THE EXPRESSION AND ROLE OF CIRCULATING GALECTINS IN COLORECTAL CANCER

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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DECLARATION

All the techniques and experiments performed and described in this thesis were undertaken by myself as a PhD student between February 2008 and February 2011, with the exception of Fluorescent Activated Cell Sorter (FACS) analysis which was carried out by Dr. Qicheng Zhao, and the detection of Anti-Xa activity of chemically modified heparins which was carried out by Professor Jerry Turnbull.

Neither this thesis nor any part of it has been submitted in support of an application of another degree or qualification of this or any other University or other institute of learning.

Hannah Elizabeth Barrow, 2011

Dedicated to my family, friends, and the people of the North West Cancer Research Fund

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ABBREVIATIONS

AFGP	antifreeze glycoprotein
Ala	alanine
ANOVA	analysis of variance
APC	adenomatous polyposis coli
ASM	asialo-submaxillary mucin
Asn	asparagine
ATIII	antithrombin III
BSA	bovine serum albumin
CEA	carcinoembryonic antigen
C1GalT	Core 1 β1-3 glycosyltransferase
CRC	colorectal cancer
Cosmc	Core 1 β1,3-gal-T-specific molecular chaperone
CRD	carbohydrate recognition domain
CSC	cancer stem cell
DiO	3,3'-dilinoleyloxacarbocyanine
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulphoxide
ECM	extracellular matrix
EGM	endothelial growth medium
ELISA	enzyme-linked immunosorbant assay
ER	endoplasmic reticulum
FAP	familial adenomatous polyposis
FCS	foetal calf serum
Fuc	fucose
Gal	galactose

GalNAc	N-acetylgalactosamine
GDP	guanosine diphosphate
Glc	glucose
GlcNAc	N-acetylglucosamine
GSL-II	*Griffonia Simplicifolia* lectin II
HMVEC-L	human microvascular endothelial cells-(lung).
HNPCC	hereditary nonpolyposis colorectal cancer
HUVEC	human umbilical vein endothelial cells
ICAM1	intracellular adhesion molecule 1
Lac	lactose
LacNAc	N-acetyllactosamine
LMWH	low molecular weight heparins
Man	mannose
MCP	modified citrus pectin
OPD	o-phenylenediamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PNA	peanut agglutinin
Pro	proline
dsRNA	double stranded RNA
mRNA	messenger RNA
siRNA	small interfering RNA
RNAi	RNA interference
RISC	siRNA-induced silencing complex
RLC	RISC loading complex
SD	standard deviation
SDS	sodium dodecyl sulphate
Ser	serine
S-Tn	sialyl-Tn antigen

TF antigen	Galβ1-3GalNAcα-Ser/Thr
	Thomsen-Friedenreich antigen
Tn antigen	GalNAcα-Ser/Thr
Thr	Threonine
TEMED	tetra-methyl-1,2-diamino-ethane
UC	ulcerative colitis
UDP	uridine diphosphate
VNTR	variable number tandem repeat

ABSTRACT

Adhesion of circulating tumour cells to the blood vascular endothelium is a pivotal step in metastasis. This study shows that the levels of free circulating galectin-2, -3, -4, and -8, but not galectin-9 and -1, were markedly increased up to 31-fold in the bloodstream of colon and breast cancer patients and in particular those with metastasis. The presence *in vitro* of each of these galectins at pathological concentrations induced dose-dependent increases of cancer cell adhesion to monolayers of human macro- and micro-vascular endothelial cells, an effect that was abolished by the presence of galectin inhibitor, by pre-fixation of the cells, or by pre-treatment of the cells with *O*-glycanase to remove cell surface TF (Galβ1,3GalNAcα-) antigen. Suppression of the TF-expressing mucin protein MUC1 by siRNA reduced, while overexpression of MUC1 increased, the galectin-mediated cancer cell adhesion. Higher levels of circulating galectin-2 were associated with a significantly increased mortality risk in colorectal cancer patients and this association was diminished by serum co-existence of auto-anti-MUC1 antibody specifically against the TF epitope of MUC1. Thus, the increased circulations of galectin members are common features in cancer and promote metastatic spread. Circulating galectins therefore represent a novel class of therapeutic targets for the development of effective agents to reduce metastasis and increase patient's survival. The possible role of modified heparins as inhibitors of the galectin3-ligand interaction that leads to increased vascular adhesion was therefore investigated. ELISA assays showed that chemically modified heparin derivatives successfully blocked galectin-3 adhesion to asialo-bovine mucin, and also galectin-3-mediated cellular adhesion to endothelial cell monolayers and extracellular matrix components, which suggests a possible role for heparin derivatives in cancer therapeutics. Finally in this study, the functional importance of Core 1 Gal-transferase (C1GalT) was investigated. It has long been presumed that there is a competition between Core 1 Gal-transferase (C1GalT), Core 3 GlcNAc-transferase (C3GnT) and sialyl-transferase (ST6GalNAc-T) for elongation of O-linked mucin-type glycans that initiate with GalNAcα-Ser/Thr. However, evidence that supports such a competition among these glyco-transferases is surprisingly lacking. This study shows that selective suppression of the C1GalT caused over 80% reduction of Galβ1,3GalNAcα- (Core 1, Thomsen-Friedenreich, TF antigen) expression in human colon cancer HT29 and SW620 cells. Suppression of C1GalT was also associated with 198±8%, 136±24% and 231±6% increase of sialyl-GalNAcα- (sialyl-Tn), GlcNAcβ1,3GalNAcα- (Core 3) and GalNAcα- (Tn) expression in HT29 and 174±11%, 155±37% and 200±5% increase in SW620 cells. These results provide direct evidence of a competition between C1GalT, C3GnT and ST6GalNAcT transferases for modification of the GalNAcα-Ser/Thr in O-glycan biosynthesis. As Tn, TF and sialyl-Tn are all oncofetal carbohydrate antigens and over-expressed in up to 90% of all human cancers, this information may also be useful for future development of glyco-transferase-targeted therapeutic strategies for cancer treatment.

CHAPTER 1

INTRODUCTION – The Development of Colorectal Cancer

1.1 Colorectal Cancer

Colorectal cancer is the third most common cancer and the fourth most common cause of cancer-related deaths worldwide. In the Western World, Colorectal cancer is the second most common cancer, with a cumulative lifetime risk of approximately 5% (1). Colorectal cancer may develop sporadically, or may manifest due to familial or hereditary cancer syndromes. Underlying inflammatory bowel diseases confer an increased risk for the development of colorectal cancer (1, 2).

Ninety to ninety-five per cent of colorectal cancer cases are the result of sporadic development, which reflects the importance of environmental factors (3, 4). However, 20% of these cases may be contributed to by some form of familial risk. 5-10% of colorectal cancers are manifested due to hereditary cancer syndromes (5). Approximately 30% of deaths related to ulcerative colitis and Crohn's disease are due directly to the subsequent development of colorectal cancer. The level of risk is dependent upon the extent of inflammation (2).

1.1.1 The Molecular Causes of Colon Cancer

Cancer can arise due to genetic changes through random replication errors, exposure to carcinogens, or faulty DNA repair or an inherited germ line mutation. There are two classes of mutated genes that are critical in the development of cancer. Firstly, proto-oncogenes are genes that promote normal cell growth, and these can be transformed to oncogenes by gain-of-function mutations. Secondly, tumour suppressor genes, which normally restrain the process of cell growth, can be nullified by loss-of-function mutations (6). The adenoma-carcinoma development in colorectal cancer can be defined by two pathways, gatekeeper and caretaker pathway. The two main forms of hereditary colorectal cancers are hereditary nonpolyposis colorectal cancer (HNPCC), which represents an abnormality in the caretaker pathway, and familial adenomatous polyposis (FAP), which represents an abnormality in the gatekeeper pathway (5).

FAP is an autosomal-dominant disease, and in the majority of cases, is a germ line mutation that can be located in the adenomatous polyposis coli (APC) gene. APC is defined as a tumour-suppressor gene, and is thus classified as a gatekeeper gene, which acts through the Wnt signalling pathway. The cell-signalling glycoprotein family of Wnt factors interact with Frizzled cell-surface receptors, which, in turn, activate the intracellular protein dishevelled. Dishevelled subsequently associates with axin, and is able to prevent glycogen synthase kinase 3B (GSK3B) from phosphorylating its substrates, which are present in a complex with the GSK3B protein, axin, APC and beta-catenin. Due to the inactivation of GSK3B, the beta-catenin is no longer degraded and it begins to accumulate in the cytoplasm and nucleus, thus binding to members of the T cell factor/lymphocyte enhancer factor family. This beta-catenin/TCF complex may bind DNA to activate the transcription of

target genes (6). In 80% cases of FAP, the mutation is found as a germ line mutation within the gene for APC. APC mutations are very frequent in sporadic colorectal cancers, and inactivating mutations in APC have been reported in 34–70% of sporadic colorectal cancer patients (7, 8). The APC gene is located on the FAP locus on chromosome 5 and consists of 16 exons. This gene encodes a 312-kDa protein of 2843 amino acids, which is expressed ubiquitously. Most somatic APC mutations in colorectal cancer are grouped together in a central domain, called the mutation cluster region (MCR). The mutated protein product is truncated at the C-terminus (9) and lacks the axin-binding SAMP motifs, and most of the beta-catenin binding motifs. This mutation inevitably results in the hyper proliferation of colon crypt cells and the subsequent formation of polyps. It is these polyps that transform into malignancy, following further independent genetic events, such as mutations in K-RAS and p53 (10). FAP patients can develop more than 100 colorectal adenomas if left untreated, and cancer arises in almost all patients by 40 years of age (11) (**Figure1.1**).

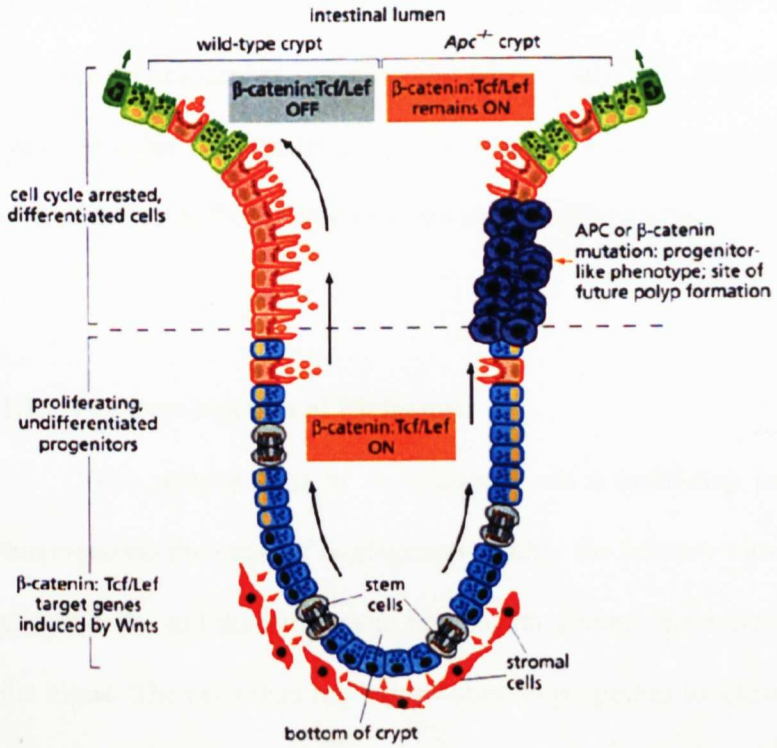


Figure 1.1. The biological layout of a healthy colonic crypt compared to an APC-/- mutant and the effect upon future polyp formation. From Weinberg, *et al.* The biology of cancer (12).

HNPCC is an autosomal-dominant disease stemming from germ-line mutations of mismatch repair genes, resulting in the molecular characteristic of microsatellite instability in which there are frequent mutations within the short repeated DNA sequences, and is thus an example of a pathway to cancer through abnormalities in caretaker genes (13).

These two pathways represent the principal mechanisms of pathogenesis for a primary colorectal tumour; however it is the possibility of metastasis that forms the

most fearsome aspect of cancer. Despite significant improvements in diagnosis, surgical techniques and local and systemic adjuvant therapies, most deaths from cancer are due to metastases that are resistant to conventional therapies (14) and the overall mortality from colorectal cancer approaches 50%.

1.1.2 The Development of Metastasis

The pathogenesis of metastasis is via a multi-step pathway. This typically encompasses the steps of angiogenesis within the primary tumour, detachment of the cancer cells, and the subsequent intravasion through the extracellular matrix and into the blood. The cells then rely upon adhesion properties to allow adhesion to the blood vessel endothelium, and aggregation for the formation of tumour cell emboli. It is widely accepted that metastasis arises when tumour cells interact with a specific microenvironment. In 1889, Stephen Paget, an English surgeon, produced a report 'Distribution of secondary growths in cancer of the breast', in which he attempted to answer 'What is it that decides what organs shall suffer in a case of disseminated cancer?' In this report, Paget made a distinct reference to the high incidence of metastasis in the liver, ovary, and specific bones, and the low incidence in the spleen of 735 women with terminal breast cancer (15). This analysis of the non-random pattern represented the distinct possibility that the target of a specific type of cancer did not arise solely due to chance. This process was introduced as the 'seed and soil' hypothesis, with the primary tumour cells and the target organ microenvironment equating to the seed and soil, respectively.

