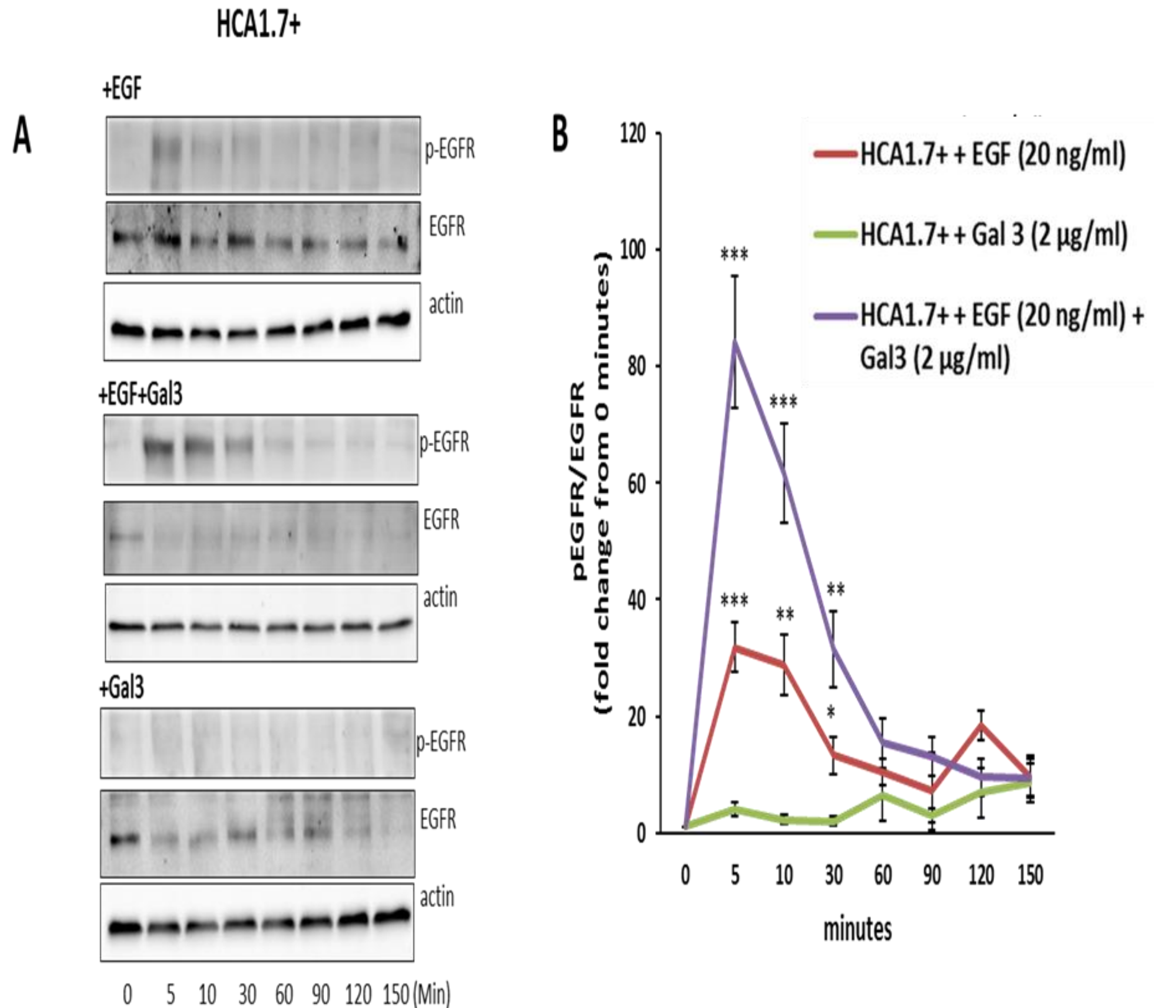


Figure 5.3: Galectin-3 MUC1 interaction enhances EGFR activation in MUC1 full transfected breast epithelial cells. MUC1 full transfected HBL100 cells (HCA1.7+) were simulated with either 20 ng/ml EGF, 2 µg/ml galectin-3 or a combined 20 ng/ml EGF and 2µg/ml galectin-3 for various time points before EGFR phosphorylation was analysed by immunoblotting. The blots were also probed with anti EGFR antibody and anti-actin antibody for protein loading. Densitometry scanning of the bands from three independent experiments is shown in B as fold-changes (mean ± SEM) of p-EGFR/EGFR ratio. A combined simulation with both EGF and galectin-3 enhanced and prolonged EGFR activation compared to simulation by EGF alone. Galectin-3 alone had very minimal effect on EGFR activation, suggesting that galectin-3 in presence of EGF promoted MUC1 mediated EGFR activation but not galectin-3 on its own. (N=3). Two-way ANOVA with Sidak post-hoc test (*P<0.05, ***P<0.001 and ****P<0.0001 vs untreated control at the same time point).

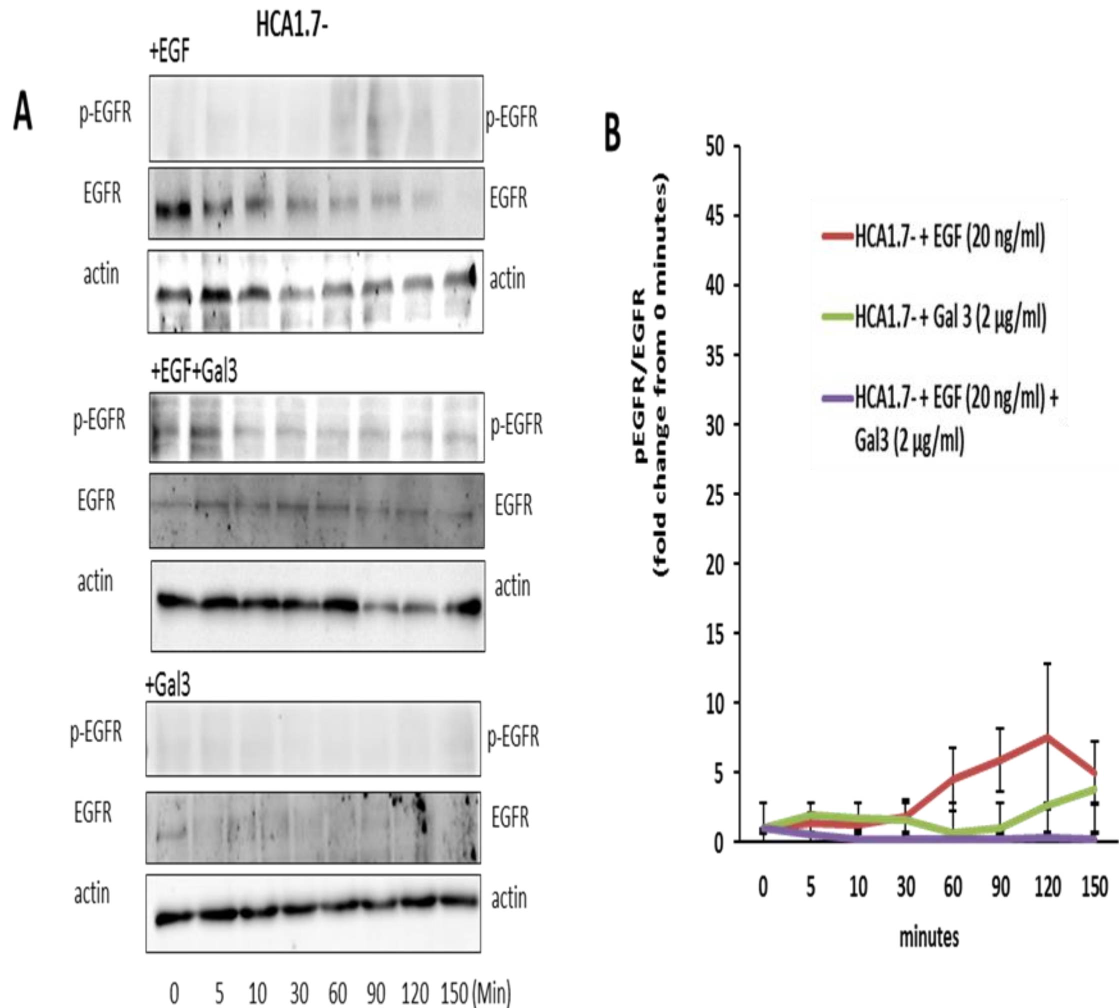


Figure 5.4: MUC1 negatively transfected breast epithelial cells have very minimal EGFR activation. Compared to HBL100 MUC1 positive cell (figure 5.3) HBL100 MUC1 neo cells showed minimal increase in EGFR activation following simulation with 20 ng/ml EGF. Addition of 2 µg/ml galectin-3 with EGF did not enhance EGF-induced EGFR activation. Galectin 3 on its own had the least effect on EGFR activation. The fact that negatively transfected MUC1 cells had minimal effect on EGFR activation suggest that EGFR activation is boosted by the presence of MUC1 and MUC1-galectin-3 interaction enhances and prolongs this effect. Representative blots are shown in A and densitometry scanning of the bands from three independent experiments is shown in B, as fold-changes (mean ± SEM) of p-EGFR/EGFR ratio. (N=3)

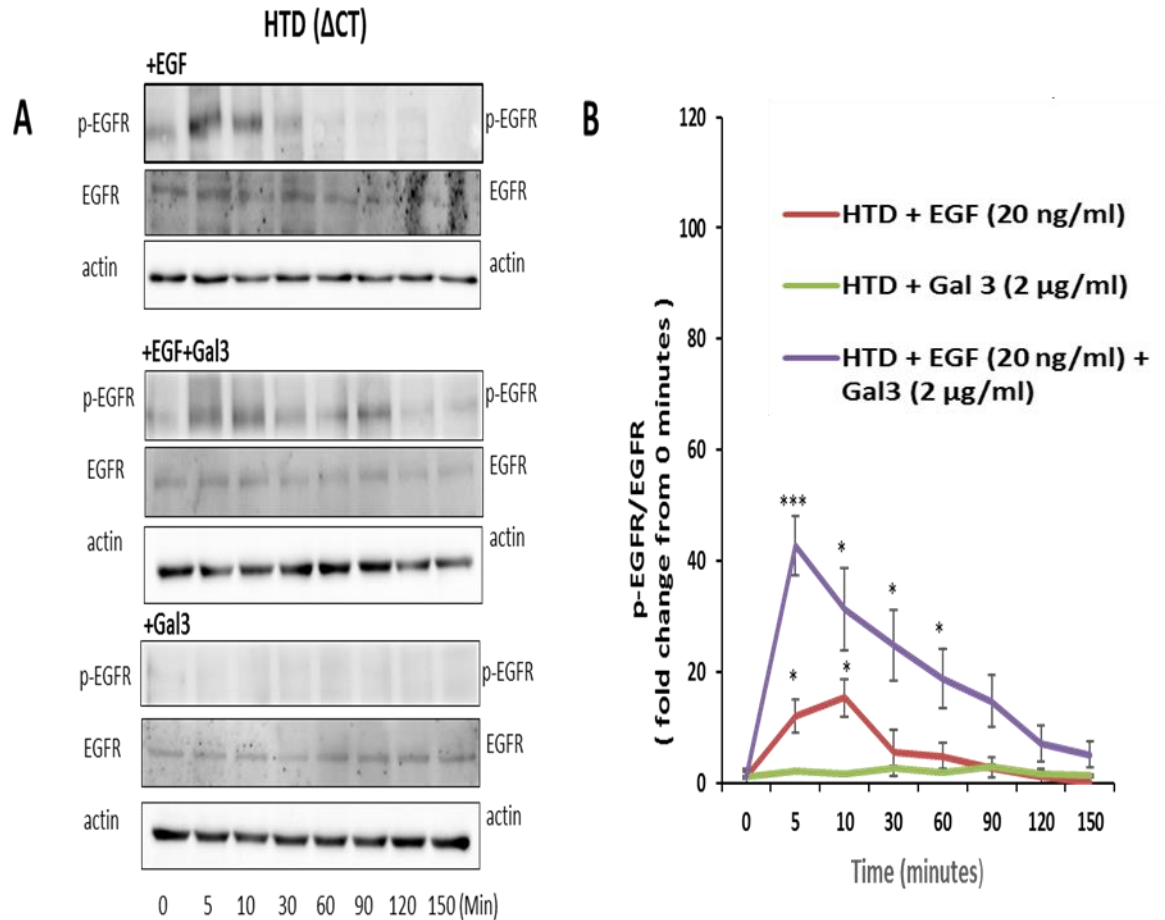


Figure 5.5: Galectin-3 interaction with MUC1 extracellular domain increases EGFR activation. HBL100 cells transfected with MUC1 ΔCT had a much higher EGFR activation, compared to MUC1 neo transfectant when simulated with 20 ng/ml of EGF. Simulation by both 2 µg/ml galectin-3 and 20 ng/ml EGF further enhanced this effect, with galectin-3 on its own having no effect, Representative blots are shown in A and densitometry scanning of the bands from three independent experiments is shown in B, as fold-changes (mean ± SEM) of p-EGFR/EGFR ratio. (N=3). Two-way ANOVA with Sidak post-hoc test (*P<0.05, ***P<0.001 and ****P<0.0001 vs untreated control at the same time point).