Although this theory prevails today, the 'seed and soil' hypothesis did not go unchallenged. In 1929, James Ewing eschewed this concept and proposed that the

metastatic distribution occurred due to mechanical factors alone, that arose due to the anatomical structure of the vascular system (16). Nevertheless, the 1970s brought about the definitive proof of the 'seed and soil' hypothesis. During this time, studies were undertaken which grafted samples of kidney, ovary and lung tissue into the subcutis or muscle of syngenic mice, followed by the intravenous injection of melanoma cells. The outcome of these experiments showed that the tumour cells did indeed reach the vascular system of all organs, but the metastases developed in the orthotropic and grafted lungs and ovaries, but not in the kidneys (17).

The 'seed and soil' hypothesis is widely cited and has withstood 120 years of scrutiny and critical analysis. The 'seed' has now been defined as a progenitor, and the 'soil' is defined as the host factors and microenvironment, and ultimately the cross talk between tissues has been placed in the forefront of research into metastasis.

1.1.3 The Pathogenesis of Metastasis

Thus metastasis is a step-wise procedure, with each step rate-limiting (17) (**Figure 1.2**). Coman *et al.* found that, upon intravascular injection of tumour cells into animals, metastases were produced in some, but not all organs. It was found that in the organs affected, the tumour cells were lodged in the capillaries, implying the importance of embolus formation and arrest in secondary organs (18). This was supported by Zeidman *et al.* who demonstrated embolic arrest in the capillaries of rabbits (19). It was seen that some of the tumour cells distorted and passed through the capillary wall. Others seemed to remain non-pliable and remained trapped. This initiated the concept of morphological factors as a basis for metastatic dissemination. These experiments showed that some cells passed through the vessel walls

immediately, and of those that succeeded, only some produced metastasis and the unsuccessful cells merely died (19). Labelling of the cells with ^{125}iodine-iodo-deoxyuridine showed that less than 0.1% (one thousandth) of the tumour cells remain viable upon entry into the circulation, but only 0.01% (one ten thousandth) of the cells introduced into the circulation survived for longer than 24hrs and give rise to metastases (20).

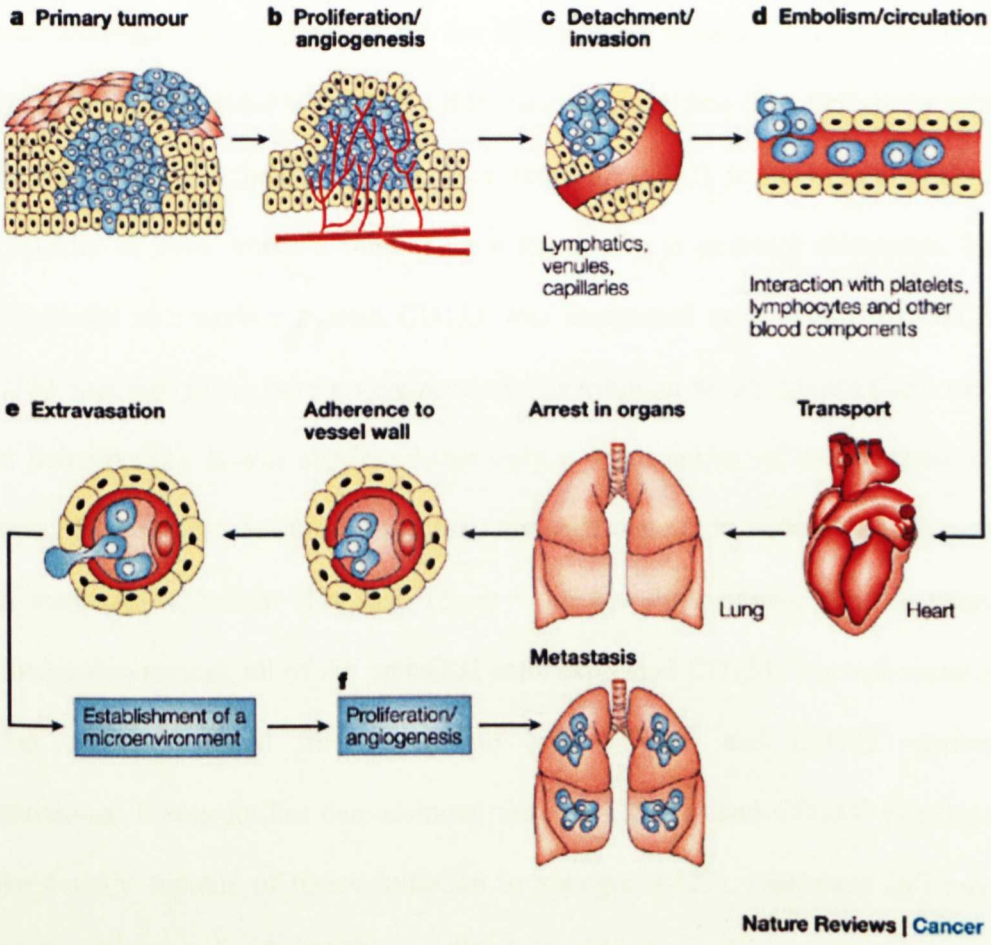


Figure 1.2. A brief overview of the key metastasis steps. From Fidler *et al* (14).

The process of metastasis selectively chooses cells that express, or over-express certain genes and proteins required for proliferation, angiogenesis, cohesion,

motility and invasion. This requires the cells within the embolus to possess the inherent abilities to succeed at invasion, survival in the circulation and arrest in a target capillary bed with the ability to undergo extravasion into the receptive parenchyma. Upon implanting a variety of cells from a single tumour within the subcutaneous tissue, muscle or organs of mice, not all the cells have the ability to disseminate. Successful cells were extracted and expanded, and then compared to the cells of the parent tumour in order to compare the behavioural changes and define which alterations are responsible for the ability to metastasise. This technique was originally used for B16-F10, from the B16 melanoma cell line (21). Selectivity within cells was reflected in the concept of cancer stem cells (CSC), which explains that only a minority of cells within a tumour have the ability to generate metastasis. Until recently the cell surface protein CD133 was designated as a marker of CSC, as CD133 was thought to have a very restricted distribution within tissues (both mouse and human) (22). It was suggested that only a small subset of colon cancer cells express CD133, and that those were the only cells that could initiate tumour growth and metastasis (23, 24). However, recent work has demonstrated that, in primary human colon tumors, all of the epithelial cells expressed CD133, whereas metastatic colon cancers isolated from liver had both $CD133^+$ and $CD133^-$ epithelial populations. It was further demonstrated that the $CD133^+$ and $CD133^-$ populations were equally capable of tumor initiation in xenografts (22). Questions still remain regarding the concept of CD133 as a CSC. It is currently not known at which stage of colon cancer progression the $CD133^-$ cells emerge, and whether the $CD133^-$ cells are descended from the $CD133^+$ cells. If they are descendents, it could remain a possibility that CD133 could still be considered a marker of metastatic stem cells.

The heterogeneity of cancer cell populations strongly implies the selective nature that the metastatic process infers, and the diversity of phenotype. There is a genetic and, therefore, phenotypic instability present in all subpopulations of tumour cells, which drives the metastatic development forwards. In order to identify whether all metastases arise from a singular clone or if different metastases originate from different progenitor cells, a series of experiments were carried out utilising gamma-irradiation as a source of random chromosome breaks and rearrangements that function as markers. Talamage *et al.* investigated the incidence of lung metastases arising from K-1735 mouse melanoma cells that had been gamma-irradiated to induce chromosomal damage. In 10 of the metastases, the chromosomes were all unchanged, which could not reveal whether they were of uni- or multicellular origin. In other metastases, unique markers were found within the chromosomes and they indicated that each lesion arose from a single progenitor cell (25). Further experiments revealed that metastases were all of unicellular origin, when heterogeneous clumps of two different melanoma cell lines were injected intravenously (26). When combined, these results indicated that whether a metastasis is hetero- or homogeneous, they still arise from a single deranged cell.

1.1.4 Colorectal Cancer and Liver Metastasis

In a population-based study, it has been shown that the proportion of patients with synchronous liver metastases was 14.5%. Age-standardized incidence rates were 7.6 per 100,000 in males, 3.7 per 100,000 in females. The 5-year cumulative metachronous liver metastasis rate was 14.5% (27). Liver metastases are the major cause of death in colorectal cancer patients. By the time of detection of a primary

tumour, 15-25% of the patients present with liver metastases, and a further 20% go on to develop these metastases post-treatment of the primary (28). Without treatment, the median survival is approximately 9 months, varying based on the extent of the disease upon diagnosis (29). Unexpectedly, resections of liver metastasis from colorectal origin have been shown to result in long-term survival and the possibility of cure. In selected patients with metastatic lesions confined to the liver, 5-year survival rates are reported at 35-40% dependent upon the extent of liver involvement. However, only a minority of patients (10-15%) with liver metastasis are considered to be candidates for resection (30).

These concepts present a new facet in the study of colorectal cancer, and the requirement for novel data to reveal and assess the key elements within colorectal cancer and the subsequent metastasis to the liver.

1.1.5 Colorectal Cancer and Lung Metastasis

About 10-25% of patients with colorectal cancer have lung metastasis, making pulmonary spread the second most common after liver metastasis. However, only 2-4% have metastasis confined just to the lungs (31, 32). Treatment options include chemotherapy and surgery. Although several novel chemotherapeutic and targeted agents have been developed for treatment of unresectable metastatic colorectal cancer, very long-term survival is uncommon (33, 34). Surgical lung resection has been considered to be the only effective treatment for isolated pulmonary metastatic tumour from colorectal cancer (35). Published data on 5-year survival rates in resectable pulmonary metastasis from colorectal cancer ranges from 27% to 56% (36-41).

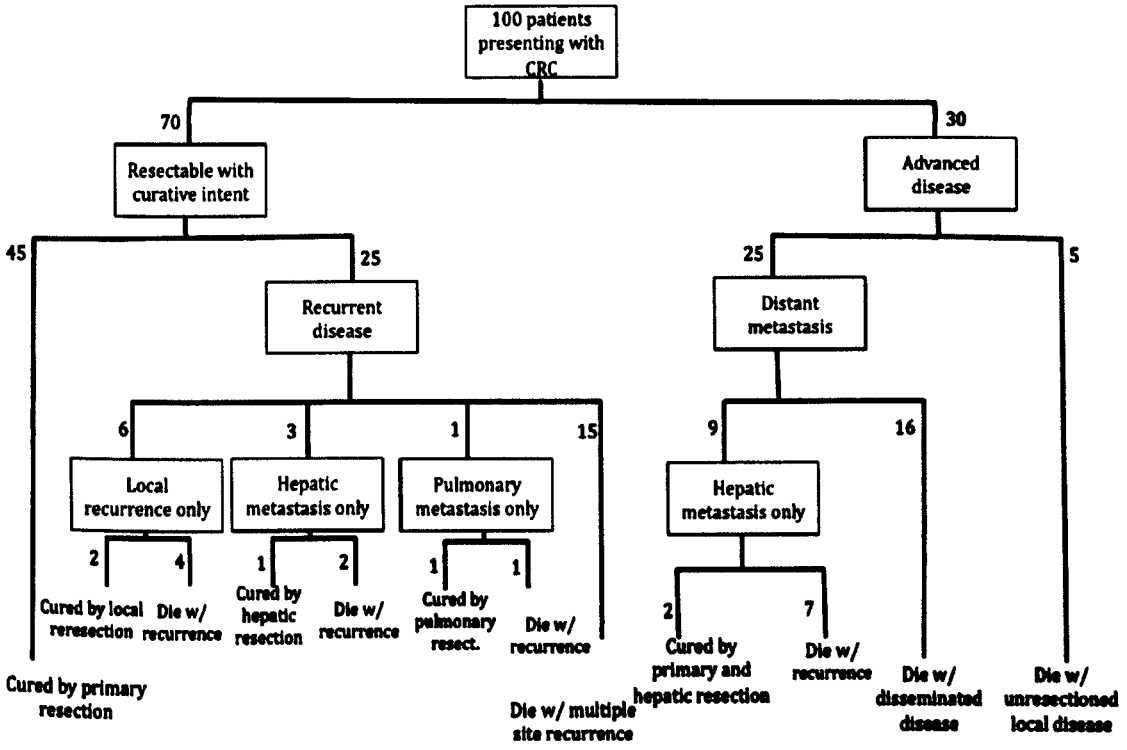


Figure 1.3. Patterns of failure in 100 patients with large bowel cancer. Adapted from August *et al* (42).

CHAPTER 2

INTRODUCTION – Glycosylation, Glycans and Carbohydrate-Binding Proteins

2.1 The Glycome

'Glycome' is a term that describes the complete collection of glycans and glycoconjugates in the cell. The term 'glycan' is often used to describe oligosaccharides, polysaccharides and carbohydrates, and the field of 'Glycomics' was subsequently developed, which refers to the profiling and study of the glycome itself. The field of glycobiology seeks to understand the structure, biosynthesis and biological function of glycans and their derivatives (43). The history of this field began with research into the basic carbohydrates produced within cells, and then grew into initial clinical applications with the discovery of human blood antigens when blood samples from individuals exhibited agglutination (44). Whereas the genome is, for the larger part, established and fixed in most cells, the proteome and glycome are dynamic, and glycomics endeavours to understand how a collection of glycans correlates with a biological event or process (45).

2.1.1 Glycoproteins

A glycoprotein is a glycoconjugate in which a protein bears one or more glycan structures that are covalently attached to the polypeptide backbone via *N*- or *O*-linkages (43). Unlike proteins, carbohydrate structures are not encoded directly by the genome. Due to the presence of enzymes and transporters responsible for the biosynthesis of the carbohydrate sequence of the glycan chains, there is a multitude of sequence combinations that can be achieved by sequential and competing transferases and glycosidases in the compartmentalized structure of the endoplasmic recticulum and the Golgi apparatus. The enzymes that are involved in the production and modification of glycans are estimated to comprise 1% of genes in the mammalian genome (46). Moreover, environmental changes can trigger significant variances in the glycan structures produced by cells. It is this dynamic nature that can result in such complexity and diversity, and even with complete understanding of the cell and genes involved, it is complex and difficult to successfully predict accurate structures at any given time. Over half of the proteins expressed in eukaryotic systems are found to be glycosylated (47), and the main post-translational modifications involving carbohydrates are *N*-linked glycosylation, and *O*-linked glycosylation.

2.2 *N*-Glycosylation

N-glycosylation occurs when a glycan modification is attached to an asparagine (Asn) residue through an N-glycosidic bond by a step-wise series of enzyme-catalysed reactions. The synthesis of eukaryotic N-glycans is initiated on the surface of the endoplasmic reticulum, where a lipid-linked oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ –P-P-Dol is transferred to an Asn residue (48). All steps of this

pathway leading up to the generation of $Man_9GlcNAc_2$-P-P-Dol occur at the cytosolic side of the ER membrane, using activated nucleotide sugars as donor substrates. The formation of the carbohydrate structures that will be eventually attached to the Asn residues is initiated by the addition of two GlcNAc monosaccharides followed by five mannose monosaccharides for which the donors are UDP-GlcNAc and GDP-Man, respectively (49). Synthesis of the oligosaccharide is completed at the luminal side of the endoplasmic reticulum, when the $Man_5GlcNAc_2$-P-P-Dol then flips across the lipid bilayer and a further four mannoses and 3 glucose monosaccharides are added. This results in the completed oligosaccharide precursor that can be transferred from the lipid to the nascent polypeptide chain by the multisubunit enzyme complex, oligosaccharyltransferase (OST), which forms a complex with other proteins to form a functional unit (50).

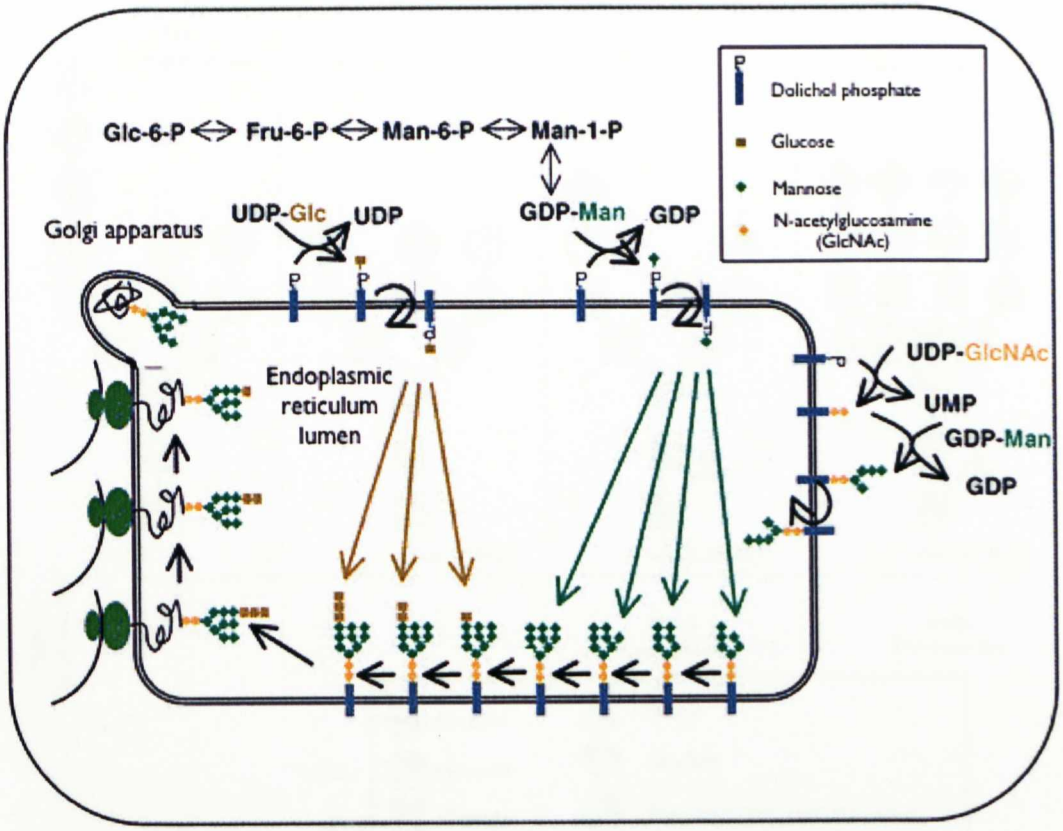


Figure 2.1. The N-glycosylation pathway. (Adapted from Jaeken *et al.*) (51)

Attachment of the N-glycan occurs at the site Asn-X-Thr/Ser, where X is any amino acid except proline. Extensive modification through trimming and elongation can occur to remodel the N-glycan. Oligosaccharide trimming starts immediately after transfer from glycolipid to the nascent polypeptide chain through the action of glucosidases I and II, which are present in the lumen of the ER. Glucosidase I acts specifically on the single α1-2-linked terminal glucose. In a manner preserved in eukaryotes, the three glucose residues are cleaved sequentially, which is shown to be crucial in protein folding (52). These modifications give rise to the production of three main oligosaccharide types (**Figure 2.2**).

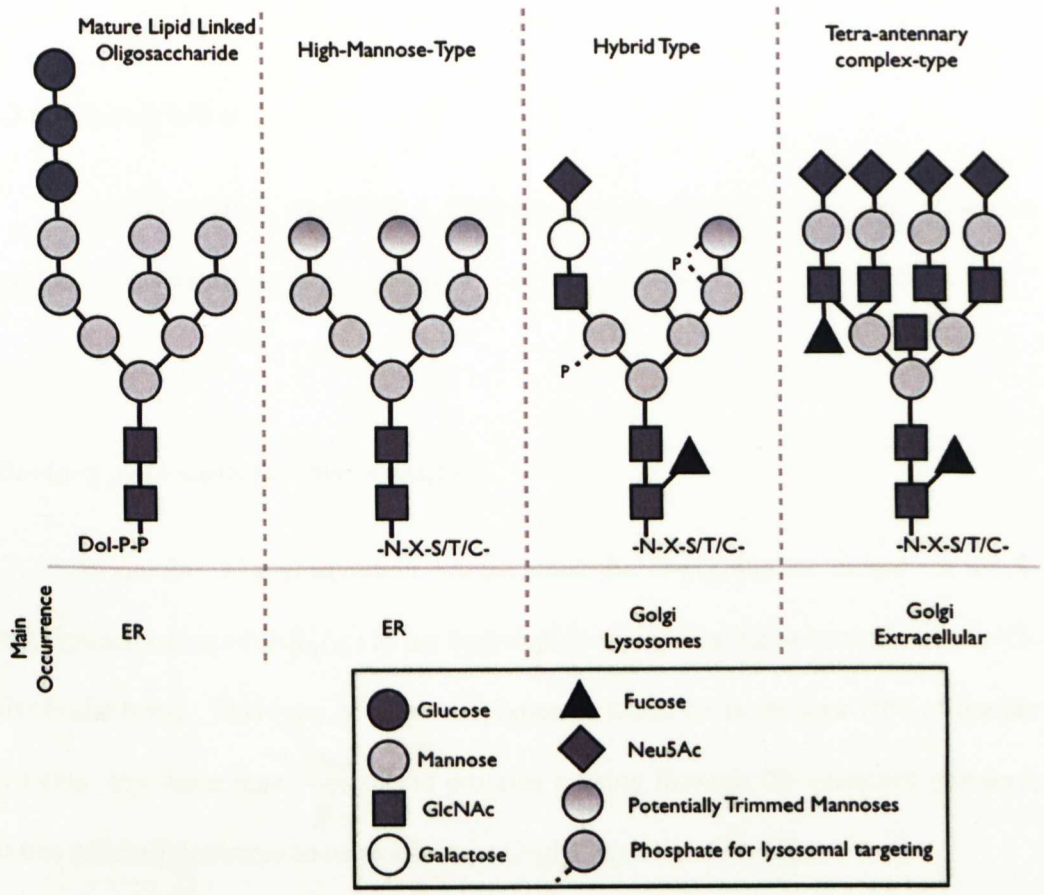


Figure 2.2. The Diversification of N-glycans. (Adapted from The Sugar Code, Wiley-Blackwell Publishing.) (53)

All N-glycan types have a pentasaccharide core, but present different sequences of sugar residues in the branches, which are altered by the activity of mannosidases or glycosyltransferases. The high-mannose type has only mannose residues, whereas hybrid types have GlcNAc substituted on one branch while the other branch contains mannose. Finally, the complex types have both of the branches substituted with GlcNAc. These differing structures provide specificity, which ensures only certain enzymes to act upon them, allowing a great range of diversity amongst the N-glycans.

2.3 *O*-Glycosylation

O-glycosylation describes a covalent modification of serine and threonine residues of mammalian glycoproteins.

Mucin-type *O*-GalNAc Glycosylation:

In mucins, *O*-glycosylation occurs when the *O*-glycans are linked via an *N*-acetylgalactosamine (GalNAc) to the hydroxyl group of a serine or threonine by an O-glycosidic bond. This type of *O*-glycosylation is found on more than 10% of human proteins, and more than 50% of the proteins passing through the secretory pathway, and is generally referred to as mucin-type O-glycosylation (54, 55).

Other types of *O*-glycosylation have been identified.

O-GlcNAc Glycosylation:

In this form of *O*-glycosylation, the monosaccharide *N*-acetylglucosamine is attached in a β-linkage to serine and threonine hydroxyl groups of proteins (56, 57). There are rarely any further carbohydrate modifications following the addition of *O*-GlcNAc. *O*-GlcNAc glycosylation occurs in the nucleoplasm, cytoplasm and to some extent in the mitochondria, which breaks the dogma that protein glycosylation is restricted to the endoplasmic reticulum and golgi apparatus (58). *O*-GlcNAc is a reversible modification and is modulated by two enzymes, *O*-GlcNAc transferase (OGT) and *O*-GlcNAc hydrolase (OGA), which attach and remove *O*-GlcNAc from proteins, respectively (59). *O*-GlcNAc has been found in all eukaryotes examined,

and appears as wide-spread as phosphorylation (60, 61). No consensus motif for O-GlcNAc attachment has been identified, but many sites are identical to those used by serine/threonine kinases, and there is a 'yin-yang' relationship between *O*-GlcNAc modification and phosphorylation. Phosphatase inhibitors and kinase activators decrease overall levels of O-GlcNAc and conversely kinase inhibitors increase levels of O-GlcNAc (62). Furthermore, on a select few proteins the mapped site for glycosylation and phosphorylation are identical, such as c-Myc (63), the estrogen receptor (64), and SV-40 large T antigen (65). This apparent reciprocity again emphasizes that proteins exist in a variety of forms through combinatorial post-translational modification.

_O_-fucose and _O_-glucose Glycosylation:

O-fucose and *O*-glucose modifications have stimulated interest due to the effects they mediate upon the Notch family of receptors and ligands, and the Cripto/FRL/Criptic (CFC) family of proteins. These modifications are important as they regulate the signal transductions involved in early development and the growth of some cancers (45). *O*-fucosylation occurs at Ser or Thr in the consensus sequence C_2X_{4-5}-S/TC_3 between the second and third Cys of Notch EGF repeats. The fucose residue is transferred by the enzyme protein *O*-fucosyltransferase-1 (POFUT1), and the *O*-fucose may be extended with *N*-acetylglucosamine (GlcNAc) transferred by a Fringe β1,3GlcNAcT from a UDP-GlcNAc, and subsequently by β1-4 galactose and α2-3 or α2-6 sialic acid in mammals (66).