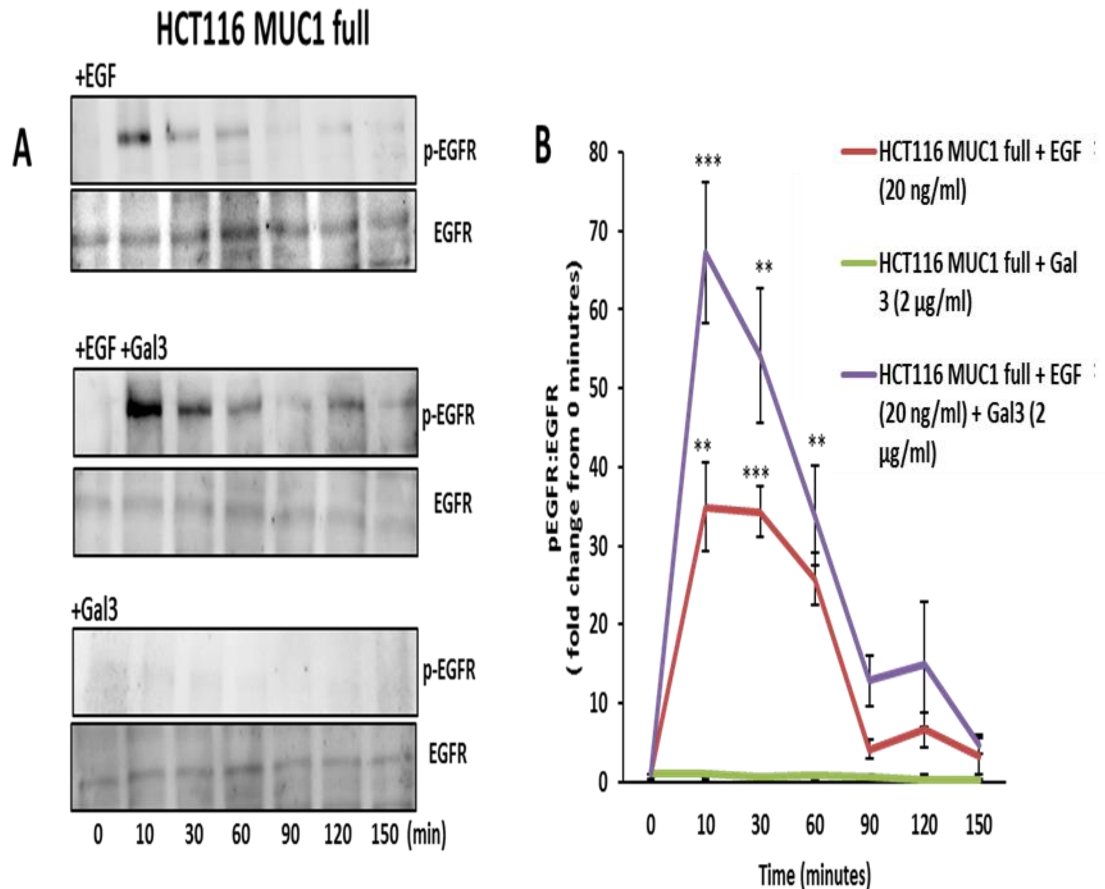


Figure 5.6: Galectin-3 MUC1 interaction enhances EGFR activation in MUC1 full transfected colon cancer cells. MUC1 full transfected HCT116 cells (HCT116 MUC1 full) were simulated with either 20 ng/ml EGF, 2 µg/ml galectin-3 or a combined 20 ng/ml EGF and 2 µg/ml galectin-3 for various time points before EGFR phosphorylation was analysed by immunoblotting. The blots were also probed with anti-EGFR antibody and anti-actin antibody. Densitometry scanning of the bands from three independent experiments is shown in B as fold-changes (mean ± SEM) of p-EGFR/EGFR ratio. Like the effect noticed in HCA1.7+ cells (figure 5.4) a combined simulation with EGF and galectin-3 enhanced EGFR activation compared to simulation by EGF alone. Galectin-3 had very minimal effect on EGFR activation (N=3). Two-way ANOVA with Sidak post-hoc test (*P<0.05, ***P<0.001 and ****P<0.0001 vs untreated control at the same time point).

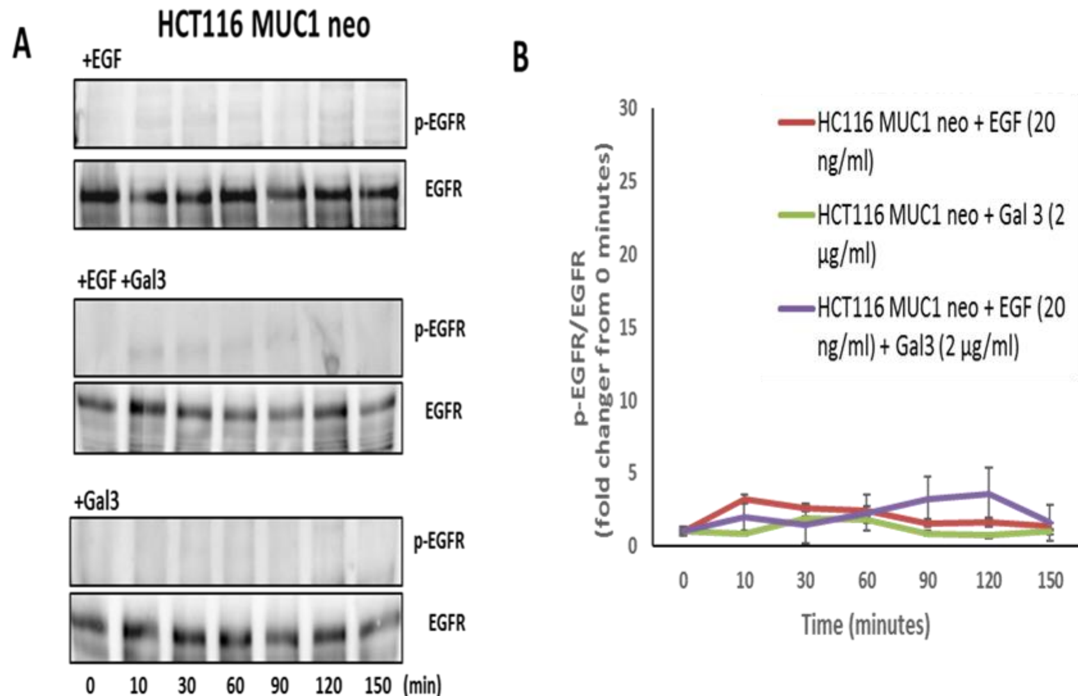


Figure 5.7: MUC1 negatively transfected HT116 MUC1 neo cells showed much lower EGFR activation compared to MUC1 full version simulated with EGF. HCT116 MUC1 neo cells showed small increase in EGFR activation following simulation with 20 ng/ml EGF. Addition of 2 µg/ml galectin-3 with EGF did not enhance the EGFR activation. Galectin 3 on its own had no effect on EGFR activation. Representative blots are shown in figure A and densitometry scanning of the bands from three independent experiments is shown in B, as fold-changes (mean ± SEM) of p-EGFR/EGFR ratio. N=3

5.5.2 MUC1 transfection has no effect on EGFR expression

There is evidence in the literature to suggest that MUC1 expression influences EGFR expression (339) and a reciprocal effect was also reported in uterine adenocarcinoma and pancreatic cancer origins, where activated EGFR drive high level MUC1 expression (430). Given the dramatic effect noted on EGFR activation across our MUC1 transfected cells, we also tested if MUC1 expression or MUC1 domain deletion influenced EGFR expression in our transfected cells.

Lysates from HCT116 MUC1 full, MUC1 neo, MUC1ΔCT and MUC1ΔTR cells and HCA1.7+, HCA1.7- and HDT cells were probed with anti-EGFR antibody to check for EGFR expression level (figure 5.8). The same lysates were probed for actin to ensure equal loading. When adjusted for actin, our result shows similar level of EGFR expression across the different transfects, suggesting that MUC1 transfection had little to no effect on EGFR expression on these cells.

This, combined with the other data in this chapter, MUC1 expression increases EGF-induced EGFR activation in epithelial cancer cells.

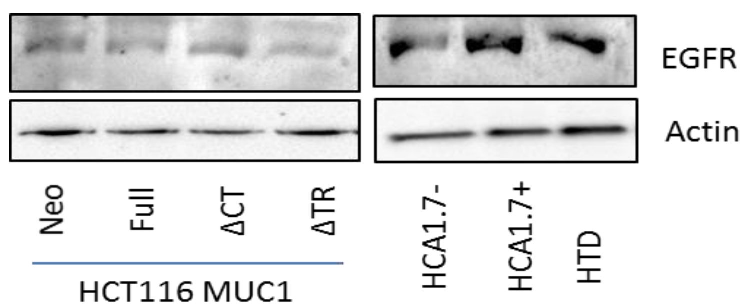


Figure 5.8: MUC1 transfection does not influence EGFR expression. Lysates from HCT116 MUC1 transfectants and HBL 100 MUC1 transfectants were immunoblotted and probed with anti EGFR and anti actin antibody. EGFR expression were the same across the MUC1 transfectants of the same cell type, suggesting that MUC1 transfection has minimal to no effect on EGFR expression. this suggests that difference in EGFR activation noted across different MUC1 transfectants is not due to altered EGFR activation in these cells. Representative image from 2 blots.

5.6 Summary of Results

1. MUC1 expression increases EGF-induced EGFR activation in MUC1-positive human colon HCT116 MUC1 full and breast HCA1.7+ cells

2. Both MUC1 intracellular and extracellular domains contribute to MUC1 mediated EGFR activation. However, the greater influence is from the MUC1 extracellular domain.

3. The presence of galectin-3 with EGF increases and prolongs EGFR activation in HCT116 MUC1 full, HCA1.7+ and HTD cells but not in MUC1-negative cells.

5.7 Discussion

This part of the study shows that EGFR activation in response to EGF binding in human breast and colon epithelial cells is substantially increased by expression of the transmembrane mucin protein MUC1. Both the MUC1 intracellular and extracellular domains are shown to contribute to the effect of MUC1 on EGFR activation but the predominate influence comes from the MUC1 extracellular domain. Addition of a combination of EGF and galectin-3 leads to a further increase in EGFR activation and this effect is seen in MUC1 ΔCT cells.

MUC1 is a transmembrane mucin protein and is ubiquitously expressed on the surface of epithelial cells. Over-expression of MUC1 is a common feature of epithelial cancer cells (442). MUC1 is reported to be interacting with EGFR in epithelial cancer such as breast (338, 425), pancreatic (474), endometrial (339) and lung. Blocking MUC1-C dimerization or silencing MUC1-C expression has been shown previously to suppress EGFR activation-associated cell signalling and survival in non-small cell lung cancer cells (440). Interaction of MUC1 with EGFR in the nucleus of breast epithelial cancer cells was shown to promote accumulation of chromatin-bound EGFR and co-localization of EGFR with phosphorylated RNA polymerase II (338). This study shows that MUC1 expression increases EGF-induced EGFR activation in human breast and colon cancer cells. Depletion of either the MUC1 intracellular or extracellular domain could only partly abolish MUC-associated effect on EGFR activation. This suggests that while both the MUC1 cytoplasmic and extracellular domains contribute to EGFR activation, the effect of MUC1 intra- and extra-cellular domain on EGFR activation

can be considered relatively independent. The fact that depletion of the MUC1 extracellular domain resulted in bigger reduction of MUC1-associated EGFR activation than depletion of the MUC1 intracellular domain indicates that a predominate influence of MUC1 on EGFR may derive from its extracellular domain. MUC1 (486) and EGFR (487) have both been shown to be associated with lipid rafts on cell membrane. It is possible that the expression of MUC1 and its association with EGFR in the lipid raft on the cell surface may increase the proximity of inactive forms of EGFR in the lipid raft microdomains for them to be in a better position to form homo- and hetero-dimers in response to ligand banding.

Galectin-3 is a chimera type galectin that binds to the Thomson-Friedenreich antigen (galactose beta 1,3 N acetyl galactosamine) on the MUC 1 extracellular domain. Earlier studies (25, 79) and studies presented in Chapter 3 have shown that galectin-3 binding to MUC1 leads to MUC1 cell surface polarization. Polarization of MUC1 exposes underling cell surface molecules. Galectin-3 addition only has an effect on EGFR activation in the presence of an EGFR ligand. The enhanced EGFR activation by galectin-3 only in MUC1 positive but not negative cells F suggests that this effect is likely linked to change of MUC1 cell surface localization in response to galectin-3 binding. It is possible that galectin-3 mediated MUC1 polarization leads to a better accessibility of EGFR ligands (e.g EGF) to EGFR on cell surface leading to EGFR activation or perhaps MUC1 polarization on the cell surface enables EGFR receptors to come in close proximity to each other through MUC1-EGFR interaction, leading to

closer association and therefore a better chance to dimerize and phosphorylate following ligand binding.

The effect of galectin-3 on EGFR signalling is rather controversial. It has been reported that galectin-3 expression enhances EGFR activation in mouse keratinocytes (485); in contrast, galectin-3 negatively regulates EGFR and ERK activation in a pancreatic cancer cell line, Capan-1 (474). Recent work done by Kuo *et al* (484) has shown that galectin-3 plays a positive role in EGFR activation in lung cancer cells., the effect of galectin-3 on the EGFR signalling pathway thus might be related to cell types. EGFR activity has been reported to be regulated by cell glycosylation (488). Galectin-3 has been shown to interact with EGFR through β-galactoside-containing polysaccharide chains of EGFR (489). Kuo *et al*, has therefore proposed that the differential effects of galectin-3 on EGFR activation across different cell lines might be due to the different glycosylaytion status of EGFR in those cells. Our results here point towards a complex interaction between galectin-3, MUC1 and EGFR leading to enhanced EGFR activation in cell response to EGF.

6 Investigating the mechanism of the effect of MUC1-galectin3 interaction on EGFR activation

6.1 Hypothesis

MUC1-galectin-3 interaction-associated EGFR activation may be associated with change of MUC1 cell surface localization.

6.2 Aim

To determine the molecular mechanism and downstream signalling involved in the effect of MUC1-galectin3 interaction on EGFR activation.

6.3 Introduction

Epidermal growth factor receptor (EGFR), also known as ERbB1, HER1 in humans, belongs to the ERbB family of receptor tyrosine kinases that contain 4 closely related members ERbB1-4. These transmembrane receptors couple the binding of extracellular growth factor ligands to intracellular signalling pathways that regulate various biological responses, including proliferation, differentiation, migration, adhesion and cell survival. At present, six EGFR ligands are known. These are Epidermal growth factor (EGF), Amphiregulin, Transforming growth factor alpha (TGF-alpha), Betacellulin, Heparin binding EGF-like growth factor (HB-EGF), and Epiregulin.

EGF is a high affinity ligand and is the most studied ligand of EGFR. Binding of EGFR ligand to the extracellular domain of ERbB leads to receptor homo-or hetero-dimerization. ErbB2 is a unique member of the ERBB family in that it does not bind any of the known ligands with high affinity. However, it is the preferred heterodimeric partner for other EGFRs (360, 490). The ligand induced receptor dimerization leads to autophosphorylation of tyrosine residues intracellularly, creating docking sites for various membrane-targeted proteins. Adapter proteins such as the SHC transforming protein 1 (Shc), Growth factor receptor-bound protein 2 (GRB2), Cas-Br-M ecotropic retroviral transforming sequence (c-Cbl), Docking protein 2 (DOK2) and NCK adaptor protein (NCK1) along with enzymes such as Phospholipase C gamma 1 (PLC-gamma 1), v-Src sarcoma viral oncogene homolog (c-Src) and PTK2 protein tyrosine kinase 2 (FAK1) are known to bind to EGFR phosphotyrosine residue. Following ligand mediated EGFR phosphorylation, these

adapter proteins lead to a signalling cascade which leads to cell proliferation and growth.