Only a few proteins are known to have the *O*-glucose modification. *O*-glucose modifiations are observed on human factor VII, factor IX, and protein Z, fetal antigen

1 and Notch (45, 67). The concensus sequence for the addition of *O*-glucose is C^1XSXPC^2 where C^1 and C^2 are the first and second concerve cysteines of the EGF repeat, respectively, and X can be any amino acid (68). The glucose residue is added in a β-linkage to a serine by protein *O*-glucosyltransferase (POGLUT) (66). Similarly to POFUT, POGLUT is localized in the ER, where it acts upon the properly folded EGF repeats (69, 70). The modification in its monosaccharide form can be seen, but the *O*-glucose glycan can be further elongated by consecutive addition of two xylose sugars to form a mature disaccharide, Xyl-α1,3-Xyl-α1,3-Glc (71). Although activities of the enzymes responsible for addition of these xyloses have been detected, the xylosyltransferases have not yet been identified (72, 73). Like *O*-fucosylation, *O*-glucosylation plays a role in the modulation of Notch signaling. Elimination of specific *O*-glucose sites on mouse Notch1 alters Notch activation (45).

_O_-mannose Glycosylation:

O-Mannosyl glycans linked to Ser and Thr residues were first identified in the late 1960s in bakers' yeast (74), and was believed to be restricted to yeast and fungi. In all yeasts and fungi studied so far, the reducing terminal mannose residue of *O*-mannosyl glycans is α-linked to Ser or Thr and may be extended to form an α1,2-linked mannose trisaccharide (Manα1-2Manα1-2Manα1-Ser/Thr) (75). This core structure is further processed according to the yeast/fungi species. Although highly abundant in fungi, protein *O*-mannosylation is less frequent in metazoa. While fungal *O*-mannosyl glycans consist mainly of mannosyl residues, the majority of mammalian *O*-mannosyl glycans are variations of the tetrasaccharide NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-Ser/Thr with different lengths and fucose (α1,3-linked to GlcNAc) contents (76-78).

Branched structures with 2,6-di-substituted mannose (GlcNAc-linked β1,2 and β1,6) have also been reported (79).

The biosynthesis of *O*-mannosyl glycans in *S. cerevisiae* is initiated at the ER (80). Dolichyl phosphate-bound mannose (Dol-P-Man) is a phosphate activated monosaccharide (81), and serves as the mannosyl donor for the initial transfer reaction in the *O*-mannosylation pathway (82). Dol-P-Man is synthesized from GDP-Man and Dol-P on the cytosolic face of the endoplasmic reticulum (ER) membrane. Dolichyl phosphate-D-mannose:protein O-mannosyltransferases catalyze the transfer of a mannosyl residue from Dol-P-ß-D-Man to Ser and Thr residues of secretory proteins (83-86). As the transfer reaction catalyzed by Dol-P-Man:protein *O*-mannosyltransferases has been demonstrated in mammals and insects (87, 88), as well as yeast and fungi, it is apparent that the initial steps of *O*-mannosylation are conserved between the fungal and the animal kingdom.

The elongation of *O*-mannosyl glycans in mammals is only partially understood. The transfer of GlcNAc to mannose in the 2-OH position is catalyzed by *O*-mannose *N*-acetylglucosaminyltransferase 1 (POMGnT1 (89, 90)), while β1,6-N-acetylglucosaminyl transferase (GnT-IX) links GlcNAc to mannose in the 6-OH position (91). The addition of GlcNAc in β1,2-Man linkage by PomGnT1 is required before GnT-IX can add GlcNAc, strongly suggesting that GnT-IX is responsible for the formation of 2,6-branched structures in brain O-mannosyl glycans (91). Further enzymes directly involved in the elongation of O-mannose saccharides remain to be identified.

2.3.1 Biosynthesis of *O*-Glycans

O-linked glycosylation biosynthesis is arguably a simpler process than that of *N*-linked glycosylation as there is no need for a lipid-linked oligosaccharide precursor. No specific consensus sequence for the attachment of *O*-glycan chains has been identified, but the sites of attachment do have characteristic features. For example, proline-rich regions of the polypeptide chain are preferentially chosen, as the serine/threonine residues found in these areas are more physically accessible (92).

O-linked glycosylation is initiated when the monosaccarides are added in a step-wise fashion by the sequential action of glycosyltransferase enzymes. In cancer cells, many of these enzymes are up- or down-regulated (93, 94). In *O*-glycan pathways, each individual monosaccharide is transferred from a specific nucleotide sugar donor through the action of a membrane-bound glycosyltransferases (95). These polypeptide glycosyltransferases have a type II membrane protein structure, which has a short cytoplasmic tail (amino terminus), a membrane anchor, and a catalytic domain (carboxy terminus), which enters the lumen of the Golgi (96). It is the addition of the first monosaccharide from a nucleotide donor, UDP-GalNAc, to the serine/threonine in the fully folded proteins chain through the action of the polypeptide GalNAc transferase (GalNAcT) that initiates the synthesis of all mucin-type glycans, and further distinguishes it from other types of glycosylation, including alternative *O*-glycosylations, such as *O*-GlcNAc (57), *O*-mannose and *O*-fucose (68).

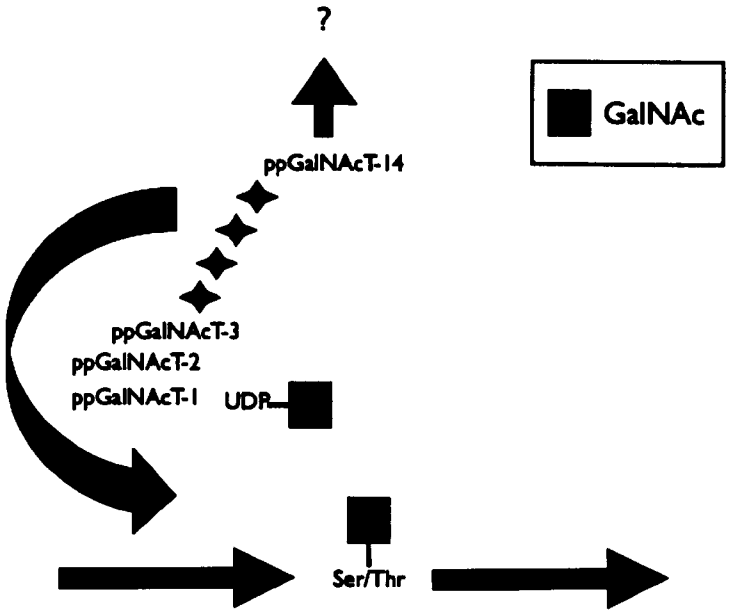


Figure 2.3. The family of ppGalNAcTs facilitating the addition of the GalNAc unit to the serine/threonine residues within the folded protein.

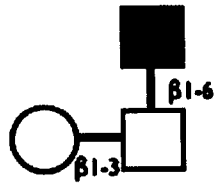
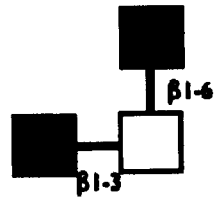
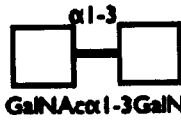
It has been shown that rather than a single GalNAcT, as cloned from the cDNA of bovine GalNAcT (97), there is a family of GalNAcTs that mediate the addition of GalNAc to the threonine and serine residues (98, 99), and 20 members have been identified to date (100) (**Figure 2.3**). Furthermore, it has been demonstrated that various GalNAcTs are expressed in a tissue-specific manner, which changes dynamically during development (101, 102).

This transfer of GalNAc to the protein is thought to occur in the Golgi apparatus (103, 104), certain regions of the endoplasmic reticulum or an inter ER-Golgi compartment (105, 106). The GalNAc monosaccharide is then acted upon in a step-wise manner by other transferases to produce various core structures, which can then be further modified to yield an extensive variety of carbohydrate moieties.

2.3.2 *O*-Glycan Core Structures

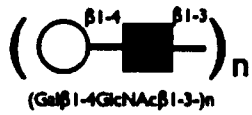
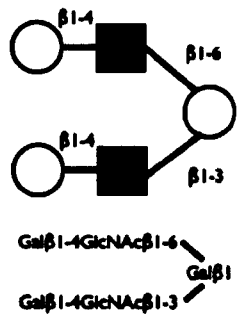
Arbitrarily, three regions of the oligosaccharide structure have been assigned: the core region, which is composed of the two or three innermost monosaccharides of the chain proximal to the peptide, the backbone region, which contributes heavily to the length of the chains when formed by uniform elongation, and the peripheral region, which demonstrates a high degree of structural complexity (54). The structure of *O*-linked mucin-type oligosaccharides is highly heterogeneous, but despite this, they can be classified in accordance to their core structures. All mucin-type *O*-glycan synthesis is initiated by the attachment of GalNAc to a serine/threonine residue, catalyzed by the enzyme *N*-acetylgalactosaminyltransferase, which is constitutively expressed in all mammalian cells. This produces the Tn epitope, GalNAcα1→Ser/Thr. Following the addition of further saccharide units, the Tn epitope can be extended into a number of core units, of which eight have been identified (107) (**Table 2.1**).

Table 2.1. The Core structures found in *O*-glycans. (The Sugar Code, Wiley-Blackwell Publishing.) (108)

Core type	Structure
1	
2	
3	
4	
5	
6	
7	
8	

The most abundant of these structures are core 1 and core 2, which are the disaccharide Galβ1-3GalNAc and the trisaccharide Galβ1-3(GlcNAcβ1-6) GalNAc respectively (54), whereas core 3 and core 4 have a lower abundance, and a restricted tissue-based expression in the bronchus (109), colonic epithelium (110, 111) and in foetal mucins (112). In the backbone region, this section can often be formed by repetitive addition of the disaccharide Galβ1-3/4GlcNAc to the core structure, which can form branches through substitution at the C6 of galactoses (54). These lactosamine-type formations can reach sizes up to 20 monosaccharides long, and can be elongated in a mono or biantennary form (**Table 2.2**).

Table 2.2. *O*-glycan chain extension as backbone structures. (The Sugar Code, Wiley-Blackwell Publishing.) (108)

Type	Structure	Name
Type 1		N-acetyllactosamine
Type 2		
Poly N-acetyllactosamine type 2		i-antigen
Branched N-acetyllactosamine type 2		I-antigen

Further to this, an enormous carbohydrate variety is produced by modification of the core and backbone units. These modifications include fucosylation, sialylation, sulfation, acetylation and methylation.

Table 2.3. Blood group and related antigens on O-linked cores.

TF (Thomsen Friedenreich antigen)	Galβ1-3GalNAcα-Ser/Thr
Disialyl TF	Neu5Acα2-3 Galβ1-3GalNAcα-Ser/Thr α2-6 NeuAc
Sialyl Tn	Neu5Acα2-6GalNAcα1-Ser/Thr
Tn	GalNAcα1-Ser/Thr
Type 1 backbone	(Gal/β1-3GlcNAcβ1-3/6)n(Galβ1-3)GalNAcα1-Ser/Thr
Type 2 backbone	(Gal/β1-4GlcNAcβ1-3/6)n(Galβ1-3)GalNAcα1-Ser/Thr
Blood group H	Fucα1-2Galβ1-
Blood group A	GalNAcα1-3 Galβ1 Fucα1-2
Blood group B	Galα1-3 Galβ1 Fucα1-2
CAD (SD^a)	GalNAcβ1-4 Galβ1 Neu5Acα2-6
Sialyl Lewis^a	NeuAcα2-3Galβ1-3GlcNAcβ1- α1-4 Fuc
Lewis^a	Galβ1-3GlcNAcβ1- α1-4 Fuc
Lewis^b	Galβ1-3GlcNAcβ1- α1-2 α1-4 Fuc Fuc
X Antigen (SSEA-1, Le^x)	Galβ1-4GlcNAcβ1- α1-3 Fuc
Y Antigen (Le^y)	Galβ1-4GlcNAcβ1- α1-2 α1-3 Fuc Fuc

2.4 Mucins

The luminal surface of the gastrointestinal tract is coated with a viscous gel layer that acts as a protective barrier against the harsh environments seen throughout the luminal environment. Mucins form part of the interactive mucosal defensive system, are responsible for the viscoelastic properties of the mucous barrier, and are active at the surface of the gastrointestinal tract, conveying protection, lubrication and transport functions (113). The extensive glycosylation of mucins creates a hydrophilic environment, which is ideal for hydration and lubrication of epithelia. Furthermore, the large protein backbone and the dense sugar chains block access of infective agents to the epithelium below. The first barrier the pathogen encounters is the highly hydrated mucus gel that covers the mucosal surface and protects the epithelial cells against chemical, enzymatic, microbial, and mechanical insult. Mucosal surfaces are coated with a layer of viscous mucus. The mucus layer is not static but moves to clear trapped material. In the gastrointestinal tract, the outer mucus layer is continually removed by movement of the luminal contents. Underneath the mucus layer, the cells present a dense forest of highly diverse glycoproteins and glycolipids, which form the glycocalyx. Membrane-anchored cell-surface mucin glycoproteins are a major constituent of the glycocalyx in all mucosal tissues, and both the secreted and adherent mucosal barriers are constantly renewed and could potentially be rapidly adjusted to changes in the environment, for example, in response to microbial infection (114, 115).