Following the signal transduction by GBR2, GBR2 along with the adapter Shc, recruits the exchange factor SOS to form a complex consisting of Shc, GRB2 and SOS. Activated SOS activates small GTPase v-Ha-ras Harvey rat sarcoma viral oncogene homolog (H-Ras) by its conversion from the inactive GDP-bounding state to the active GTP-bounding state. The activated H-Ras stimulates v-Raf-1 murine leukemia viral oncogene homolog 1 (c-Raf-1) and Mitogen-activated protein kinase kinase 1 and 2 (MEK1 and MEK2) which subsequently activates Mitogen-activated protein kinase 1 and 3 (ERK1/2) kinase cascade. ERK activates a number of transcriptional regulators to induce cell growth and proliferation such as transcription factors ELK1, v-Myc myelocytomatosis viral oncogene homolog (c-Myc) and v-Fos FBJ murine osteosarcoma viral oncogene homolog (c-Fos). GRB2 also recruits PI3Ks, which convert Phosphatidylinositol-4,5-bisphosphate (PIP_2) to Phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 binds to AKT and recruits it to plasma membrane where activated AKT, through phosphorylation via PDK1, regulates the activity of various proteins that mediate cell survival.

As well as the signalling cascade described above, EGFR activation also leads to generation of Inositol trisphosphate (IP_3) and 1,2-Diacylglycerol (DAG). IP_3 induces the release of Ca2+ from the endoplasmic reticulum to activate calcium-regulated pathways. DAG activates protein kinase C pathway (PKC). One of the signalling modules regulated by PKC in EGFR pathway is the NFkB signalling, NFκB is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell

survival. EGFR also translocates from the plasma membrane to other cellular compartments including nucleus where it directly regulates the expression of several genes in cooperation with other transcriptional regulators such as STATs, PCNA and E2F family of proteins.

As a growth, differentiation and cell survival factor; mutations and overexpression of EGFR and/or the defective regulation of its signal transduction pathways is associated with oncogenesis. Aberrant expression of EGFR by tumors typically also confers a more aggressive phenotype and has been shown to be an indicator of poor prognosis in several types of epithelial cancer. Breast carcinoma, non-small cell lung carcinoma and colon carcinomas have all been shown to have high EGFR expression coupled with a higher expression of EGFR ligands (219). In glioblastoma multiforme tumors, EGFR is often mutated in the intracellular domain rendering the tyrosine kinase constitutively active (491). In addition, EGFR expression and mutation in tumor cells is often accompanied by production of transforming growth factor alpha (TGF-alpha) or other EGF family ligands (492). Thus, EGFR and its signalling components are promising targets for effective therapy for various cancers.

MUC1 is known to interact with various cellular proteins, through both its intracellular and extracellular domains, and influences diverse signalling pathways that are important in cell proliferation, adhesion and immunodeficiency. EGFR is one of the proteins which has recently been reported to interact with MUC1. A potential link between MUC1 expression and EGFR activation was shown in the last chapter, which showed that MUC1 expression and presence of its extracellular domain, promotes EGF-induced EGFR activation. Presence of galectin-3, a natural ligand for

MUC1, along with EGF led to a significant increase and prolonged EGFR activation. The aim of this chapter is to investigate the molecular mechanism for the impact of MUC1-galectin-3 interaction on EGFR activation.

6.4 Methods

Cell surface protein crosslinking

Sub-confluent cells were incubated in serum-free medium overnight. The cells were washed twice with Ca^{2+} and Mg^{2+} free PBS and then treated with serum free media containing BSA 2μg/ml (control), EGF (20 ng/ml) without or with galectin-3 (2 μg/ml) or galectin-3C (2 μg/ml) for 10 minutes at 37^0C and 5% CO_2. The cells were then washed with ice-cold Ca^{2+} and MG^{2+} free PBS and incubated with 3mM BS3 cross-linker in Ca^{2+} and Mg^{2+} free PBS on ice for 20 minutes. Excess BS3 was quenched with 250 mM glycine in PBS for 5 minutes at 4^0C. The cells were washed three times with ice-cold PBS, lysed in SDS-sample buffer and analysed by immunoblotting with antibodies against EGFR.

Confocal microscopy

Sub-confluent cells grown on glass coverslips in 24-well plates were incubated in serum-free medium at 37^0C overnight. The cells were treated with BSA (2 μg/ml) (control), EGF (20 ng/ml) without or with galectin-3 (2 μg/ml) for 10 minutes at 37^0 C. The cells were washed with ice cold PBS and fixed with 4% paraformaldehyde. The cells were washed with PBS and probed with anti-MUC1 B27.29 (1 μg /ml) or anti-

EGFR (D38B1) (2 µg/ml) for 2 hours at room temperature. After two washes with PBS, FITC-conjugated anti-mouse or Alexa fluor 643 conjugated anti-rabbit antibodies was applied for 1 hour at room temperature. The cells were washed twice before mounted using DAPI-containing fluorescent mounting media (Vector Laboratories, Burlingame, CA). The slides were analysed with 3i confocal microscope (Marianas SDC, 3i Imaging) and Slidebook 6 Reader version 6.0.4 (Intelligent-imaging).

Immunoprecipitation

Sub-confluent cells were incubated in serum-free medium containing 0.5 mg/ml BSA overnight. The cells were incubated in TBS with EGF (20 ng/ml), EGF (20 ng/ml) and galectin-3 (2 µg/ml), galectin-3 (2 µg/ml) or 20 ng/ml BSA (control) in serum-free media for 10 min at 37^0 C. The cells were washed with ice-cold PBS, scraped and collected into 1 ml PBS containing 1% triton-100 and protease inhibitors (Calbiochem). The cells were lysed on ice for 30 min followed by centrifugation at 10,000 *g* at 4^0C for 15 minute. The supernatants were collected and pre-cleared by adding 20 µl of the protein A/G beads and incubating at 4^0C for 30 minutes with gentle agitation. One ml lysate (protein concentration 2 mg/ml) were incubated with B27.29 (1 µg/ml), anti-EGFR (DB81) (2 µg/ml) or isotype-matched normal IgG at 4^0C with continuous agitation for 16 hours. 30 µl of protein A/G plus agarose beads were added for 4 hr and the beads were washed five times at 4^0C with ice-cold PBS. Proteins were eluted from the beads by boiling in SDS-sample buffer for 10 minutes before applied to SDS-PAGE and subsequent immunoblotting.

6.5 Results

6.5.1 MUC1-galectin-3 interaction-induced EGFR activation increases downstream ERK1/2 signalling

It is known that EGFR activation on the cell membrane triggers an array of intracellular signalling pathways (493-495). One of the common signalling pathways triggered by EGFR activation is ERK signalling (496-498). To test whether galectin-3-MUC1 interaction induced EGFR activation effectively transduces EGFR signalling to downstream EGFR effectors, we assessed ERK activation in cell response to EGF and galectin-3 in the MUC1- positive and -negative cells.

Introduction of EGF to the cells induced rapid ERK1/2 phosphorylation in MUC1-positive HCA1.7+ (Figure 6.1A). This increase of ERK1/2 phosphorylation peaked at 10 min at which point a 3.6-fold increase of ERK1/2 phosphorylation was observed in HCA1.7+ cells. Introduction of EGF also induced ERK1/2 phosphorylation of the MUC1 negative HCA1.7- and HCT116 MUC1 neo cells but to a much lower level in comparison to the MUC1-positive cells (Figure 6.1 B), consistent with the effect of MUC1 expression on EGFR activity recorded in chapter 3. At 10 min, a 1.9-fold increase of ERK1/2 phosphorylation was observed in HCA1.7- cells. When galectin-3 was introduced, EGF showed a stronger (1.9- fold further increase at 10 min) and also prolonged ERK1/2 phosphorylation in the MUC1-positive HCA1.7+ (Figure 6.1A), while ERK1/2 phosphorylation in the MUC1-negative HCA1.7- (Fig 6.1B) cells remained the same as the cells treated with EGF alone. Moreover, without the

presence of EGF, introduction of galectin-3 alone did not show any influence on ERK1/2 phosphorylation.

Further work done in our lab showed a similar effect for MUC1 positive HCT116 MUC1 full and MUC1 negative HCT116 MUC1 neo cell (499). In contrast to the enhanced ERK1/2 activation by the full-length galectin-3/EGF in the MUC1 positive cells, it was also noticed that introduction of C-terminally-truncated galectin-3 form (galectin-3C) with EGF showed no further effect on ERK-1/2 phosphorylation in comparison to the cells treated with EGF alone.

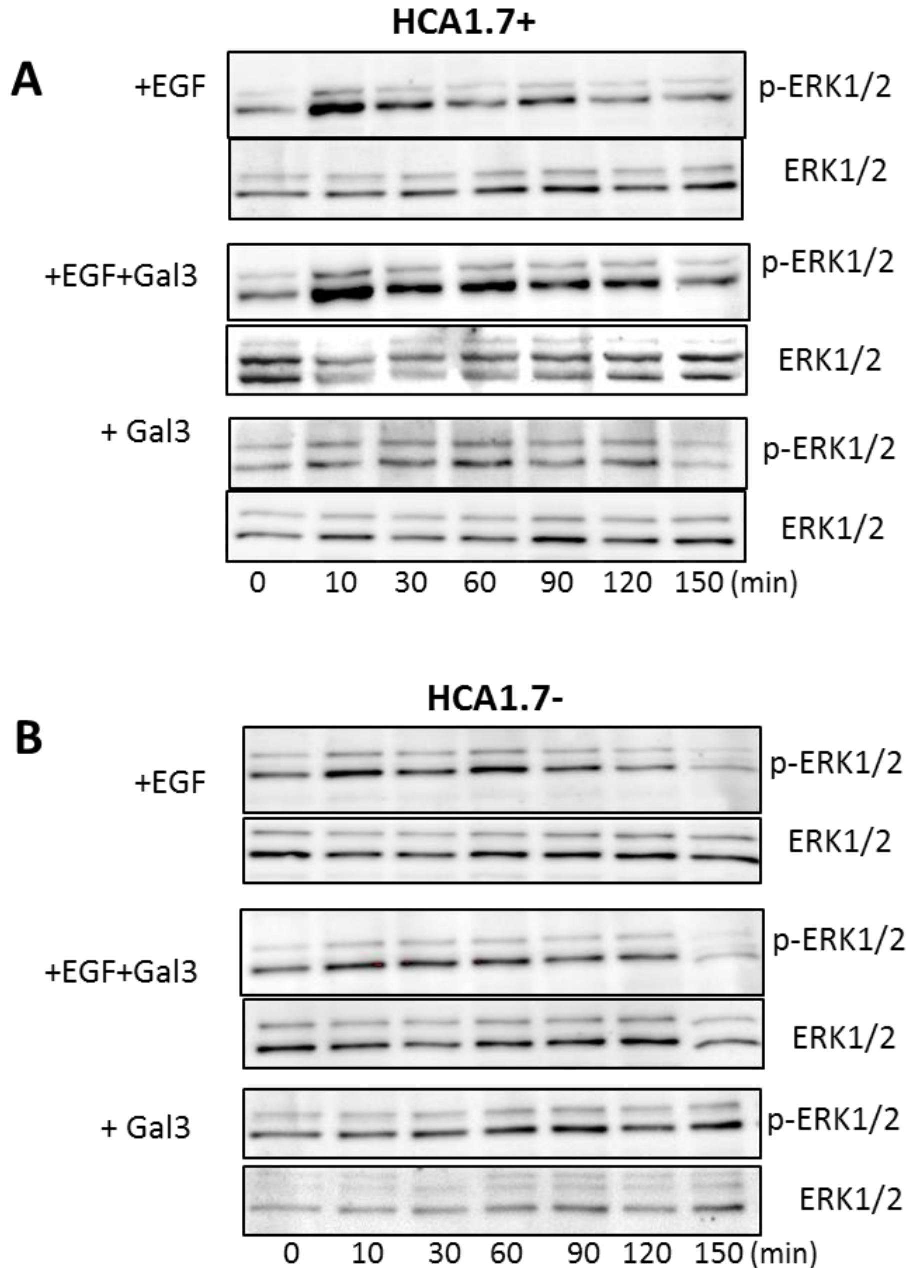


Figure 6.1: MUC1- as well as MUC1-galectin-3 interaction-associated EGFR activation increases ERK activation. MUC1-expressing HCA1.7+ (A) and MUC1-negative HCA1.7- (B) cells were treated with either 20 ng/ml EGF, 20 ng/ml EGF and 2 µg/ml galectin-3, 2 µg/ml galectin-3 or 2 µg/ml galectin-3C for various times as before the expression of p-ERK1/2 and ERK1/2 were analysed by immunoblotting. EGF treatment increases ERK1/2 phosphorylation in the MUC1- expressing HCA1.7+ and HCT116MUC1full cells. Introduction of galectin-3 further enhances ERK1/2 activation in the MUC1-expressing cells but not in the MUC1- negative cells. Representative blots from three independent experiments are shown.

These results suggest that, as predicted, EGFR activation on the cell surface induced by MUC1 expression and by MUC1-galectin-3 interaction effectively transduces the signalling to downstream EGFR effectors. The stronger and prolonged ERK1/2 activation in the MUC1-positive cells induced by galectin-3 is in keeping with the stronger and prolonged activation of EGFR in those cells in the presence of galectin-3 (Fig 6.2, Fig 6.3 and Fig 6.4). Compared to the marked effect on EGFR activation of full length galectin-3, the lack of effect of galectin-3C, in which its N-terminal ligand multimerization domain is depleted hence unable to crosslink MUC1 for cluster formation, indicates that MUC1 polarization is essential in galectin-3-MUC1 interaction-induced EGFR activation.

6.5.2 Activation of EGFR and ERK by galectin-3-MUC1 interaction is inhibited by EGFR inhibitor lapatinib

To determine whether the effect of galectin-3-MUC1 interaction on ERK activation was the consequence of EGFR activation, we tested the effect of Lapatinib, an EGFR phosphorylation inhibitor (500) on activation of EGFR and ERK in these cells. As shown above, the presence of EGF induced EGFR phosphorylation and the introduction of galectin-3 further increased EGF-induced EGFR phosphorylation of HCT116 MUC1 full (Figure 6.2A) and HCA1.7+ cells (Figure 6.3A). The presence of Lapatinib prohibited EGFR phosphorylation in response to EGF of HCT116 MUC1 full and HCA1.7+ cells and also abolished the increased EGFR activation/phosphorylation in these cells induced by the presence of galectin-3. A similar effect was observed for

ERK1/2 phosphorylation, with Lapatinib preventing activation of ERK1/2 phosphorylation of HCT116MUC1full (Figure 6.2A) and HCA1.7+ cells (Figure 6.3A). The presence of galectin-3C did not show any effect on phosphorylation of either EGFR (Figure 6.2A and 3A) or ERK1/2 (Figure 6.2B and 3B). These results suggest that the increased phosphorylation of ERK1/2 induced by MUC1 expression and by MUC1-galectin-3 interaction is the consequence of EGFR activation. It also further confirms that the effect of MUC1 and galectin-3-MUC1 interaction on EGFR activation effectively enhances downstream EGFR signalling.

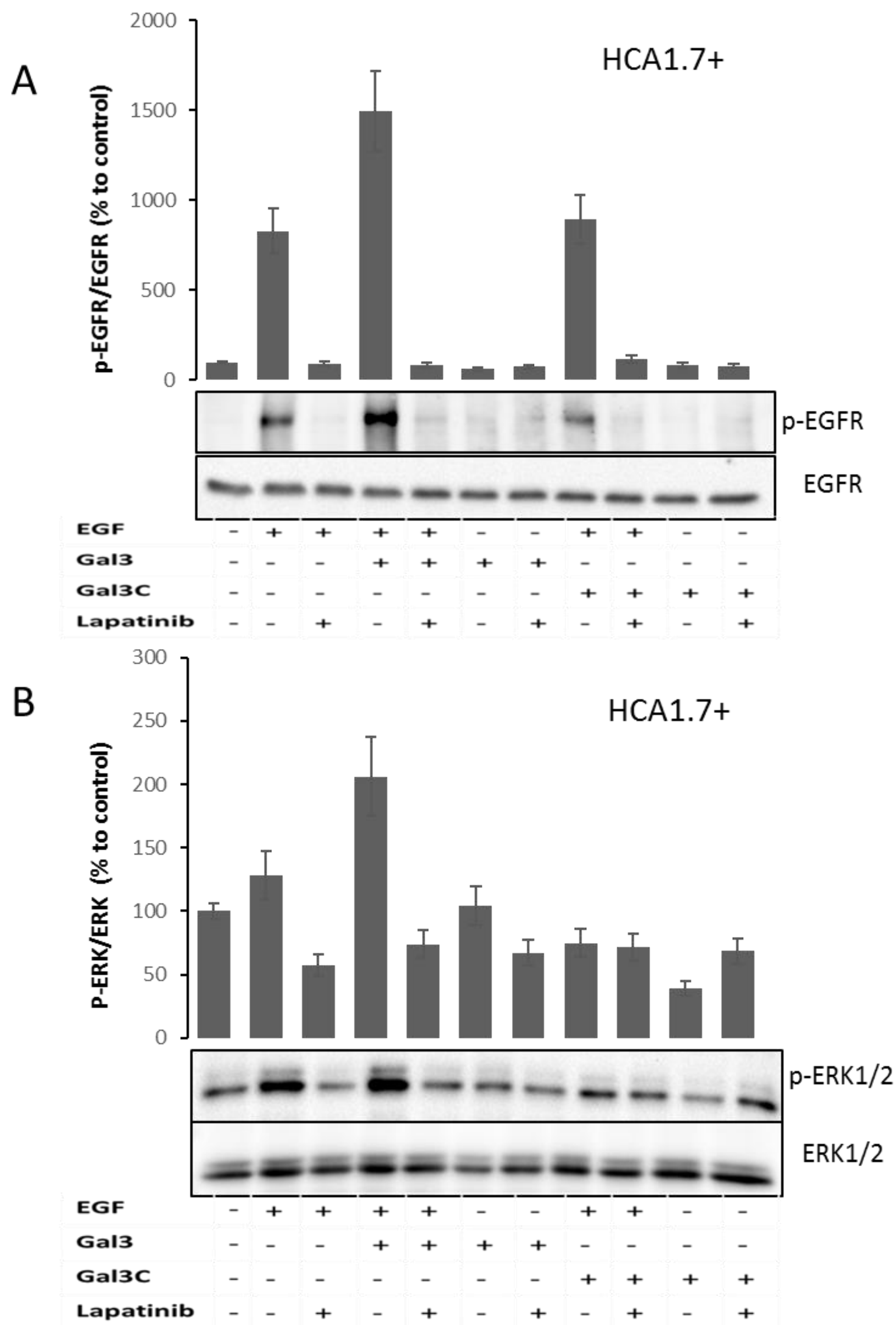


Figure 6.2: EGFR inhibitor Lapatinib inhibits EGFR and ERK activation induced by MUC1-galectin-3 interaction. HCA1.7+ cells were treated with and without EGF in the absence or presence of galectin-3, truncated galectin-3 galectin-3C, EGFR inhibitor lapatinib for 10 min before analysed by immunoblotting with antibodies against p-EGFR, EGFR (A) or pERK1/2 and ERK1/2 (B). Densitometry analysis of the bands from two independent experiments was quantified and was presented as mean percentage changes (± SD) of pEGFR/EGF and p-ERK1/2/ERK1/2, respectively, in comparison to the controls. N=2, n=2

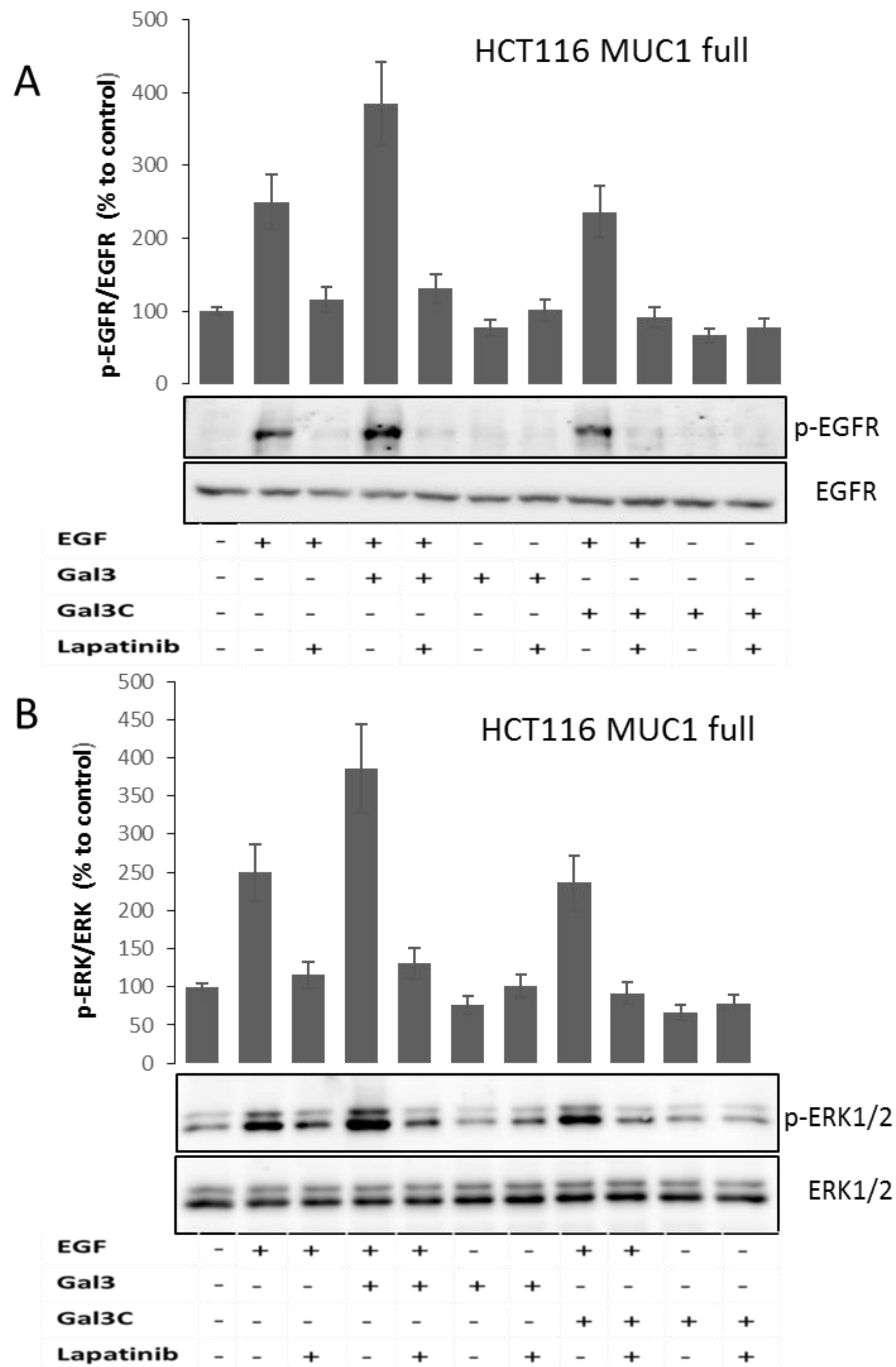


Figure 6.3: Lapatinib inhibits EGFR and ERK activation induced by MUC1-galectin-3 interaction. HCT116 MUC1 full cells were treated with and without EGF in the absence or presence of galectin-3, truncated galectin-3 galectin-3C, EGFR inhibitor lapatinib for 10 min before analysed by immunoblotting with antibodies against p-EGFR, EGFR (A) or pERK1/2 and ERK1/2 (B). Densitometry analysis of the bands from two independent experiments was quantified and was presented as mean percentage changes (±SD) of pEGFR/EGF and p-ERK1/2/ERK1/2, respectively, in comparison to the controls. N=2, n=2

6.5.3 Galectin-3-MUC1 interaction increases EGFR homo- and hetero-dimerization

In EGFR activation, an immediate event following ligand binding is EGFR dimerization, which is followed by EGFR auto-phosphorylation and internalization (501-503). As galectin-3-MUC1 interaction occurs on the cell surface and as the MUC1 extracellular domain plays a role in EGF-induced EGFR activation, we speculated that the effect of galectin-3-MUC1 interaction on EGFR activation might be linked with an effect of galectin-3-MUC1 interaction on EGFR dimerization. To test this, we treated the cells without or with EGF or galectin-3 and then crosslinked the cell surface proteins with non-cleavable crosslinker BS3 and then analysed cell EGFR by immunoblotting.

It was found that treatment of the cells with EGF induced EGFR dimerization in the MUC1-positive HCT116MUC1full (figure 6.5A) and HCA1.7+ (figure 6.4A) cells. EGFR dimerization predominately occurred as homo-dimers in HCT116 MUC1 full but hetero-dimers in HCA1.7+ cells in response to EGF. The presence of galectin-3 further increased EGFR dimerization in both cell types. Interestingly, galectin-3-induced EGFR dimerization occurred both as homo- and hetero-dimers in HCT116 MUC1 full cells but predominantly as homo-dimers in HCA1.7+ cells (figure 6.5A and figure 6.4A, respectively). Consistent with the lack of influence on activation of EGFR and ERK in the MUC1-negative cells (figure 6.2-6.4), EGF, alone or with galectin-3, showed little effect on EGFR dimerization in the HCT116 MUC1 neo (figure 6.5B) and HCA1.7- (figure 6.4B) cells. Moreover, although the presence of full length galectin-3 increased EGFR dimerization (figure 6.4A and 6.5A) and EGFR phosphorylation, the

presence of the truncated galectin-3C did not show any effect on EGFR dimerization and the levels of EGFR homo- and hetero-dimers remained the same as the EGF alone-treated HCT116 MUC1 full (figure 6.5A) and HCA1.7+ (figure 6.4A) cells.

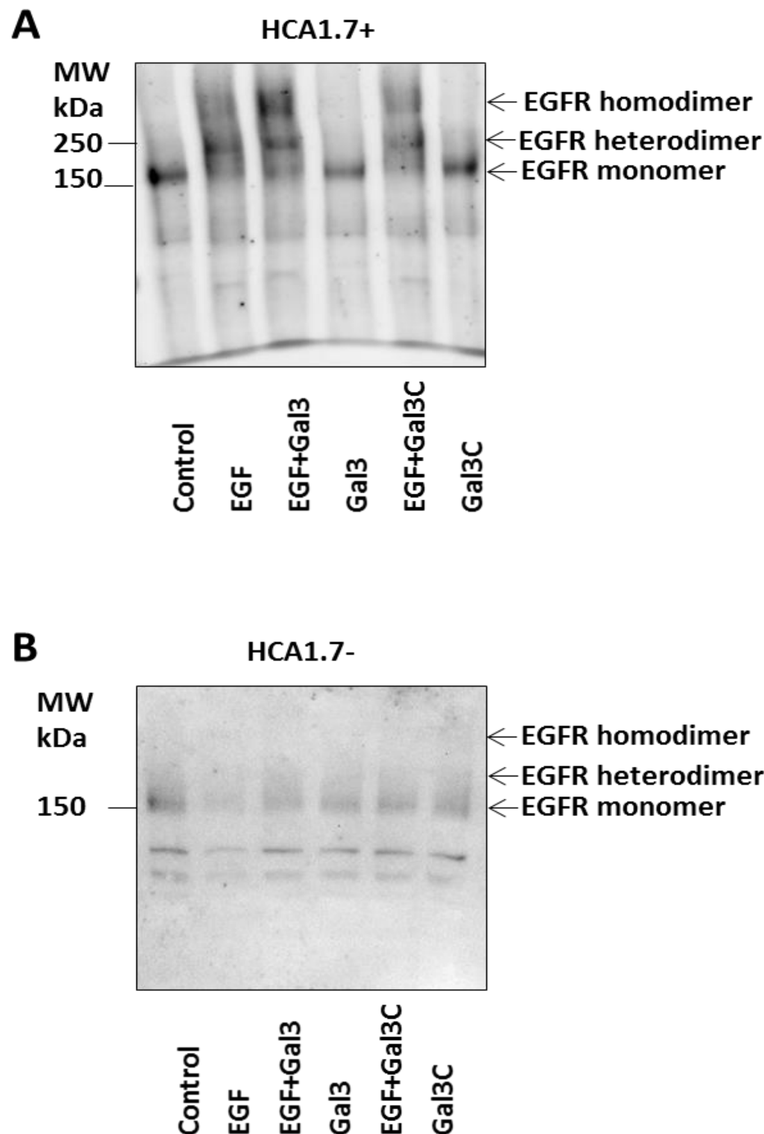


Figure 6.4: Galectin-3-MUC1 interaction promotes EGFR dimerization in human breast epithelial cells. HCA1.7+ (A) and HCA1.7- (B) cells were treated with and without EGF in the absence or presence of galectin-3 or galectin-3C for 10 minutes before EGFR dimerization were analysed using BS3 cross linker and immunoblotting. The presence of galectin-3, but not galectin-3C, increased EGFR homo- and hetero-dimerization in the MUC1-expressing, but not MUC1-negative, cells. Representative blots from 2 independent experiments, N=2.