Steric constraints cause the mucins to have rod-like structures in which the protein core is extended, and the carbohydrate chains branch out from the axis of the molecule, giving an appearance much like a bottle brush (116, 117). This allows trans-membrane mucins to extend significantly further from the cell surface, more so

than most extracellular receptors, which provides a mechanism by which mucins modulate the adhesion of cells and pathogens (118, 119).

Mucins make up a family of heavily *O*-glycosylated proteins with molecular weights usually more than 200kDa. 50-80% of the molecular weights of mucins can be due to their *O*-linked carbohydrate content (120, 121). They are widely expressed in epithelial tissues, and are characterized by functionally important peptide domains that are Ser/Thr/Pro-rich, called variable number of tandem repeat (VNTR) domains, which are an attractive site for heavy *O*-glycosylation (122-124). These areas are often protease-resistant, and this is suggested to be due to the fact that attached carbohydrate residues block access to the peptide core, as the same sequences are susceptible to protease action in the absence of the carbohydrate (125).

To date, there are approximately 20 genes that encode mucin-type proteins, and these are expressed by epithelial cells in the gastrointestinal, tracheobronchial and reproductive tracts (126) (**Table 2.4**).

Table 2.4. Currently cloned human mucin genes. From Ali, *et al.* (126)

Gene	Mucus Type	Mucin	Main Tissue Expression	Chromosomal Locus
MUC1	Transmembrane	Pan-epithelial	Breast, pancreas	1q21
MUC2	Secreted	Intestinal	Jejunum, ileum, colon	11p15.5
MUC3A	Transmembrane	Intestinal	Colon, small intestine, gall bladder	7q22
MUC3B	Transmembrane	Intestinal	Colon, small intestine, gall bladder	7q22
MUC4	Transmembrane	Airway	Airways, colon	3q29
MUC5AC	Secreted	Airway	Airways, stomach	11p15.5
MUC5B	Secreted	Airway	Airways, submandibular glands	11p15.5
MUC6	Secreted	Gastric	Stomach, ileum, gall bladder	11p15.5
MUC7	Secreted	Salivary	Sublingual and submandibular glands	4q13-21
MUC8	Secreted	Airway	Airways	12q24.3
MUC9	Both	Reproductive	Fallopian tubes	1p13
MUC11	Transmembrane	Colonic	Colon, airway, reproductive tract	7q22
MUC12	Transmembrane	Colonic	Colon, pancreas, prostrate, uterus	7q22
MUC13	Transmembrane	Colonic	Colon, trachea, kidney, small intestine	3q13.3
MUC15	Transmembrane	Colonic	Colon, airway, small intestine, prostate	11p14.3
MUC16	Transmembrane	Reproductive	Ovarian epithelial cells	19q21
MUC17	Transmembrane	Intestinal	Duodenum, colon, stomach	7q22
MUC18	Transmembrane	Airway	Lung, breast	11q23
MUC19	Secreted	Salivary	Salivary glands, trachea	12
MUC20	Transmembrane	Renal	Placenta, colon, lung, prostate, liver	3q29

The mucin family can be sub-divided based upon their formations. MUC2, 5AC, 5B, 6 and possibly 19 are secreted, gel-forming mucins, while MUC1, 3A, 3B, 4, 11 to 13, 15 to 18, and 20 are membrane bound. However, MUC7 is a secreted but not gel-forming mucin as it exists as a monomer. Both secreted and membrane-bound forms of MUC9 have been identified (126).

2.4.1 Gel-Forming Mucins

This type of mucin covers the epithelial cells in organs in all vertebrates, gills in fishes and the amphibian epidermis. Upon analysis, gel-forming mucin polypeptides were shown to have non-glycosylated, 'bare' domains adjacent to the Ser/Thr-rich glycosylated ones. It was suggested that the bare regions were areas of smaller mucin molecules that were linked to others by disulfide bonds to form larger molecules (127-129). Gel-forming MUC2, MUC5AC, MUC5B and MUC6 are found to be clustered to human chromosome 11, and each have a cysteine knot domain and thus form a mucin subfamily (130). Furthermore, purified mucins observed with an electron microscope appear as long linear structures that can be reduced to smaller subunits upon reduction of disulfide bonds (131, 132). Due to the large, carbohydrate-rich structure and polymeric nature, gel-forming mucins endow mucus secretions with a high viscosity and the chemical diversity necessary to interact, entrap and transport microorganisms, particles and chemicals (133). Furthermore, the hydrodynamic properties of gel-forming mucins in the gastric mucosa prevent damage to the mucosa by the hydrochloric acid (134). Some gastric mucins have demonstrated an antibiotic activity against *Helicobacter pylori*, one of the prominent agents responsible for the formation of gastric ulcers (135). Furthermore, it has been suggested that the gel-forming mucins act to retain antimicrobial peptides in airway mucus (136). Gel-forming mucins in the colon have been involved in protection against cancer development in an environment where the mucosa is exposed to potentially carcinogenic products (137). Deregulation of the mucins or mucus production can present strong risks towards serious health consequences. Reduction in the synthesis of intestinal mucins has been associated with a predisposition towards colitis (138). Overproduction and subsequent accumulation of gel-forming mucins has been shown

to feature in the lungs of individuals with a obstructive lung diseases, such as COPD, cystic fibrosis and asthma (139).

2.4.2 MUC1 and Transmembrane Mucins

Transmembrane mucins are generally composed of two dissimilar subunits forming a dimer, linked together by non-covalent SDS-labile bonds. The larger N-terminal subunit is extracellular, highly glycosylated and, particularly in the case of MUC1 and MUC4, mostly composed of VNTR domains. In a similar way as the gel-forming mucins, transmembrane mucins are also shown to have gene clustering, and can undergo a variety of splicing events (140). The smaller subunit is composed of a short extracellular region of 58 amino acid residues, a single-pass transmembrane domain, and the C-terminus which result in a C-terminal core peptide of around 14kDa, which increases to 25-30 kDa following glycosylation and any subsequent phosphorylation (141). In the case of MUC1, following translation, a single polypeptide is cleaved in the ER into the two subunits of the heterodimer (116, 142, 143). The peptide core of the extracellular domain ranges from 120-300kDa, while the mature protein, after glycosylation in the VTNR domains, can almost double in size dependent upon the tissue of origin and it's physiological state (141).

Cleavage of the MUC1 precursor polypeptide happens through autoproteolysis, which occurs during protein folding (144, 145). The site of cleavage is N-terminal to the serine residue in a G↓SVVV motif in the SEA (sea urchin sperm protein, enterokinase and agrin) module of the extracellular domain (146). Structurally, the N-terminus of MUC1 bears a signal peptide that directs the localization of the mature protein to the apical surface of the cell in epithelial cells,

and the first three residues (CQC) of the cytoplasmic domain are involved in retention of the mucin at the plasma membrane (147). Once at the plasma membrane, the MUC1 extracellular membrane can be shed into the lumen by tumour necrosis α-converting enzyme and possibly ADAM9, a disintegrin (148). The shedding mechanism allows the release of soluble MUC1, as does alternative splicing of the MUC1 mRNA.

In a way similar to MUC1, the heterodimeric MUC4 is derived from a single gene that is posttranslationally processed into an *O*-glycosylated extracellular subunit of about 600-800 kDa, and a mostly *N*-glycosylated fragment of approximately 120 kDa. The cleavage of MUC4 is suggested to be carried out by an unknown serine protease (149). MUC4 represents the only transmembrane mucin without an SEA module, but it contains three domains unique amongst the transmembrane-mucins. These are a nidogen homology region (NIDO), an adhesion-associated domain in MUC4 and other proteins (AMOP), and a von Willebrand factor type D sequence (VWD) (150, 151).

2.4.3 The roles of MUC1

Mucins have been shown to have both anti- and proadhesive properties. The heavy glycosylation on the mucins can create steric hindrance which is able to distrupt close contact between cells (152). However, in the case of MUC1, the peptide backbone and the carbohydrate modifications provide epitopes for a wide range of adhesion molecules (116, 153, 154). While the protein component of MUC1 interacts with intracellular adhesion molecule-1 (ICAM-1) (155), the sialyl-Lewis^a and sialyl

lewis^x carbohydrate structures are bound by the selectin family of adhesion molecules (156).

Both extracellular and intracellular domains of mucins are involved in signalling. Mucin domain interactions have been shown to interact and encourage heterocellular adhesion such as MUC1-ICAM-1 interactions and MUC16-mesothelin interactions (153, 154, 157). ICAM-1 is shown to bind to the peptide backbone of the MUC1 extracellular domain on adjacent cells, which results in a rapid increase in intracellular calcium in MUC1-expressing cells (158). Further studies have shown that this calcium response involves the Src, phosphatidylinositol 3-kinase (PI3K), and phospholipase C enzymes, and that the interaction of MUC1 with ICAM-1 increases migration of MUC1-expressing cells across a gelatin matrix in vitro, suggesting a role of MUC1 in the regulation of cellular adhesion and motility (159).

Furthermore, the MUC1 C-terminus has been shown to affect a variety of signalling pathways through interactions regulated by phosphorylation. An indication of this would be that six of the seven tyrosine residues found in the human MUC1 C-terminus are found to be 100% conserved (160). These tyrosines, as well as several serines and threonines in the C-terminus, make up recognition sites for kinases and can be phosphorylated (161-163). These kinases includes c-Src (164), all members of the epidermal growth factor receptor (EGFR or ErbB) family (161, 165), glycogen synthase kinase 3β (GSK3β) (166), and protein kinase C δ (PKCδ) (167). Various non-kinase proteins have been shown to interact with MUC1. These proteins include β-catenin (168, 169), adenomatous polyposis coli (170), Grb-2 (171), p53 (172), p120 catenin (173), estrogen receptor α (ERα) (174), and the heat shock protein HSP70 and HSP90 (175).

MUC1 has been shown to enhance the effect of tumorigenic proteins, including those of the ErbB kinase family. MUC1 overexpression increases activation of downstream extracellular signal-regulated kinases (Erks) 1 and 2 (161). Furthermore, MUC1 has been shown to enhance ErbB1 signalling in breast cancer cells and immortalized breast epithelial cells by inhibiting ErbB1 ubiquitination, and promoting ErbB1 recycling back to the cell surface following internalization (176). However, these MUC1-ErbB interactions are readily detected in non-polarised cells, but it is disputed as to whether these findings are directly applicable to normal, polarized epilthelial cells, as MUC1 is located on the apical surface, while the ErbB proteins are primarily basolateral in location (177). It has been suggested that MUC1 has the ability to activate MAPK signaling through association with Grb2, and other signalling mediators (171). Reduction of MUC1 proteins has been shown to affect the transcription of two members of the ERK pathway, MEK1 and Raf, coinciding with changes in the activation of MEK1/2 and ERK1/2 (178). Furthermore, following a decrease in MUC1 expression in Jurkat lymphoma cells, there is a reduction in signalling associated with T-cell activation, which includes ERK1/2 phosphorylation and cell proliferation (179). Finally, it has been shown that the MUC1 C-terminus can be found in the nucleus, where it is free to modulate transcription directly (169, 172, 174).

2.4.4 Cancer-Associated MUC1

MUC1 is ubiquitously expressed on the ducts and glands of secretory epithelial tissues (180, 181). MUC1 overexpression has been identified in most carcinomas, particularly in breast cancers (180, 182-185), and correlates with a high

metastastic potential and poor survival (186-189). Originally characterized as an epithelial-specific protein, MUC1 has been identified in lymphoid cells, B-lymphoblasts, plasma cells, myelomas, selected normal B cells, T and B cell lymphomas, normal pro-erythrocytes and erythroblasts in bone marrow (180, 190-194).

The changes in expression and post-translation modifications of MUC1 have instigated research into links between these changes and the behaviour of cancer cells, particularly in regards to interactions with other cells, and the extracellular matrix. MUC1 expression is localized to the apical surface bordering the lumen in epithelial cells. However, in cancer cells, the MUC1 has lost polarity and is found expressed on all surfaces of the cell (195). Furthermore, cancer-associated MUC1 has shorter and less complex *O*-glycan chains, exposing core structures (196-198). A feature of colon carcinogenesis is shown to be accompanied by an increase in carbohydrate structures of less complexity, such as the core antigens Tn, TF and their siaylated counterparts sialyl-Tn and sialyl-TF, as well as the blood group antigens Lewis^a, Lewis^b, Lewis^x and Lewis^y (199). There has been some dispute in whether cancer-associated MUC1 is over- or under-glycosylated when compared to MUC1 expressed by healthy cells. T47D, a breast cancer cell line, exhibits a high level of *O*-glycosylation upon MUC1 with carbohydrate moieties attached to 95% of available sites, while MUC1 from milk demonstrated only 55% of available sites were *O*-glycosylated (200). However, earlier studies have suggested that cancer-associated MUC1 is underglycosylated, and that this is due to reduced density of substituted sites (198).

2.4.5 MUC1 and Immunotherapy

The role of the immune system is to reject invading pathogens including bacteria, fungi, and viruses. The innate immune system recognizes molecules with a specific structural patterns, characteristic of classes of pathogen (pathogen associated molecular patterns, or PAMPS) (201), which are usually structures like mannans of yeasts, lipopolysaccharides of Gram-negative bacteria, or bacterial DNA which contains unmethylated CpG dinucleotides (202). PAMPs are quite often rich in carbohydrates and can therefore interact with lectins on antigen presenting cells (APCs), which, in turn, can activate the cells to increase their expression of molecules such as MHC, co-stimulatory molecules and cytokines. These molecules stimulate the adaptive immune response, which is mediated by T- and B-cells.

The glycosylation of MUC1 is of importance in regards to any subsequent antibody response, and lectin-mediated interactions with the effector cells of the immune system. The multiple *O*-glycans presented on MUC1, which have the capability of acting as a PAMP, as well as enhancing any cell-cell interactions. For example, MUC1 was first recognized as a tumour-associated antigen due to its dominance of inducing antibodies in mice (203). In humans, the first example of MUC1's immunogenicity was when cytotoxic T-cells (CTLs) isolated from breast and ovarian cancer patients killed MUC1-expressing cells in a non-human leukocyte antigen (HLA) restricted fashion (204). Furthermore, these studies suggested that only the cancer-associated glycoform of MUC1 was recognized by the CLTs (205) and that the intact MUC1 molecule formed multiple interactions with the T-cell receptors (TCR), particularly through the epitopes found on the VNTR domain (206). It was then reported that the antibodies that recognized the sequences in the VNTR are found in cancer patients (207, 208).

Since the 1980s, anti-MUC1 antibodies have been available, and were used to carry radioactive elements to the peritoneum (209). Anti-MUC1 antibodies have been used in breast cancer management to detect MUC1 in serum, which attempted to detect cancer relapse before clinical symptoms appear (203). There has been interest in using MUC1-based immunotherapy for carcinomas expressing the molecules. The majority of MUC1-based immunotherapy developments so far have been based upon synthetic or recombinant peptides, which are restricted to the VNTR domain (210-213). As there are as many as 120 tandem repeats in a MUC1 molecule, it was theorized that the VNTR domains represent the most abundant complement of epitopes. However, it has been demonstrated in non-transgenic mice that immunotherapy with MUC1 that does not have the VNTR region is as effective as immunotherapy using the entire molecule (214). Furthermore, immunization of non-transgenic mice with MUC1 peptide alone has produced very little immune response, while coupling the peptide to a large protein carrier, such as KLH (keyhole limpet hemocyanin, a metalloprotein), or diptheria toxoid (DT) (215), and injecting it along side adjuvants produces anti-MUC1 antibody and further provides protection against challenge with MUC1-baring cancer cells (212). Conversely, MUC1 transgenic mice have been shown to produce very little MUC1-specific antibody in response to MUC1 vaccination (216), but the growth of MUC1-expressing tumours can be deterred by the same MUC1 vaccination (217, 218).

The MUC1-VNTR has been developed in bacteria as a fusion protein with glutathione S-transferase (GST), a construct which contains five MUC1-VNTR per GST molecule, and allows easy purification (219). It has been shown that coating the fusion protein with the sugar mannan encourages uptake by antigen-presenting cells, due to the mannan receptors on the surface of the cell. However, when injected into

mice, it was shown that type-1 T-cell response (CLT) and subsequent anti-tumour activity was only generated when mannan was attached under oxidizing conditions. Conversely, a Th2 response, where MUC1-specific antibodies, but no CTL activity was generated, was seen when mannan was attached under reducing conditions (218, 220).

Since the 1990s, it has been well known that mice injected intramuscularly with plasmid DNA generate a strong immune response to the antigen coded within the plasmid (221, 222). This has now been applied to MUC1 immunization, and incorporation of the MUC1 gene into plasmid DNA has been assessed preclinically (223). MUC1 vaccination utilizing a viral vector has been implemented by inserting the MUC1 gene into the thymidine kinase gene of vaccinia virus (VV) (224), as disruption of the thymidine kinase gene also attenuates the viral virulence (225). VV-MUC1 constructs have been assessed in mouse experimental systems, including MUC1 transgenic mice (216, 226-228).

In more recent experiments, MUC1 has been expressed in the highly attenuated modified vaccinia Ankara (MVA) with the gene sequence of interleukin (IL)-2. This construct has shown to be effective at inducing MUC1-specific CTL in mice, which can lead to the rejection of established MUC1-expressing tumours, and is now in clinical studies (229). Futhermore, MUC1 has been expressed in adenoviral vectors, which were used to infect dendritic cells, resulting in increased expression of MUC1 on them (230, 231).

Table 2.5. Immunotherapy developments involving MUC1. From Acres *et al.* (232)

Immunogen	Name	Clinical phase	Cancer indication	Reported results
MUC 5xVNTR		I	Pancreas	Antibody response
MUC1 1xVNTR-KLH		I	Breast	Antibody response
MUC1 (2xVNTR) DT		I		Cellular and antibody response
MUC1 CTL epitope peptides/dendritic cells		I	Breast and ovarian	CTL, stable disease
Sialyl-Tn KLH	Theratope®	III	Breast	MUC1 antibody and increased survival
5xVNTR-GST-mannan	MFP	I	Breast, colon, stomach or rectum	Antibody response to MUC1
Vaccinia MUC1-IL2	TG1031	II	Breast/prostate	Partial clinical responses; decreased or stabilised PSA levels
Vaccinia CEA/MUC1/ Tricom	PanVacc®	III (in progress)	Pancreas	
MVA-MUC1-IL2	TG4010	II	Lung, prostate, kidney, breast	CTL responses, stable disease, increased survival
Liposome 1xVNTR	L-BLP-25	II	Lung	Increased survival

CEA: Carcinoembryonic antigen; CTL: Cytotoxic T-lymphocytes; DT: diptheria toxoid; GST: Glutathione S-transferase; KLH: Keyhole limpet hemocyanin; MVA: Modified vaccinia Ankara; VNTR: Variable number tandem repeat; PSA: Prostate-specific antigen.

2.5 Altered Glycosylation in Disease

Considering that it is one of the most common posttranslational modifications of proteins, it is unsurprising that alterations in glycosylation are seen in all human cancers (93, 233, 234). Furthermore, altered epithelial glycosylation in the colonic epithelium is present in pre-malignant disease, such as adenomatous and metaplastic polyps and inflammatory conditions such a ulcerative colitis and Crohn's disease (235). These changes affect the majority of glycoconjugates, including intracellular, cell-surface and secreted products.

The most abundant changes are found within the *O*-linked mucin-type glycans . These changes include truncation of the *O*-glycans, increased sialylation, reduced sulfation, and the increased expression of the core 1 structure within the O-linked glycans, the Thomsen-Friedenreich (TF) antigen (galactoseβ1,3N-acetylgalactosamine), which is α-linked to serine or threonine within the protein core (**Table 2.6**). Typically, colon cancer *O*-glycans present this TF-antigen core, while *O*-glycans from a healthy colon present predominantly express core 3 glycans (GlcNAc β1-3 GalNAc α-ser/thr) (236). These were identified within mucosal samples of colon cancer and ulcerative colitis patients (237). Colonic mucin sulphation in ulcerative colitis is reduced (238). Other changes include increased expression of short *O*-linked oncofetal antigens including sialyl-Tn (sialyl 2-6 GalNAc α-ser/thr). Sialyl-Tn and its expression represent a marker of high-risk cancer development in inflammatory bowel disease mucosa, and is also being investigated as a diagnostic marker in breast and gastric cancers (239-241). These changes are not exclusive to the colon, and have been identified in other epithelial cancers, such as the breast and pancreas.

Table 2.6. Mucin type *O*-glycans and their alterations in cancer (236).

O-glycan	Structure	Increased/decreased in cancer
Tn antigen	GalNAcα-Ser/Thr	↑
STn antigen	Sialylα2-6GalNAcα-Ser/Thr	↑
Core 1,T antigen	Galβ1-3GalNAcα-Ser/Thr	↑
Sialyl-T antigens	Sialylα2-3Galβ1-3GalNAcα-Ser/Thr Sialylα2-6(Galβ1-3)GalNAcα-Ser/Thr	↑ ↑
Core 2	GlcNAcβ1-6(Galβ1-3)GalNAcα-Ser/Thr	↑↓
Core 3	GlcNAcβ1-3GalNAcα-Ser/Thr	↓
Core 4	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcα-Ser/Thr	↓
Type 1 chain	[GlcNAcβ1-3 Galβ1-3]n	↓
Type 2 chain	[GlcNAcβ1-3 Galβ1-4]n	↑
	Poly-N-acetyllactosamines	
Sialyl-Lewis a	Sialylα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-3Gal-	↑
Sialyl-Lewis x	Sialylα2-3Galβ1-4(Fucα1-4)GlcNAcβ1-3Gal-	↑
Sialyl-dimeric Lewis x	Sialylα2-3Galβ1-4(Fucα1-4)GlcNAcβ1-3 Galβ1-4 (Fucα1-3)GlcNAcβ1-3Gal-	↑

These glycosylation changes may precede dysplasia, the common precursor of malignancy (242). The same relationship between colitis and the cancerous state exists within a species of New World monkey, the cotton-top tamarin. This species, when in captivity, commonly develops a colitis that is virtually indistinguishable from the human ulcerative colitis, and this often progresses to colon cancer. Furthermore, colon cancer has not been identified in the monkeys prior to the development of colitis (243). As recognised in human colitis, there was an increased expression of TF antigen which coincided with an increased level of binding of peanut lectin in cotton-

top tamarins with colitis (244). The over-expression of TF antigen significantly coincided with the development of colorectal carcinoma (245).

The changes in cellular glycosylation are similar in both inflammatory disease and colon cancer. This led to speculation that the glycosylation changes might be primary in both conditions however it was shown there is no increase to the risk of colorectal cancer for first-degree relatives of those with inflammatory bowel diseases, unless those individuals also suffer from a form of inflammatory bowel disease (246).

This implies that the glycosylation changes seen in inflammation and cancer are likely to be secondary phenomena.

2.5.1 Mechanisms of Altered Glycosylation

There are a variety of possibilities that could account for the glycosylation abnormalities in colon disease. These could include alterations in the activities of the glycosyltransferases within the Golgi, altered substrate availability or changes in the sequence and structure of the core protein. Glycosylation can be altered by splicing of the gene producing the target protein. It was shown that, specifically upon the high molecular weight splice variants of the adhesion molecule CD44, there is an expression of TF antigen, when the normal CD44 in healthy colorectal tissue demonstrates no expression (247). Furthermore, studies have implied a link between the CD44 splicing, altered cell surface glycosylation and tumour cell behaviour. Particularly, there has been a correlation between the glycosylation trait and metastatic ability (242). When a poorly tumourigenic rat colon cancer cell line was transfected with human H blood group antigen-forming α(1-2) fucosyltransferase

cDNA, which enforced the expression of H antigens which are selectively carried upon CD44v6, the transformed cells exhibited increased mobility and tumorigenicity (248).

The altered glycosylation seen in cancer has been shown to correlate only poorly with altered activity of the relevant glycosyltransferases (249). It has been suggested that the biggest influence may be dependent upon the altered arrangement of the transferases within the Golgi, which is accepted as a critical factor for the determination of the *O*-glycan structure (250). The organisation of the transferases has been shown to be pH-dependent, and it is speculated that this may be affected by cross-talk initiated by pro-inflammatory cytokines. There is evidence of defective Golgi acidification within cancerous cells (251, 252). It has been shown that disruption of the pH gradient of the Golgi stack not only resulted in the displacement of the glycosyltransferases to endosomal compartments or the cell surface, but also results in a decreased O-glycosylation of mucin (253).

2.5.2 Thomsen-Friedenreich Antigen

The Thomsen-Friedenreich (TF) antigen is a disaccharide, Galβ1-3GalNAcα1-Ser/Thr, and is also known as core 1 of the *O*-linked glycans (254). This structure is rarely seen under normal circumstances, and acts as an oncofetal antigen, being expressed only in embryonic tissues and cancerous cells. Typically, the TF antigen is concealed by other structures and moieties forming branches within the glycan, such as other carbohydrate units, sulphates and sialic acids, which add to the complexity of the glycans (233). This is not the case in cancer, or pre-cancerous

conditions when the core 1 structure is often unsubstituted, and the expression therefore increased (**Figure 2.4**).