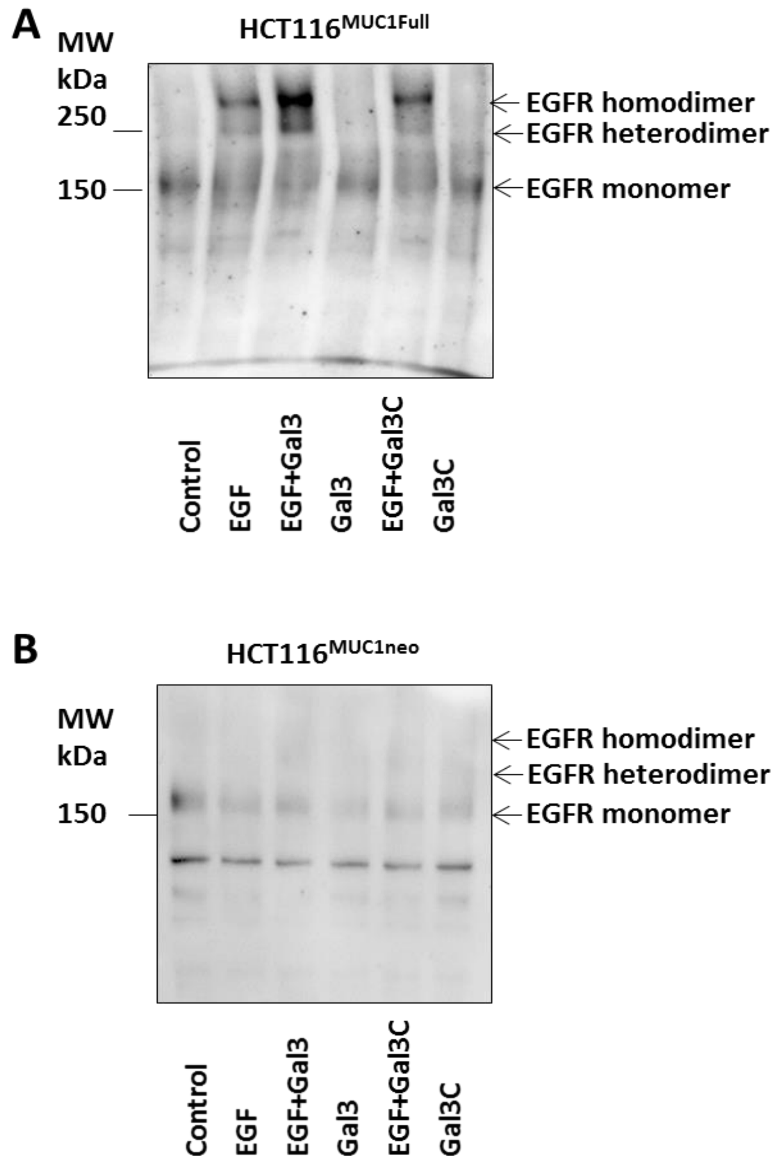


Figure 6.5: Galectin-3-MUC1 interaction promotes EGFR dimerization in human colon cancer cells. HCT116 MUC1 full (A) and HCT116 MUC1 neo (B) cells were treated with and without EGF in the absence or presence of galectin-3 or galectin-3C for 10 minutes before EGFR dimerization were analysed using BS3 cross linker and immunoblotting. The presence of galectin-3, but not galectin-3C, increased EGFR homo- and hetero-dimerization in the MUC1-expressing, but not MUC1-negative, cells. Representative blots from 2 independent experiments, N=2.

These results suggest that EGFR activation by galectin-3-MUC1 interaction is associated with its promotion of EGFR dimerization. The lack of effect of the truncated galectin-3C on EGFR dimerization in comparison to the full-length galectin-

3 provides further support to an essential role of galectin-3-induced ligand clustering in EGFR activation.

6.5.4 Galectin-3 increases interaction of MUC1 with EGFR

To gain further insight into the action of galectin-3-promoted EGFR activation, we determined the association of MUC1 with EGFR in response to galectin-3. It was found that treatment of HCA1.7+ cells with galectin-3, regardless of the presence or absence of EGF, resulted in more EGFR to be co-immunoprecipitated with MUC1 (figure 6.6A and B) in comparison to the control untreated or the EGF-alone treated cells. Treatment of the cells with EGF did not have any effect on MUC1-EGFR interaction. This suggests that galectin-3-MUC1 interaction promotes physical interaction of EGFR with MUC1 and this increased MUC1-EGFR interaction is a key component of galectin-3-associated EGFR activation.

As galectin-3 has been previously reported to be able to interact directly with EGFR, we also assessed whether direct interaction of galectin-3 with EGFR is involved in this action. Minimal galectin-3 was found to be co-immunoprecipitated with EGFR in these cells (figure 6.7 F). In comparison to EGF alone treated cells (figure 6.7F, lane 2), introduction of galectin-3 and EGF did not increase galectin-3 presence in EGFR immunoprecipitates (figure 6.7F, lane 3), thus supporting a role of galectin-3 EGFR interaction in this action of EGFR activation.

We initially immunoprecipitated galectin 3 and immunoblotted for EGFR and found that galectin-3 has little to no interaction with EGFR. Using the same immunoprecipitates and immunoblotting them for MUC1 however shows that addition of galectin-3 with EGF (Figure 6.7A, lane 2) leads to an increased interaction of MUC1 with EGFR. Immunoprecipitating EGFR and immunoblotting for MUC1 shows an increased MUC1 EGFR association in presence of galectin-3 (figure 6.7 B lane 3 and 4). However, in sample treated with EGF alone, EGFR immunoprecipitation shows an interaction with both MUC1 and galectin-3, albeit to a lower extent, but still higher than control group. (figure 6.7B and C, lane 2). This suggests that there is an underlying interaction between MUC1, galectin-3 and EGFR which is enhanced in the presence of EGF and which is further enhanced in the presence of both EGF and galectin-3.

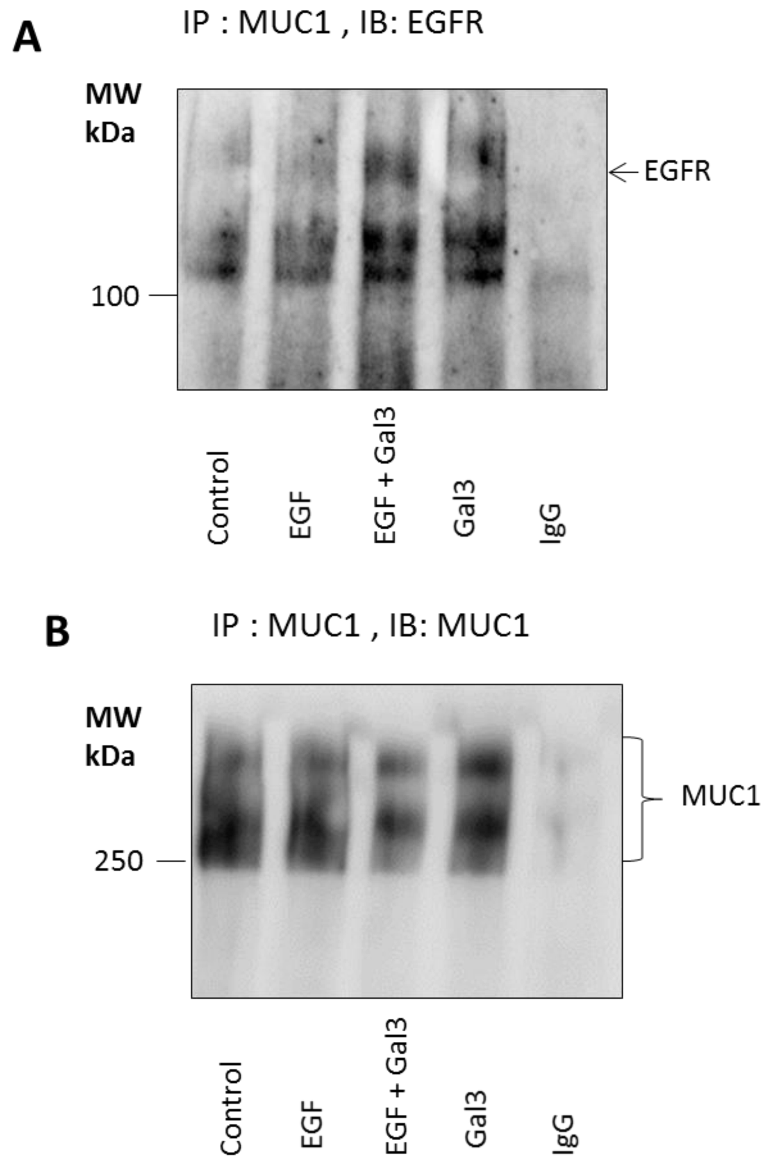


Figure 6.6: Galectin-3 enhances MUC1 interaction with EGFR. HCA1.7+ cells were treated with PBS (control), EGF with or without galectin-3 for 10 minute followed by MUC1 immunoprecipitation of the cells with B27.29 anti-MUC1 antibody. The immunoprecipitates were analysed by immunoblotting with anti-EGFR or B27.29 anti-MUC1 antibody. Representative blots from 3 independent experiments, N=3.

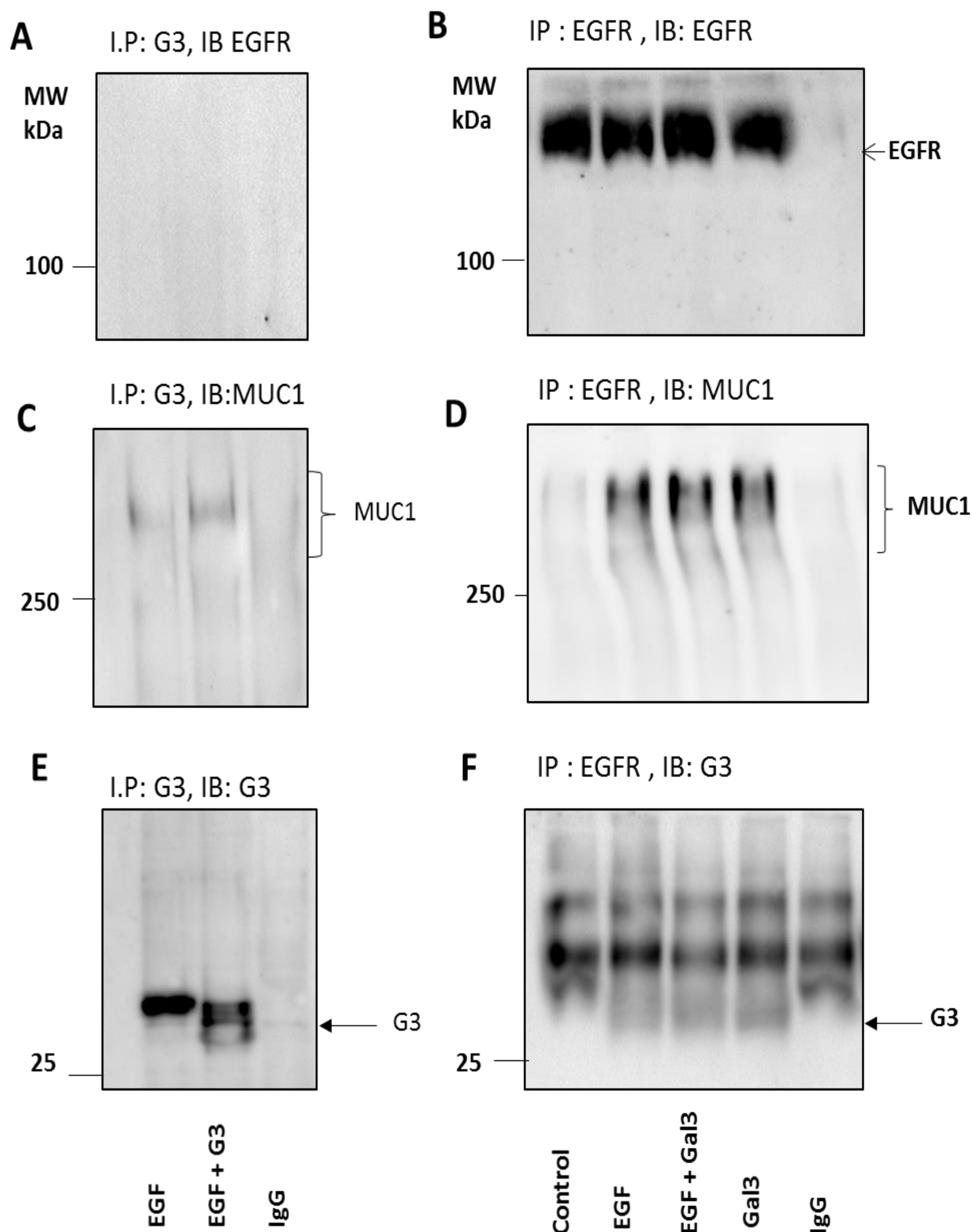


Figure 6.7: Galectin-3 enhances MUC1 interaction with EGFR. (A) HCT116 MUC1 full cells were treated with PBS (control), EGF with or without galectin-3 for 10 minute followed by EGFR immunoprecipitation of the cells with anti-EGFR antibody. The immunoprecipitates were analysed by immunoblotting with anti-EGFR or B27.29 anti MUC1 or anti galectin-3 antibodies. The same cells were treated with EGF in the presence or absence of galectin-3 for 10 minutes followed by galectin-3 immunoprecipitation of cell with anti galectin-3 antibody. The immunoprecipitates were analysed by immunoblotting with anti-EGFR or B27.29 anti MUC1 or anti galectin-3 antibodies. Representative blots from 3 independent experiments, N=3.

6.5.5 MUC1 polarization increases EGFR internalization

Following EGFR dimerization and auto-phosphorylation in response to ligand binding, EGFR internalization is an essential next step in EGFR signalling. In both HCT116 MUC1 full and HCA1.7 cells, EGFR appeared both on the cell surface and inside the cells (figure 6.9 A and B). Addition of EGF resulted in disappearance of EGFR from cell surface and accumulation inside the cells in both HCT116 MUC1 full cells (figure 6.9A) and HCA1.7+ (figure 6.9B) cells. MUC1 localization was uniformly spread on the cell surface and was not affected by the absence or presence of EGF (431).

Introduction of MUC1 polarization inducing agents galectin-3 and anti-MUC1 antibody 214D which has shown previously to induce MUC1 polarization (chapter 3), changes MUC1 cell surface localization (as illustrated by disruption of the uniform localization). Presence of galectin-3 and 214D with EGF also increased EGFR internalization (more disappearance of EGFR from the cell surface and stronger appearance in the cytoplasm) in comparison to the cells treated with EGF alone. Interestingly, internalized EGFR induced by galectin-3/EGF or 214D/EGF was seen to be in a more clustered appearance inside the cells than that induced by EGF alone. Introduction of galectin-3 without addition of EGF did not show any effect on EGFR localization compared to the control cells. This, together with the lack of effect of full length galectin-3 and 214D on EGFR activation in MUC1-negative cells along with the lack of effect of truncated galectin-3C on EGFR activation in the MUC1-

positive cells, indicate that MUC1 polarization mediated EGFR activation is associated with its effect on alteration of MUC1 cell surface localization.