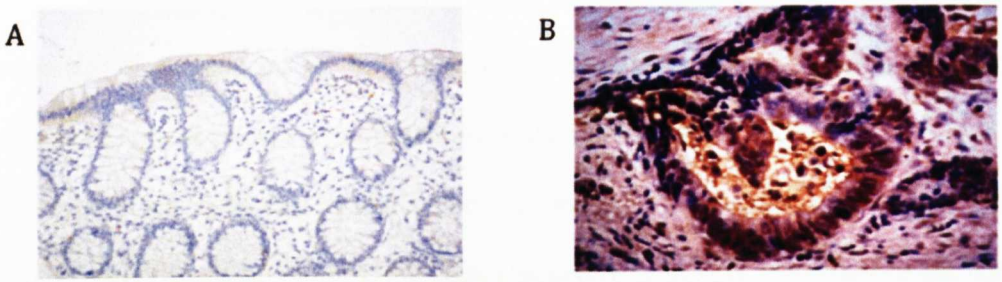


Figure 2.4. PNA histochemistry. A. Section from irritable bowel syndrome that demonstrates no TF expression. B. Section from colon cancer that demonstrates an increased level of TF expression (brown staining) (235, 255).

It is particularly noted that within colon cancer tumours, TF-positive tumours present a 4-fold increase in risk of metastasis when compared to TF-negative tumours (244). Furthermore, the naturally occurring anti-TF antibodies that occur in the patient sera are significantly reduced in the sera obtained from colorectal cancer patients (256). Although it is widely acknowledged that the level of TF expression is significantly increased in cancer patients, the mechanism of this increase is not completely understood. The first facet of this development could be linked to the activity of the glycosyltransferases. However, the significance of the glycosyltransferase activities in the expression of the TF antigen is disputed, and it would be incorrect to assume that increased expression of glycosyltransferases would automatically correlate with the increased expression of the TF antigen. Recent studies have demonstrated that there is similar glycosyltransferase expression and

activity in both normal and cancerous tissues (257). Therefore, it would seem, for at least gastric cancer tissues, the glycosyltransferase gene expression is not the most influential factor.

In eukaryotic *O*-glycosylation, sugar moieties are added individually, and the sequence is influenced by the position of glycosyltransferases within the Golgi apparatus compartments, and alterations of the relative activities will, in turn, alter the structure of the *O*-glycan products that are ultimately destined for the surface of the cell. In healthy epithelial cells, the core 1 structure is rapidly converted to the core 2 structure by the addition of N-acetyl glucosamine to the GalNAc portion of core 1. This process is catalysed by core 2 β1,6-GlcNAc-transferase. However, in breast cancer tissues the expression of the core 2 enzyme is significantly reduced (258), and this would correlate with a reduced core 1 to core 2 conversion, and an increase in TF antigen on the cell surface. This concept can be translated specifically to colorectal cancer. In normal colorectal epithelium, TF structures may be concealed by *O*-sulphate esters. But in the cancerous colon tissue, the sulphotransferase is reduced, thereby contributing to the increased occurrence of the TF antigen (259). It is further hypothesised that an elevated availability of the UDP-galactose substrate may increase the biosynthesis and expression of the TF antigen. This nucleotide sugar substrate is typically located in the cytosol and is transported into the Golgi apparatus for the carbohydrate biosynthesis by the UDP-Gal transporter in the Golgi membrane (233). This elevated level of UDP-Gal is identified in cancer, where it is on average 3.6-fold greater in cancerous mucosa when compared to healthy mucosa.

The activity of these glycosyltransferases are also affected by specific chaperones. The core 1 β1,3 Gal-transferase reaction is modulated by the ER-based

molecular chaperone Cosmc (Core 1 β1,3-Gal-T-specific molecular chaperone). This chaperone promotes the folding and stability of the core 1 β1,3 Gal-transferase (260). In the absence of Cosmc, Core 1 β1,3 Gal-transferase is targeted for degradation by proteasomes, and mutations in the Cosmc gene coincide with increased TF expression (261). Initial investigations have demonstrated that cervical tumour cells that possess heavy Tn and Sialyl Tn expression typically possess Cosmc mutations (262). Furthermore, disruption of C1GalT in mice results in embryonic lethality, typically caused by defective angiogenesis and lymphangiogenesis (263, 264).

2.6 Lectins

The term 'lectin' is derived from the Latin *lectus*: to gather or select (265), and is defined as a carbohydrate binding protein of non-immune origin. Lectins are found in widely in nature, in plant and animal systems, and are involved in a diverse range of biological functions.

Plant lectins are tightly globular in structure, and have resistance to digestion by mammalian enzymes. This allows them to pass through the gut lumen where they retain their biological activity. Lectins have been classically described as agglutinating proteins, with two carbohydrate-binding sites that allow the lectins to precipitate the sugar-containing structures they bind to. However, it has been demonstrated that agglutination is not a defining characteristic of a lectin. The specificity of binding in a lectin is defined by the monosaccharides and oligosaccharides that have the ability to inhibit their activity. Through this, lectins have been classified into five groups based on which monosaccharide ligand

demonstrates the most affinity (266). These groups are mannose, galactose/N-acetylgalactosamine, N-acetylglucosamine, fucose and N-acetylneuraminic acid.

Insights into the functional dimension of lectin binding to cellular glycans have strongly contributed to the emerging importance of glycosylation structures as binding targets for lectins. In regards to lectin architecture, binding sites, ranging from shallow grooves to deep pockets, have developed sugar-binding capacity (267).

2.6.1 Lectin-Based Interactions

It has been indicated through a number of studies that the increased level of TF antigen on the cell surface may increase tumour cell growth, through the recruitment and interaction with exogenous or endogenous carbohydrate-binding lectins to the mucosa, including ones that may not ordinarily bind. The lectins may be of microbial, dietary or human origin. Dietary lectins are often found in legumes and are tightly globular and highly resistant to heat and digestion (268), and can be detected in the active form in faeces, retaining haemagglutinating and pro-proliferative activity (269).

A range of non-toxic, dietary-based lectins have been assessed for their functional effects upon the human colon epithelial cells, particularly with focus upon binding the TF antigen. The largest family of lectins is the legume family, which has around 100 members. Generally, they consist of two or four identical subunits of 25-30 kDa each with Ca^{2+} or Mn^{2+} binding sites. They are characterized by a high content of β-sheets and lack any α-helices (270). A legume lectin of significant importance is peanut agglutinin (PNA), isolated from *Arachis hypogaea*, which is a tetramer - a dimer of a dimer, similar to the lectin *Griffonia simplifolia* (271). Peanut lectin is one

of the most commonly ingested dietary lectins, and is able to remain active in the digestive tract, as PNA extracted from the faeces is still able to agglutinate desiaylated red blood cells (272).

Peanut lectin has shown pro-proliferative properties in colon cancer cell lines such as HT29 human colonic epithelial cells (272), SW837 rectal adenocarcinoma and HCT-15 human colonic carcinoma cells (273), and produces a 40% increase in rectal mucosal proliferation in individuals who consumed peanut butter or a packet of peanuts per day for five days (272). Moreover, the opposite effect can be seen with the edible mushroom lectin, ABL (*Agaricus bisporus*). This lectin also binds TF, and sialylated TF, but, unlike peanut lectin, causes the inhibition of cell proliferation through the internalisation of the lectin, and in turn, causes the inhibition of nuclear localisation sequence-dependent protein import mechanism (274, 275).

Studies were carried out to investigate the effect of PNA on abnormal colonic epithelium, which express TF. Ulcerative colitis, Crohn's disease and colonic polyp biopsy samples demonstrated an average 25% greater crypt cell proliferation rate (CCPR) upon treatment with 25 μg/ml PNA when compared with normal colonic biopsies. Ulcerative colitis samples almost doubled in CCPR when treated with PNA (269). Inflamed and neoplastic colonic tissue, due to altered mRNA splicing, express a high molecular weight variant of CD44 called CD44v6 which expresses TF, as previously described. PNA can bind this, and stimulate phosphorylation of the hepatocyte growth factor c-MET which is known to associate with CD44v6. This is followed by downstream activation of p44/p42 mitogen activated protein kinase (MAPK). This PNA-induced signalling can be inhibited by MAPK inhibitor PD98059. These effects where notable in HT29 and T84 colonic cancer cell lines, but not CaCo2 colonic cancer cells which do not express CD44v6 (276).

It would seem that these dietary lectins might act in the same way as endogenous human lectins, inducing different responses due to the subtleties of the lectin-carbohydrate interactions. An example of these includes the family of galactose-binding galectins.

2.7 Galectins

Galectins are a family of 15 mammalian galactoside-binding proteins that share a consensus amino acid sequence in their carbohydrate recognition domains (CRDs) (277). Based on their structural differences, members of the galectin family are classified into three subgroups, the prototype, chimera-type and tandem-repeat type. The prototype galectins include galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15. Members of the prototype galectins contain a single CRD in their polypeptide sequences and are often identified as non-covalently linked homodimers. The chimera-type galectin, which has only one member (galectin-3) at the moment, is composed of a non-lectin domain linked to a CRD and can precipitate as a pentamer with its carbohydrate ligands. The tandem-repeat-type galectins include galectin-4, -6, -8, -9 and -12 and members of this subgroup of galectins contain two CRDs in a single polypeptide chain and these two CRDs are often different from each other (278). All galectins are bivalent or multivalent molecules in physiological or pathological conditions, as a result of the presence of either two CRDs within a single polypeptide chain or molecule polymerization, and are able to form arrays and lattice structures on the cell surface with their carbohydrate ligands (279). Through this recognition and binding ability, galectins have been shown to control immune cell processes through binding to specific glycan structures on pathogens and tumors or by

acting intracellularly via modulation of selective signaling pathways. Recent findings demonstrate that various galectin family members influence the fate and physiology of different innate immune cells including polymorphonuclear neutrophils, mast cells, macrophages, and dendritic cells. Moreover, several pathogens may actually utilize galectins as a mechanism of host invasion (280).

2.7.1 Expressions of Galectins in Colorectal Cancer

The human digestive tract is rich in galectins. In the colon and rectum, four galectins, galectin-1, -3, -4 and -8, are known to be present (281-283) (**Table 2.7**).

Table 2.7. The expression of galectins in the human intestine and rectum.

Galectins	Locations	Expression in cancer
Galectin-1	Large intestine	Increased in colorectal cancer(281)
Galectin-3	Small and large intestines	Increased in colorectal cancer(284-286) Increased in sera of colon cancer(287, 288)
Galectin-4	Small and large intestines	Decreased in colorectal cancer(282, 289)
Galectin-8	Small and large intestines	Decreased in colorectal cancer(283)

Galectin-1 is expressed weakly in the normal human colonic epithelia and its expression is increased in inflammatory and cancerous conditions. The increased expression of galectin-1 is often seen to be correlated with a metastatic phenotype of colorectal cancer (290). Immunohistochemistry studies have shown expression of

galectin-1 in 12% of normal colonic mucosa but in 40% of adenomas and 84% of carcinomas (281). Furthermore, 91% of adenocarcinomas and 33% of mucinous adenocarcinomas have demonstrated strong stromal galectin-1 expression (281).

Galectin-3, one of the most extensively studied galectins so far, is widely expressed in the human gastrointestinal tract including the colon and rectum. Normal colonic mucosa shows strong nuclear expression of galectin-3. The cells at the base of the crypt show a weak or negative galectin-3 expression, and the intensity of nuclear galectin-3 expression increases progressively from the base towards the surface of the gland (281). In the nucleus, galectin-3 is localised in the dense fibrillar component of the nucleolus, as well as the periphery of the fibrillar centres (291). Galectin-3 has also been demonstrated in the interchromatic spaces and along the borders of condensed chromatin of the nucleoplasm (291). These areas are suggested to be the location both for mRNA synthesis (292) and for the early events in pre-mRNA splicing (293). Altered expression of galectin-3 is common in inflammatory and cancerous conditions. Cancer metastases are also seen to express higher levels of galectin-3 than the primary tumours from which they arose (294). In addition, galectin-3 shows a change of subcellular localization from nucleus to the cytoplasm in the progression from colorectal adenoma to carcinoma (295).

Galectin-4 is a hydrophobic molecule due to its high content of apolar amino acids in its linker sequence (residues 151-175). Galectin-4 is expressed in the human intestinal and colonic mucosa (278, 282) and its expression is generally lower in cancer and malignant tissues than in healthy ones (282, 289, 296). Galectin-4 has a tendency to be associated with generally insoluble tissue components (297, 298). Interestingly, cancers that express high levels of galectin-4 tend to have a poor prognosis (289). There is a progressive reduction of the dense supra-nuclear galectin-

4 aggregates and a subsequent increase in diffuse cytosolic galectin-4 throughout the progression of colorectal malignancy (282). Analysis of galectin-4 protein expression in cultured cell lines shows that galectin-4 is specifically expressed in highly differentiated cell types, such as the human colon adenocarcinoma T84 where it represents 38-60% of the total galectin content (298).