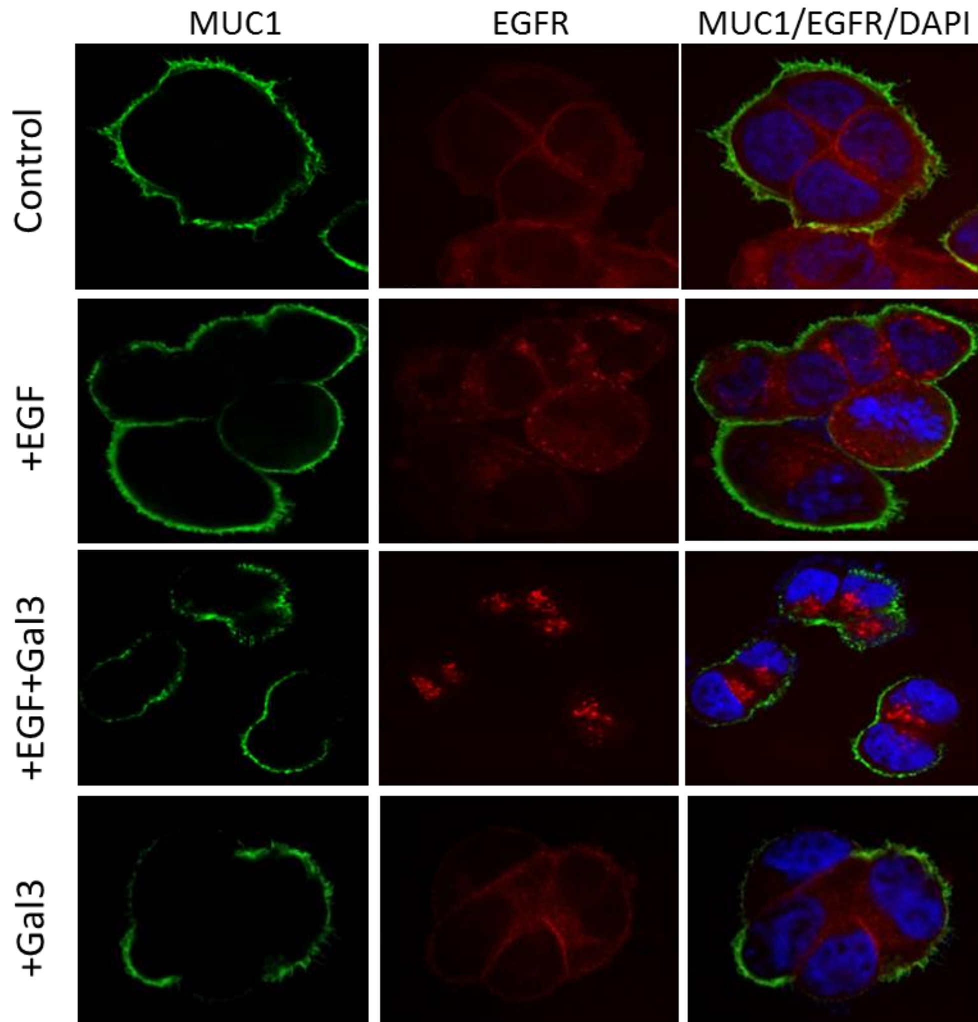


Figure 6.8: Galectin-3-MUC1 interaction enhances EGFR internalization in human colon cancer cells. HCT116 MUC1 full cells were treated with PBS (control), EGF with or without galectin-3 for 10 minutes before localization of MUC1 (green) and EGFR (red) were determined by fluorescent immunohistochemistry and analysed by confocal microscopy. The cell nucleus was stained with DAPI (blue). Galectin-3 changes MUC1 cell surface localization (as illustrated by disruption of uniform MUC1 localization). More intense and clustered EGFR localization were seen inside the cell in cells treated with both galectin-3 and EGF than in the EGF alone treated cells. Representative images of 20 random field observations from 3 experiments (100 x magnification).

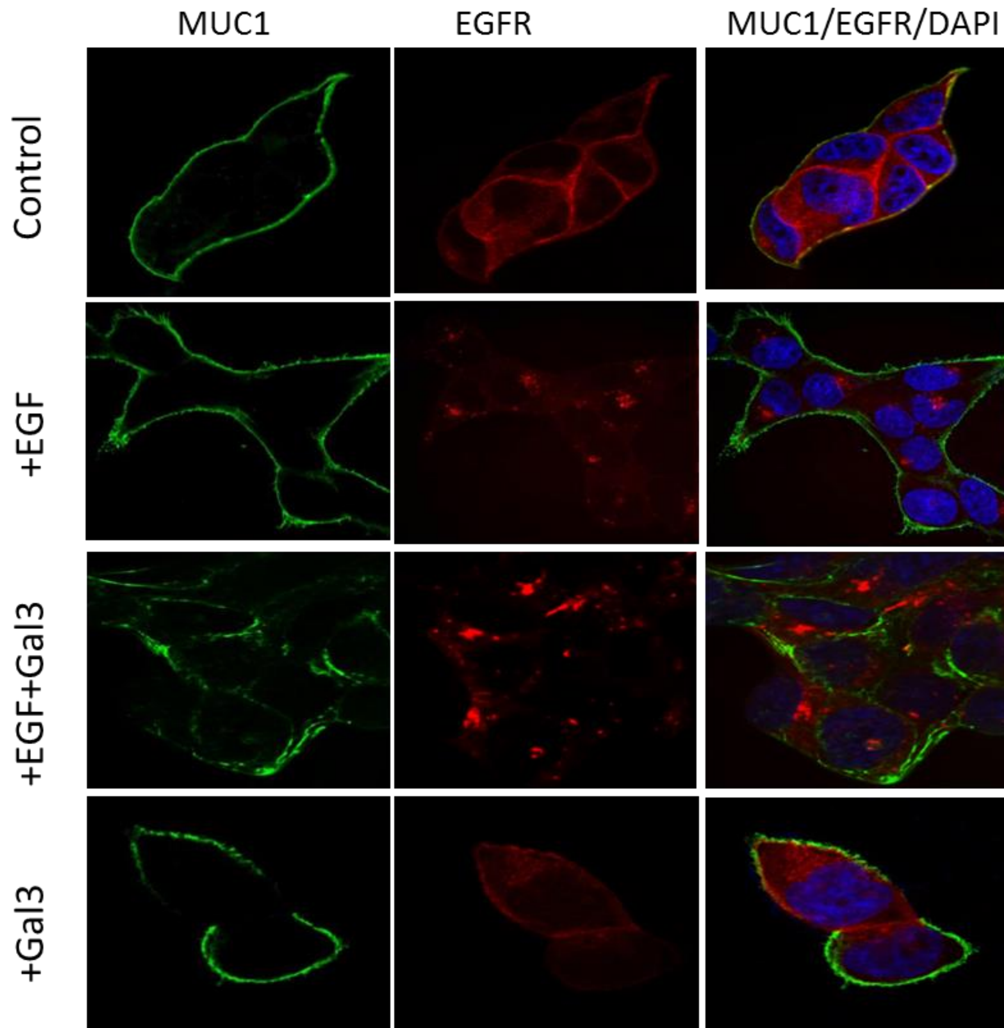


Figure 6.9: Galectin-3-MUC1 interaction enhances EGFR internalization in human breast epithelial cells. HCA1.7+cells were treated with PBS (control), EGF with or without galectin-3 for 10 minutes before localization of MUC1 (green) and EGFR (red) were determined by fluorescent immunohistochemistry and analysed by confocal microscopy. The cell nucleus was stained with DAPI (blue). Galectin-3 changes MUC1 cell surface localization (as illustrated by disruption of uniform MUC1 localization). More intense and clustered EGFR localization were seen inside the cell in cells treated with both galectin-3 and EGF than in the EGF alone treated cells. Representative images of 20 random field observations from 3 experiments (100 x magnification).

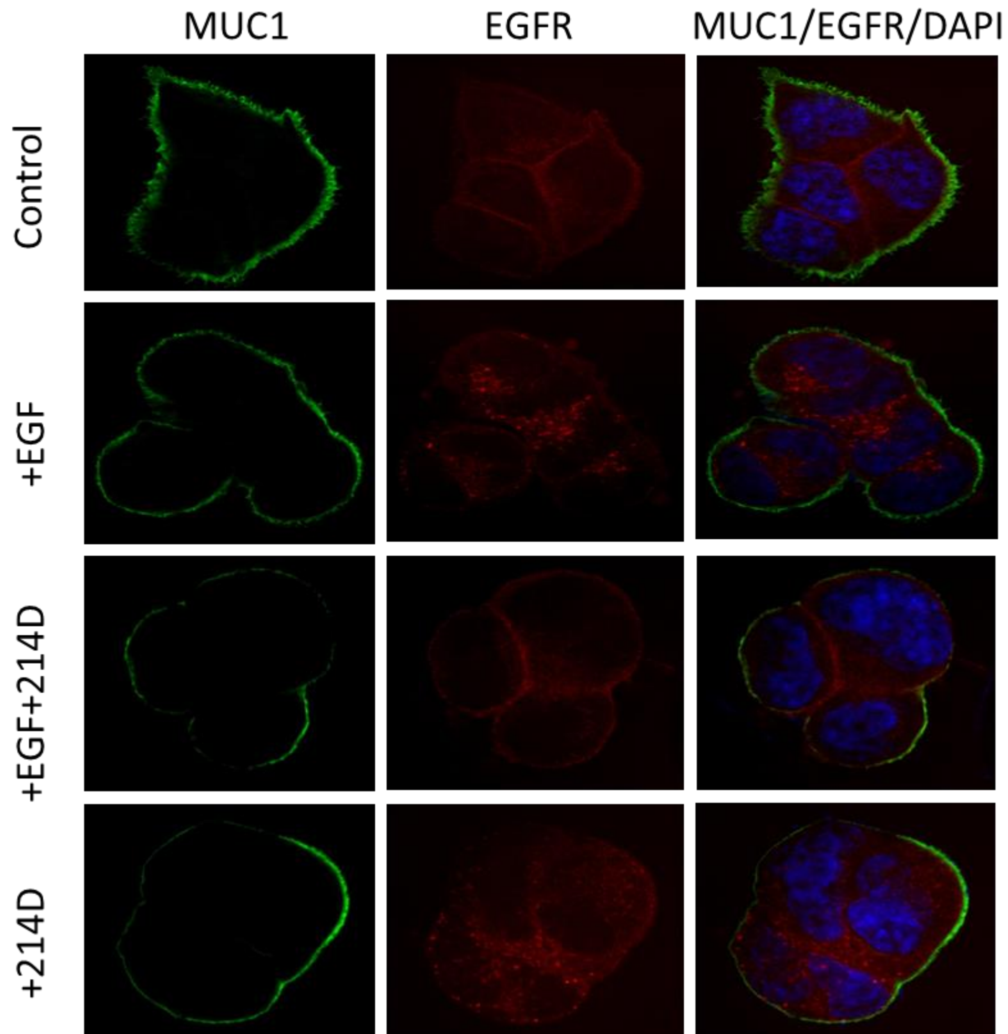


Figure 6.10: Effect of 214D anti-MUC1 antibody on MUC1 polarization and EGFR internalization. HCT116 MUC1 cells were treated with PBS (control), EGF with or without MUC1 polarizing 214D for 10 minutes before localization of MUC1 (green) and EGFR (red) were determined by fluorescent immunohistochemistry and analysed by confocal microscopy. The cell nucleus was stained with DAPI (blue). 214D changes MUC1 cell surface localization (as illustrated by disruption of uniform MUC1 localization). More intense and clustered EGFR localization was seen inside cells treated with both 214D and EGF than in the EGF alone treated cells. Representative images of 20 random field observations from 3 experiments (100 x magnification).

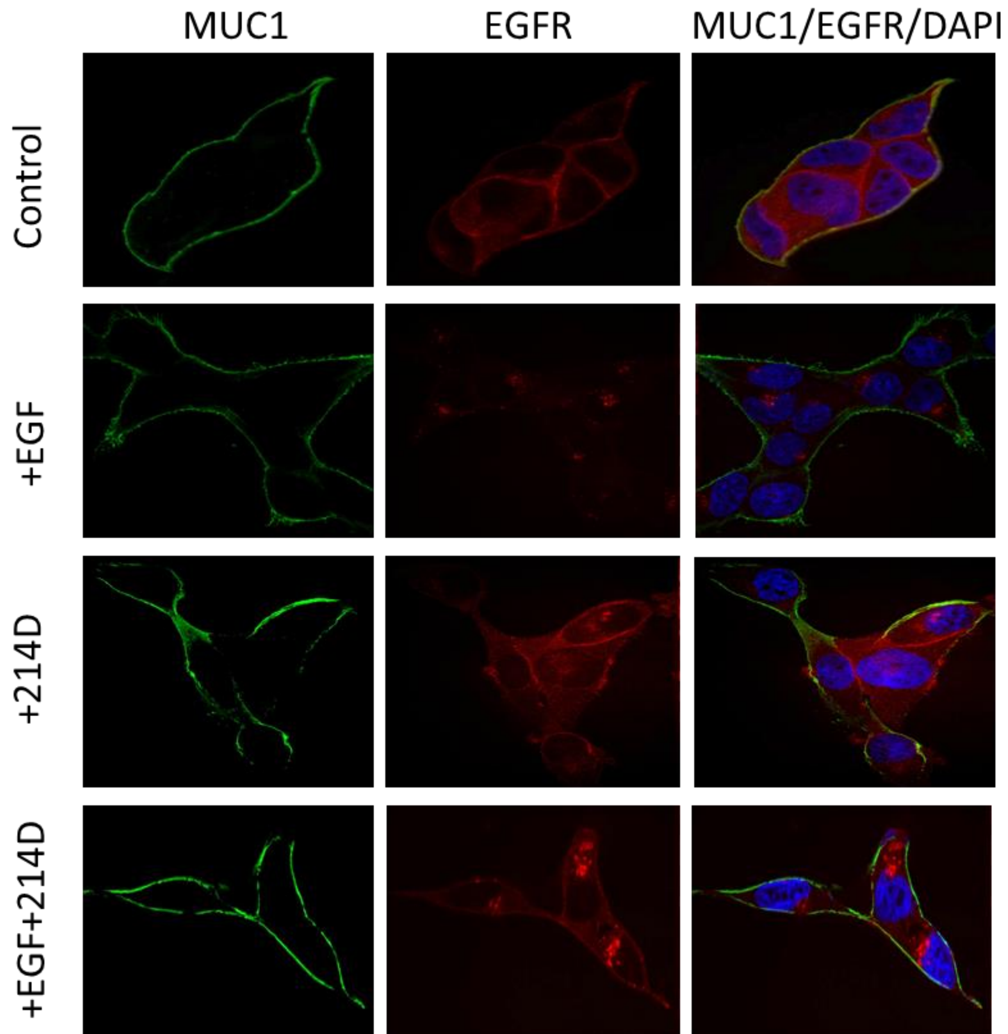


Figure 6.11: Effect of 214d anti-MUC1 antibody on MUC1 polarization and EGFR internalization in human breast epithelial cells. HCA1.7+ cells were treated with PBS (control), EGF with or without MUC1 polarizing 214D for 10 minutes before localization of MUC1 (green) and EGFR (red) were determined by fluorescent immunohistochemistry and analysed by confocal microscopy. The cell nucleus was stained with DAPI (blue). 214D changes MUC1 cell surface localization (as illustrated by disruption of uniform MUC1 localization). More intense and clustered EGFR localization were seen inside the cell in cells treated with both 214D and EGF than in the EGF alone treated cells. Representative images of 20 random field observations from 3 experiments (100 x magnification).

6.6 Summary of results

1. MUC-1-galectin-3 interaction-induced EGFR activation is associated with activation of downstream ERK1/2

2. EGFR inhibitor Lapatinib inhibits galectin-3 -MUC1-mediated effect on EGFR activation and Erk1/2 activation

3. Galectin-3 promotes EGFR homo- and hetero-dimerization induced by EGF

4. Galectin-3 enhances MUC1 cell surface polarization and interaction with EGFR

5. 214D anti-MUC1 antibody showed a similar effect on MUC1 polarization and EGFR internalization

6.7 Discussion

Binding of galectin-3 to cell surface MUC1 is shown in this study to markedly increase EGF-induced EGFR activation. Interaction between MUC1 and galectin-3, and between MUC1 and anti-MUC1 antibody 214D, are known to induce MUC1 cell surface polarization (25, 79, 337). The effect of galectin-3 or 214D on MUC1 cell surface localization was clearly shown in this study (Fig 6.8 and 6.9 for galectin-3, Fig 6.10 and 6.11 for 241D). Change of MUC1 localization in galectin-3-treated cells was seen in the absence or presence of EGF (Fig 6.8 and 6.9) but only in those with the presence of EGF did it enhance EGFR activation with subsequent induction of down-stream signalling (Fig 6.2 and 6.3). This indicates that galectin-3-induced change of MUC1 cell surface localization alone is not sufficient to induce EGFR activation, the presence of EGFR ligand is essential. MUC1 co-immunoprecipitation showed an increase of MUC1 association with EGFR in galectin-3-treated cells, with or without the presence of EGF (Fig 6.6), but only those with the presence of EGF enhanced EGFR activation. This, together with the discovery that the presence of galectin-3 alone did not induce EGFR dimerization, suggests that galectin-3-MUC1 interaction is essential in galectin-3-mediated EGFR activation. The importance of galectin-3-mediated change of MUC1 localization in EGFR activation is further supported by the discovery that, in contrast to the presence of full length galectin-3, the presence of a truncated form of galectin-3 (galectin-3C), which lacks the N-terminal domain responsible for galectin-3-induced ligand clustering thus could not induce MUC1 polarization (Fig 3.11), did not show any effect on EGFR dimerization (Fig 6.4 and 6.5), activation of ERK signalling (Fig 6.2 and 6.3), in the presence of EGF in MUC1-positive cells.

There was an earlier study reporting binding of galectin-3 to EGFR in pancreatic cancer cells to induce ERK activation (474). In our study, galectin-3 did not show any effect on EGFR phosphorylation, EGFR dimerization, or ERK activation in the MUC1-postive cells in the absence of EGF, nor showed any effect on activation of the molecules in the MUC1 negative cells even in the presence of EGF. This suggests that a direct binding of galectin-3 to EGFR, even if exists, does not contribute to galectin-3-mediated EGFR activation in those cells. It is noted that a recent study has reported a role of galectin-3 in promoting sphere formation activity of lung cancer cells through activation of EGFR (484). Although that study did not identify the galectin-3 binding ligand in producing the effect, their discovery of the requirement of galectin-3 carbohydrate recognition domain in the effect is broadly in keeping with an effect of galectin-3-MUC1 interaction on EGFR activation shown in the present study.

EGFR activation on the cell membrane is known to trigger activation of an array of intracellular signalling pathways (219), commonly including Ras/MEK/ERK signalling. EGFR activation induced by either MUC1 expression or by MUC1-galectin-3 interaction is shown in this study to be accompanied by an increase of ERK activation. This indicates that the MUC1 expression and MUC1-galectin-3-mediated EGFR activation effectively passes the signalling to downstream EGFR effectors. It is noted that galectin-3-MUC1 interaction-mediated EGFR activation is also associated with a prolonged activation of ERK phosphorylation (Fig 6.1). It is generally believed that EGFR activation is terminated primarily through endocytosis of the receptor-ligand

complexes which are either degraded in the endosomes or recycled to the cell surface. It has been shown that if recycled EGFR is unable to reach the cell surface or to the lysosomal compartment but accumulates in the early endosomes, it will lead to prolonged signalling and increased activation of ERK (504). This notion seems supported by our study. We found that following EGFR activation, more EGFR was seen to be located in a clustered patter inside the cells in the galectin-3/EGF treated cells than in EGF alone treated cells (Fig 6.8 and 6.9). There was a much weaker EGFR cell surface localization in the galectin-3/EGFR treated cells than in the other groups including EGF-alone treated cells. This indicates that the galectin-3/MUC1/EGF-mediated EGFR activation and subsequent EGFR endocytosis is associated with slower recycling of EGFR to the cell surface. This may provide an explanation to the prolonged activation of ERK in those cells (Fig 6.1). This conclusion is in keeping with an earlier study showing that MUC1 expression inhibits EGFR degradation in response to ligand binding but was accompanied by an increase of EGFR internalization in breast epithelial cells (505).

As a key therapeutic target for cancer treatment, development of anti-EGFR strategies is a crucial area of clinical study for the treatment of solid tumours. Currently, main strategies include monoclonal antibodies directed towards the extracellular domain of EGFR, small molecule tyrosine kinase inhibitors targeting the catalytic kinase domain of EGFR and strategies to disrupt receptor trafficking to the cell surface. The discovery that the expression of MUC1 and its interaction with galectin-3 promotes ligand-dependent EGFR activation has implications in EGFR-

targeted therapies in cancer treatment. MUC1 and galectin-3 are both well known to be commonly over-expressed by solid tumours. Overexpression of MUC1 and galectin-3 and their effects on EGFR activation may therefore not only have an influence on EGFR-mediated tumourigenesis and cancer progression, but may also have an impact on the effectiveness of EGFR-targeted therapy. It is conceivable that a closer localization of EGFR with MUC1 on the cell surface induced by galectin-3 may limit the access of anti-EGFR antibodies to cell surface EGFR due to the massive size of MUC1 that easily protrudes over EGFR on the cell surface. An enhanced EGFR dimerization induced by galectin-3-MUC1 may also limit the treatment effectiveness of anti-EGFR antibody as well as kinase inhibitors due to a slower recycling of EGFR to the cell surface. It is possible therefore that in cancer patients who have higher tumour expression of galectin-3 and/or MUC1, a combined treatment that targets EGFR as well as galectin-3 and/or MUC1 may improve treatment effectiveness.

Thus, MUC1 expression and its interaction with galectin-3 make important contributions to EGFR activation in epithelial cells by promoting EGFR dimerizaton. As over-expression of MUC1 and over-expression of galectin-3 are both common in epithelial cancer, the influence of MUC1 and MUC1-galectin-3 interaction on EGFR activation likely contributes to EGFR-associated tumorigenesis and tumour progression and to the effectiveness of EGFR-targeted therapy. A combined therapy that targets EGFR as well as galectin-3 and/or MUC1 may improve effectiveness of EGFR-targeted cancer treatment.

7 General Discussion and future research

7.1 Key findings

- MUC1 expression confers epithelial cell resistance to anoikis in response to loss of cell adhesion.
- Inhibition of O-glycosylation by stable suppression of C1GT expression significantly increased anoikis of MUC1-positive, but not MUC1-negative cells.
- EGF-induced EGFR activation is substantially increased by expression of the transmembrane mucin protein MUC1 in human breast and colon epithelial cells.
- Binding of galectin-3 to cell surface MUC1 increases EGFR activation and this effect requires not only the galectin-3 C-terminal CRD domain but also its N-terminal ligand polarization domain.
- MUC1-galectin-3 mediated EGFR activation is translated downstream into ERK 1/2 activation

7.2 Discussion

It was found in this study that MUC1 expression in epithelial cells leads to a significant decrease in anoikis compared to MUC1 null cells in melanoma, breast epithelial and colon cancer cells. Both the intracellular and extracellular domains of MUC1 were found to contribute to this effect. My work shows that this effect is largely attributed to the elongated and heavily glycosylated extracellular domain of MUC1. The extracellular domain of MUC1 protrudes high above the cell membrane and hence prevents activation of the cell surface anoikis-initiating molecules such as integrins, cadherins and death receptors by providing them a mechanically 'homing' microenvironment.

Both intrinsic and extrinsic apoptotic pathways are known to have an important role in anoikis and many of the intrinsic apoptotic signalling proteins (for example, Bcl and p53 family members) are involved in regulation of anoikis process (124). Several earlier studies have reported a role of the MUC1 cytoplasmic domain in regulation of apoptosis in cells growing under adhesion (anchored) conditions through interaction with a number of intracellular signalling proteins (30). For example, MUC1 cytoplasmic tail has been reported to interact with mitochondrial membrane, p53 and β-catenin and prevent mitochondrion-mediated apoptosis in cell response to DNA damage (108). Additionally, interaction of the MUC1 cytoplasmic tail with FADD have been reported to blocks caspase-8 recruitment to the death-inducing signalling complex in response to TNFα-induced apoptosis (508). Evidence of MUC1 expression having a potential role in anoikis is seen in the array results presented in chapter 3

where expression levels of P53, pro-capsase-3 and Fas/TNFSF6 were noted to be several folds higher in MUC1 expressing HCA1.7+ cells compared to MUC1 negative HCA1.7- cells. The ability to interact with key intracellular anoikis proteins is potentially one of the mechanisms MUC1 expression leads to anoikis resistance.

Data presented in chapter 3 also highlights MUC1 extracellular domain has an inhibitory effect on anoikis. The extracellular domain of MUC1 is large and heavily glycosylated and its sheer size is able to dwarf many cell surface proteins. Normally the expression of MUC1 is controlled and is limited to a cells apical surface only. However, in cancer state this apical polarization is lost and MUC1 expression is substantially increased (up to 10 fold). Our earlier work has shown that this overexpression of MUC1, together with the large and heavily glycosylated extracellular domain, plays a key role in initiation of anoikis and on antibody accessibility to cell surface anoikis initiating molecule. The importance of MUC1 extracellular domain is further highlighted by the shRNA suppression of C1GT, as detailed in chapter 4. C1GT is a key glycosyltransferase in the biosynthesis of O-linked mucin type glycans, responsible for the formation of the Core 1-related complex O-glycans. Suppression of C1GT blocks Core-1 O-glycosylation and results in increased expression of the short glycans GalNAcα (Tn) and sialyl-Tn (51). Data presented in chapter 4 shows that suppression of C1GT in cancer cell line HCT116 MUC1 full and SW620 decreased the size of MUC1 by over 25%. Using the C1GT knockdown variant and the control variant of the same cell line in anoikis (suspension) culture, this change led to a significant increase in anoikis in only the MUC1 positive cells but not the MUC1 negative cells. Further analysis showed that under suspension conditions,

reduction of the MUC1 O-glycosylation increased ligand/antibody accessibility to cell surface anoikis-initiating molecules such as E-cadherin, integrinβ1 and Fas and increased caspase-8 activity in response to exogenous introduction of Fas-L to suspended cells.

The extracellular matrix plays a key role in maintaining tissue homeostasis by ensuring that under proper adhesion conditions, a cell receives appropriate signals to grow and divide. The extracellular matrix has an abundance of large and heavily glycosylated proteins such as laminins and fibronectins. In normal epithelium, interactions between these ECM glycoproproteins and cell surface anoikis initiating molecules acts as trigger/sensor for anoikis activation. MUC1 extracellular domain shares two key characteristics with these ECM glycoproteins; it is large and has complex glycosylation. It is likely that that overexpression and abnormal glycosylation of MUC1, noted in epithelial cancer cells, provides a similar 'home' microenvironment to anoikis initiating molecules on cell surface as the ECM glycoproteins, thereby preventing their activation following cell detachment and facilitating anoikis resistance. A model of proposed mechanism is presented in figure 7.1.

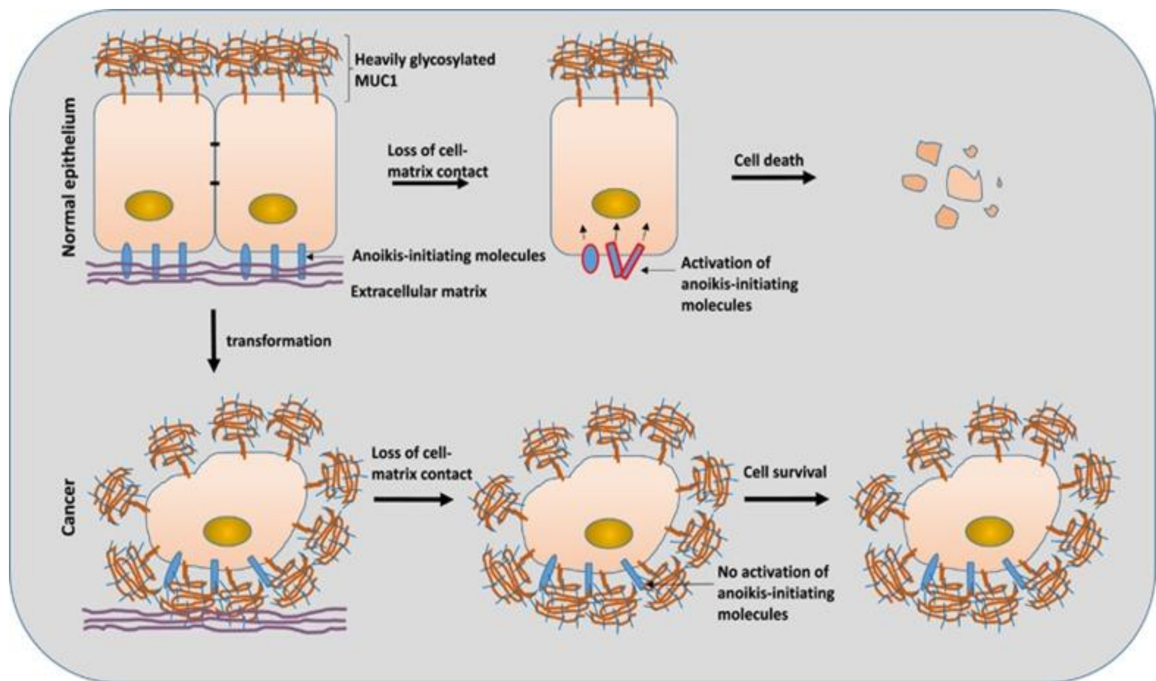


Figure 7.1: Model of the MUC1-mediated epithelial cancer cell resistance to anoikis. In normal epithelia, MUC1 polarizes at the apical side and has no influence on activation of the cell surface anoikis-initiating molecule. Loss of cell-Matrix contact leads to the activation of anoikis initiating molecules leading to cell death. In epithelial cancer, MUC1 is overexpressed over the entire cell surface and thus is able to interact with the cell surface anoikis-initiating molecules, preventing their activation during loss of cell-matrix contacts, leading to anoikis resistance. adapted from (506) (open access article distributed under the terms of the Creative Commons CC BY license)

It is possible that other members of mucin transmembrane family which are large in size and are heavily o-linked glycosylated may also have a similar effect on anoikis of epithelial cancer cells. The work presented in this thesis highlights the importance of cell surface glycosylation, altered in majority of epithelial cancers, in cancer cell progression and metastasis.

One of the hallmarks of cancer is the ability to maintain sustained proliferative signalling. Increased EGFR phosphorylation has been reported in numerous epithelial cancers and is now understood to be one of way to ensure autocrine proliferative signalling. The present study (chapter 5 and 6) shows that MUC1 expression increases EGF-induced EGFR activation in human breast and colon cancer cells. Depletion of either the MUC1 intracellular or extracellular domain could only partly abolish MUC1-associated effect on EGFR activation. This suggests that while both the MUC1 cytoplasmic and extracellular domains contribute to EGFR activation, some of the effect mediated by the MUC1 intra- and extra-cellular domain on EGFR activation is also relatively independent. The fact that depletion of the MUC1 extracellular domain resulted in greater reduction of MUC1-associated EGFR activation than depletion of the MUC1 intracellular domain indicates that the predominate influence of MUC1 on EGFR activation derives from its extracellular domain. MUC1 (487) and EGFR (488) have both been shown to be associated with lipid rafts on cell membrane. It is possible that the expression of MUC1 and its association with EGFR in the lipid raft on the cell surface might increase the proximity of EGFR molecules in the microdomains of lipid raft for them to be in a better position to form homo-/hetero-dimers in response to ligand banding.

Interaction of cell surface MUC1 with galectin-3 is seen in this study to induce changes of MUC1 cell surface localization and increases MUC1-EGFR interaction. This leads to an increase of EGFR homo-/hetero-dimerization and subsequently increased EGFR activation and downstream signalling. This effect of galectin-3 occurs only with the full length but not the truncated galectin-3 form that lacks its N-terminal domain

responsible for galectin-3-mediated receptor clustering. Thus, MUC1 interaction with galectin-3 enhances EGFR dimerization and activation in epithelial cancer cells. The effect of galectin-3 on MUC1 cell surface localization was indeed visible in this study, irrespective of the presence or absence of EGF. However, galectin-3 presence enhances EGFR activation only when EGF is also present. This indicates that galectin-3 cannot activate EGFR without the presence of an EGFR ligand. MUC1 cell surface polarization induced by MUC1-galectin-3 interaction has been shown previously to expose underlying smaller cell surface molecules (24, 76, 334). The discovery that EGF showed much weaker effect on EGFR activation in the MUC1-negative than in the positive cells, irrespective of the presence of galectin-3 (Fig 5.3, Fig 5.4, Fig 5.6 and Fig 5.7), indicates that exposure of cell surface EGFR for easy EGF access is unlikely a mechanism of the MUC1-galectin-3 interaction-associated EGFR activation.

MUC1 co-immunoprecipitation showed a weak presence of EGFR in MUC1 immunoprecipitates but a substantial increase after addition of galectin-3, with or without the presence of EGF. This, together with the discovery that the presence of galectin-3 alone did not induce EGFR dimerization, suggests that galectin-3-MUC1 interaction is essential for galectin-3-associated, EGF-induced EGFR activation. The importance of galectin-3-mediated change of MUC1 cell surface localization in EGFR activation is supported by the discovery that the presence of a truncated form of galectin-3 (galectin-3C), which lacks the N-terminal domain responsible for galectin-3-induced ligand clustering hence could not induce MUC1 polarization, did not show any effect on EGFR dimerization and activation or ERK signalling, in the presence of EGF in MUC1-positive cells.

An earlier study has proposed formation of a bridge formed by galectin-3 between MUC1 and EGFR in cancer cells (513). In our study, very minimal galectin-3 was co-immunoprecipitated with EGFR and addition of exogenous galectin-3 also showed no effect on EGFR association with galectin-3 and in the cells (chapter 6). Addition of galectin-3 also did not show any effect on EGFR phosphorylation, EGFR dimerization, or ERK activation in the MUC1-positive cells in the absence of EGF, nor did it show any effect on EGFR activation in the MUC1-negative cells even in the presence of EGF. These findings indicate that a direct binding of galectin-3 to EGFR, even if it occurs, does not contribute to galectin-3-MUC1-associated EGFR dimerization and activation in those cells. It is noted that a recent study has reported a role of galectin-3 in promoting spheroid formation of lung cancer cells through activation of EGFR (490). Although that study did not identify the galectin-3 binding ligand related to the effect, their discovery of the requirement of the galectin-3 carbohydrate recognition domain in its effect is broadly in keeping with an effect of galectin-3-MUC1 interaction on EGFR activation, which requires galectin-3 CRD domain, shown in the present study.

EGFR activation on the cell membrane is known to trigger activation of an array of intracellular signalling pathways (214) including commonly Ras/MEK/ERK signalling activation. EGFR activation induced by either MUC1 expression or by MUC1-galectin-3 interaction is shown in this study to be accompanied by an increase of ERK activation. It is noted that in addition to increase EGFR activation, galectin-3-MUC1 interaction also causes a prolonged activation of EGFR and ERK activation. It is

generally believed that EGFR activation is terminated primarily through endocytosis of the receptor-ligand complex which are either degraded in the endosomes or recycled to the cell surface. It has been reported that if recycled EGFR is unable to reach the cell surface or to the lysosomal compartment but accumulates in the early endosomes, it will lead to prolonged signalling and increased activation of ERK (506). This does seem to be supported in our study. We found that following EGFR activation, more EGFR was seen to be located in a clustered pattern inside the cells in the galectin-3/EGF treated cells than in EGF alone treated cells. There was a much weaker EGFR cell surface localization in the galectin-3/EGFR treated cells than in the other groups including EGF-alone treated cells. This indicates that the galectin-3/MUC1-mediated EGFR activation and subsequent EGFR endocytosis is associated with slower recycling of EGFR to the cell surface. This may provide an explanation for the prolonged activation of EGFR and ERK in those cells. This is also in keeping with an earlier study showing that MUC1 expression inhibits EGFR degradation in response to ligand binding but was accompanied by an increase of EGFR internalization in breast epithelial cells (507).

We noted that the presence of EGFR phosphorylation inhibitor Lapatinib completely inhibited EGFR phosphorylation but detectable level, similar as the controls, of ERK activity remained in the cells irrespective of the presence or absence of EGF. This indicates that endogenous, non-EGFR-related ERK activity exists in the cells. ERK is a one of the vital signalling pathways in cell proliferation and is known to be regulated by a variety of growth factors and molecules (516). Expressions of either galectin-3(479, 517, 518) or MUC1 (454) in cancer cells has been shown to induce ERK

activation. It is most likely therefore the inability of lapatinib to completely inhibit ERK activity in the cells is due to the existence of non-EGFR-related actions of endogenous molecules such as galectin-3, MUC1 or other molecules expressed or secreted by the cells.

7.3 Final conclusions

MUC1 overexpression is a common feature in epithelial cancer cells and is shown in this study to enhance cancer cell resistance to anoikis. Overexpression of MUC1 has been shown previously to inhibit E-cadherin-mediated cell–cell interactions and to increase the ability of the cancer cells to detach from adjacent cells at primary tumour sites and to promote tumourigenesis (56, 476). Interaction of cancer-associated MUC1 with circulating galectin-3, a galactoside-binding protein whose concentration is markedly increased up to 30-fold in the bloodstream of cancer patients (483), via expression of the oncofetal TF antigen on MUC1 (76), induces MUC1 cell surface polarization and exposure of the cell surface adhesion molecules. This consequently results in increased homotypic aggregation and heterotypic adhesion of circulating tumour cells to the blood vascular endothelium and tumour cell spread (24, 334). Thus, overexpression of MUC1 in epithelial cancer cells can influence several steps in tumourigenesis and metastasis and each of these is influenced not only by the MUC1 protein expression but also by the MUC1 localization/depolarization, its glycosylation patterns and the presence of its interacting proteins in the tumour microenvironment.

MUC1 expression is also shown in this study to increase EGFR activation in epithelial cancer cells and this effect is further enhanced by its interaction with galectin-3. EGFR represents a key therapeutic target for cancer treatment. Development of anti-EGFR strategies is a crucial area of clinical study for the treatment of solid tumours. Currently, main strategies include monoclonal antibodies directed towards the extracellular domain of EGFR, small molecule tyrosine kinase inhibitors targeting the catalytic kinase domain of EGFR and strategies to disrupt receptor trafficking to the cell surface. The discovery in this study that the expression of MUC1 and its interaction with galectin-3 promotes ligand-dependent EGFR activation has implications in EGFR-targeted therapies in cancer treatment. MUC1 and galectin-3 are both well known to be commonly over-expressed by solid tumours. Over-expressions of MUC1 and galectin-3 and their increased interaction on EGFR activation may therefore not only have an influence on EGFR-mediated tumourigenesis and cancer progression, but may also have an impact on the effectiveness of EGFR-targeted therapy.

7.4 Implication for future research

While this study has identified that MUC1-galectin-3 interaction leads to increased EGFR activation and reduction in MUC1 size (through reduced *O*-glycosylation) leads to increased anoikis, there are still a few unanswered questions. For example, I wasn't able to assess if MUC1 polarization has any effect on anoikis and on caspase 3/7 level following suspension culture. As noted in this thesis, MUC1 polarisation leads to an increased access to underlying molecules. This could in theory lead to an increased level of anoikis (as anoikis initiating molecule on cell surface will more readily be activated) or reduce anoikis due to formation of tumour emboli. This could shed further light on potential MUC1-galectin3 interaction on anoikis. Additionally, we still don't know if reduction in MUC-1 *O*-glycosylation has an effect on EGFR activation, following simulation with EGF. Since depletion of the MUC1 extracellular domain resulted in greater reduction of MUC1-associated EGFR activation compared to intracellular domain, the finding from this experiment could further identify the underlying mechanism behind MUC1 and EGFR interaction.

One of the key finding in that thesis is that MUC1-galectin-3 interaction leads to increased EFFR and ERk activation following simulation with the ligand EGF. There are still a few limitations with this finding. Notably, EGF is one of the seven known ligands for EGFR. Other ligands such as TGF-α and amphiregulin are also noted to be overexpressed in tumour state. Further work is needed to assess if MUC1-galectin-3 interaction leading to increased EGFR phosphorylation can be replicated with these ligands, Additionally, this study has mainly focused on EGFR/ErbB1 interaction with

MUC1. Due to similarity in structure of ErbB family of proteins and their involvement in development of various cancers, further study is warranted to assess if other members of ErbB family interacts with MUC1. Finally, this study only focused on MUC1-galectin-3 interaction on EGFR activation/phosphorylation on cell surface and did not explore further into receptor internalization, recycling and degradation. Further work in this area could lead to a better understanding of MUC1-EGFR interaction.

7.4.1 Potential for translation into clinical practice

Work done in this study also has possible therapeutic potential that could be explored further. Both galectin-3 and MUC1 are reported to be overexpressed by in epithelial cancer. Work done in our lab has identified circulating galectin-3 to be increased by up to 30 folds in patients with metastasising tumours. These patients are also reported to have an increased level of autoantibodies against MUC1 in circulation along with fragments of shed MUC1 itself. These molecules in circulation presents a novel target to be used as biomarkers for early detection of potential metastatic tumours.

Targeting MUC1 and galectin-3 in circulation also presents as a potential therapeutic target against metastatic tumour. MUC1 has already generated a lot of interest in the research community and currently ranks second among 75 antigen candidates for cancer vaccines. Combining anti-MUC1 treatment with anti-galectin-3 therapy presents a novel way to therapeutically combat tumour metastasis in future. Several

compounds including modified heparin, gold–nanoparticles and modified antibiotics are currently under development as a potential means to target increased galetin-3 in circulation.

Increased EGFR expression and activation, seen in many cancers is currently targeted by anti-EGFR therapy such as with tyrosine kinase inhibitors (TKI) gefitinib, erlotinib and afatinib. Most patients receiving anti-EGFR therapies benefit from the treatment, but in some cases and especially in patients with prolonged use of TKI, the tumor ultimately develops drug resistance (507). In these patients combining therapy with anti MUC1 and anti-galectin-3 therapy presents a triple target to supress tumour proliferation and could perhaps increase the effectiveness of the TKI.

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9 Appendix

9.1 Appendix 1

9.1.1 Research output

Data presented in this thesis has been published in following Publications

Piyush T, Rhodes JM, Yu LG. MUC1 O-glycosylation contributes to anoikis resistance in epithelial cancer cells. Cell death discovery. 2017;3:17044. Epub 2017/07/21. doi: 10.1038/cddiscovery.2017.44.

Piyush T, Chacko AR, Sindrewicz P, Hilkens J, Rhodes JM, Yu LG. Interaction of galectin-3 with MUC1 on cell surface promotes EGFR dimerization and activation in human epithelial cancer cells. Cell death and differentiation. 2017. Epub 2017/07/22. doi: 10.1038/cdd.2017.119.

Zhao Q, **Piyush T**, Chen C, Hollingsworth MA, Hilkens J, Rhodes JM, Yu LG. MUC1 extracellular domain confers resistance of epithelial cancer cells to anoikis. Cell death & disease. 2014;5:e1438. Epub 2014/10/03. doi: 10.1038/cddis.2014.421.

Poster presentation

Piyush T and Yu LG. MUC1 interaction with galectin-3 promotes EGFR dimerization and activation in epithelial cancer cells. Poster session presented at the national cancer research institute conference, NCRI 2016, Liverpool 6-9 Nov 2016