In T84 human colon adenocarcinoma cells, the localization of galectin-4 is distinctly different from the patterns of galectin-3. Whereas galectin-3 is seen in subapical regions, galectin-4 is often seen in a thick layer formation near the basal membrane. The accumulation of galectin-4 in the leading edge of lamellipodia, in conjunction with the two galectin-4 substrate-binding sites, suggests that galectin-4 is involved in cell-substrate interactions (282, 298, 299). Following Ca^{2+} depletion, the accumulation of galectin-4 is observed at break sites in the T84 cell monolayer, indicating that galectin-4 may be involved in initial cellular reattachments and the spreading of cells within a disrupted monolayer (298).

Galectin-8 is expressed widely in the gastrointestinal tract. Low basal levels of galectin-8 are found in the intestine and colon, with a non-uniform micro-clustering pattern (300, 301). In healthy or benign colon tissue, galectin-8 can be detected both in the nuclei and cytoplasm, while in colon cancer galectin-8 is found exclusively in the cytoplasm (283). Overall expression of galectin-8 is reduced in cancer and is inversely related to the rates of tumour growth and migration (283).

2.7.2 Galectins in Tumour Angiogenesis

There is evidence that galectin-1 and -3 is involved in the regulation of tumour angiogenesis as a result of their influence on endothelial cell proliferation and

capillary formation. Over-expression of galectin-1 is observed in the connective tissue surrounding the cancer cells in high-grade colon carcinomas (302) as well as in tumour-associated vascular endothelial cells (TAVEC) (302-304). Galectin-1 over expression has been shown to increase the proliferation of the angiogenically active endothelial cells as a result of the galectin-1 interaction with neuropilin-1, a key mediator of angiogenesis (302). Introduction of recombinant galectin-3, albeit at much higher than patho-physiological relevant concentrations, to human umbilical endothelial cells (HUVEC) in cell culture has been shown to stimulate capillary tube formation of these cells (305).

2.7.3 Galectins in Cancer Cell Adhesion and Metastasis

There is strong evidence that galectins are active players in cancer cell adhesion and metastasis. Cell-surface associated-galectin-1 has been shown to promote cancer cell interactions with Extracellular Matrix (ECM) *in vitro* as a result of its interactions with ECM components (e.g. laminin and fibronectin) (**Table 2.8**) (303, 304).

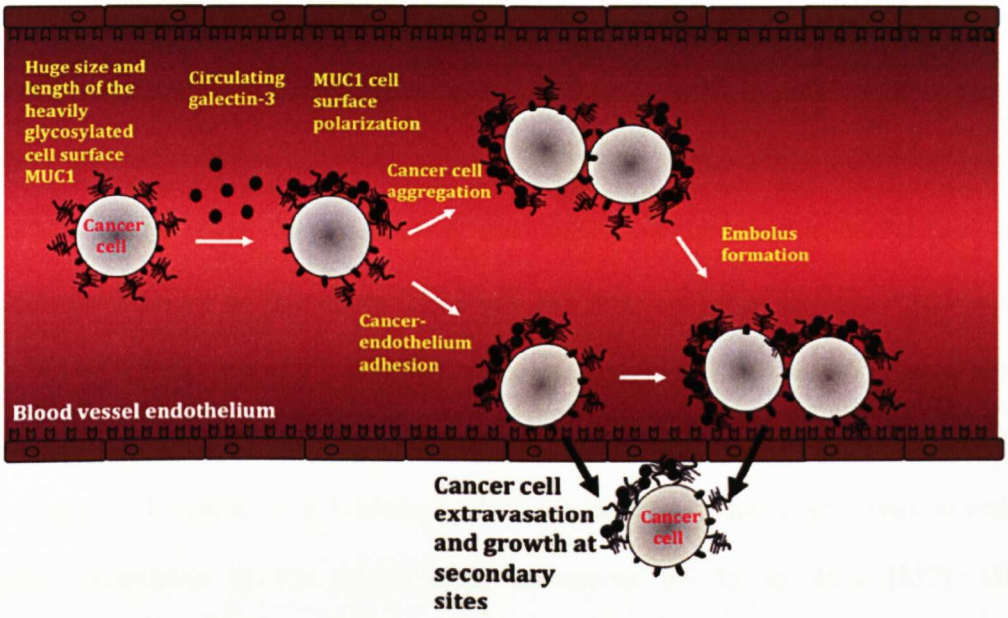
Table 2.8. Galectin binding ligands and the influences of galectin-ligand interactions in colorectal cancer.

Galectins	Ligand	Location/Cell type	Influences
Galectin-1	CEA, Lamp-1, Lamp-2	Colon cancers	Promotes cell adhesion(306)
	CD2, CD3, CD7, CD43, CD45, Fas/CD95	T cell surface	Increases apoptosis and signalling (307-310)
	Laminin, Fibronectin Neruropilin-1	Extracellular matrix Endothelial cell surface	Promotes cell adhesion(304) Promotes endothelial proliferation, adhesion and migration(302)
Galectin-3	Bcl-2, Synexin	Variety of carcinomas	Prevents apoptosis and promotes cell growth(311-314)
	MUC1/TF	Colorectal cancers	Promotes cell adhesion, aggregation and metastasis(315, 316)
	Fas/CD95		Prevents apoptosis(311)
	CD7/CD29,CD45,	T cell surface	Induces apoptosis(313, 317)
	CD71	T cell surface	Enhances cell adhesion(318)
	CEA, laminin,	Colon cancers	Promotes cell adhesion(319)
	Integrins	Endothelial cells	Promotes angiogenesis(320)
	aminopeptidase	Endothelial cells	Enhances cell adhesion(321)
	N/CD13	Cell cytoplasm	Unknown(322)
	Mac2-BP/90K	Serum of colon cancer patients	Unknown(323)
	Haptoglobin βsubunit	Serum of colon cancer patients	
Galectin-4	CEA	Colon cancers	Unknown(324)
	Glycosphingolipids	Colon cancer	Unknown(325)
	Wnt	Colon cancer	Inhibits cell proliferation, adhesion and migration(326)
Galectin-8	Integrins	Variety of cancers	Inhibits cell adhesion(327)

Expression of galectin-3 on the cell surface, as a result of galectin-3 secretion through the non-classical secretory pathway, acts as an adhesion molecule and regulates cell-cell and cell-matrix interactions and plays a very important role in cancer cell metastatic spread from primary to secondary tumour sites (305, 328). Reduction of galectin-3 expression using anti-sense technology before tumour cell inoculation in mice is associated with a marked reduction in liver colonisation and spontaneous metastasis by the high-metastasing colonic adenocarcinoma cells LSLiM6 and HM7 whilst increase of galectin-3 expression is associated with an increase in liver metastasis by the low-metastasising colonic LS174T cells (329).

Galectin-3 is also found in the blood circulation. The concentrations of circulating galectin-3 are increased up to 5-fold in the serum of colorectal cancer patients (287). Moreover, the concentrations of circulating galectin-3 are seen to be much higher in patients with metastasis than those with localized tumours (287). Recent studies in our laboratory have revealed that the increased circulation of galectin-3 in the bloodstream of cancer patients is an important promoter in cancer cell haematogenous dissemination to secondary tumour sites in metastasis (315, 330). It is found that galectin-3 interacts with the oncofetal Thomsen-Friedenreich (Galactoseβ1,3N-acetylgalactosamineα-, TF) antigens on the transmembrane mucin protein MUC1 expressed by the cancer cells (316). The galectin-3-TF/MUC1 interaction induces polarisation of MUC1 cell surface localization and exposure of the smaller cell adhesion molecules, which otherwise are concealed by the much large and heavily glycosylated MUC1. This results in increased heterotypic adhesion of the cancer cells to blood vascular endothelium (315) and homotypic aggregation of the cancer cells to form micro-tumour emboli, which prolongs the survival of tumour cells in the circulation (316) (**Figure 2.5**).

Figure 2.5. A model of the galectin-3-MUC1 interaction in promoting cancer cell haematogenous spreading.



The expression of galectin-4 by cancer cells has been shown to also influence cancer cell adhesion by binding to SO_3^-→3-Galβ1→3GalNAc pyranoside (325), a carbohydrate structure present both in O-linked glycosides and glycosphingolipids (331). When immobilised on bacterial plates, galectin-8 has been shown to promote the adhesion of HeLa cells (332) through its selective interaction with the sugar moieties on α3, α6, β1, to a lesser extent α4 and β3, of the subunit of cell surface integrins (327).

2.7.4 Galectins as Therapeutic Targets in Colorectal Cancer

There is good *in vitro* and *in vivo* evidence that tumourigenesis and metastasis can be effectively reduced by galectin inhibitors. Natural small saccharides such as β-

D-galactose, D-lactose and N-acetyllactosamine (LacNAc) are galectin ligands. Some other small saccharides such as β1,3GlcNAc, galβ1,3Ara and galβ1,4Man have shown to inhibit galectin binding two to four times more effectively than lactose (333, 334). When administered intraperitoneally every 8hr at 2mg/g body weight, D-galactose completely inhibited liver metastasis of L-1 sarcoma cells in mice (335). However, these natural ligands are very sensitive to hydrolysis, which limits their effective use as therapeutic drugs. Modification of the sugar anomeric structure to increase their biological action in inhibiting metastasis has been investigated by several groups (336). Modifications of the carbohydrate structures by thio residues have been shown to stabilize the carbohydrate structures in response to acidic and enzymatic hydrolysis. Inoculation of 1-Methyl-β-D-lactoside in mice has been shown to reduce lung metastases of the B16 murine melanoma by 35 to 45% (337). Daily intraperitoneal injections of glycoamines, a group of low molecular weight carbohydrate structures linked with a stretch of amino acids, reduced metastasis of MDA-MB-435 human breast carcinoma *in vivo* in nude mice (338).

Carbohydrate polymers have also been investigated for their potential to block galectin-mediated actions in cancer. Tree-shaped monodisperse glycodendrimers, obtained by repetitive assembly cycles with the carbohydrate ligands composing the outer sphere, have shown to effectively inhibit galectin-1 and -3 binding to other carbohydrate ligands when compared to lactose in a solid phase assay (336, 339). Small synthetic peptides, which bind specifically to the C-terminal CRD of galectin-3, have shown to inhibit galectin-3-mediated cancer cell adhesion *in vitro* and metastasis *in vivo* in mice (340).

The potential for modified citrus pectin (MCP) to inhibit galectin-3-mediated cancer promotion has also been examined. Pectin is a carbohydrate polymer found in

the peel and pulp of fruits and is composed of a complex multi-branched structure, rich in galactose. The dominant structure of pectin is the linear chain of poly-α- (1-4)-D-galacturonic acid with varying levels of carboxylic acid methylation, which makes up a 'smooth region' (336). This region of pectin is occasionally broken up by side sugar chains rich in neutral sugars, such as arabinose, galactose and rhamnose. Modified citrus pectin has been produced by degradation of the galacturonic acid chain by α-elimination followed by partial acid degradation of the natural saccharides (MCP) that is resulted in a reduced molecular weight of pectin from an average of 70-100 kDa, to an average of 10 kDa. Modified citrus pectin is more readily dissolved and absorbed by the gut (341). MCP shows inhibition of galectin-3-mediated anti-apoptotic action, and of galectin-3-mediated cell invasion, angiogenesis and cell resistance to chemotherapy (342). Injections of MCP decreased metastasis of B16-F1 melanoma cells to the lungs by more than 90%. When given orally to mice, MCP significantly reduced MDA-MB-435 breast carcinoma growth and metastasis to the lungs. It should be mentioned that galectin-3-null mice are relatively healthy (343), indicating that inhibition of galectin-3-mediated actions may present a viable and relatively safe therapeutic approach for cancer treatment.

2.8 Circulating Galectins and Metastasis

Galectin-3 is only one member of the galectin family. Members of the galectin family are often expressed in same tissue types or cells. The discovery that circulating galectin-3 level is increased in cancer patients and promotes metastasis led us to speculate that other galectin members may also altered in the circulation of cancer patients and, like circulating galectin-3, they may also affect disseminating tumour cell haematogenous dissemination in metastasis.

CHAPTER 3

HYPOTHESES AND AIMS

3.1 HYPOTHESES

1. The concentration of members of the galectin family in the bloodstream of cancer patients may be different from that in healthy individuals and such changes may be useful markers for cancer diagnosis.

2. Other galectin members that show increased circulation in the bloodstream of cancer patients may, like galectin-3, also influence adhesion of disseminating cancer cells to vascular endothelial cells and thus also promote metastasis by interaction with cancer associated MUC1.

3. Chemically modified heparins may inhibit galectin binding thus may be used as potential metastasis inhibitors.

4. Suppression of the Core 1 Galtransferase that controls the biosynthesis of TF antigen regulates galectin-mediated cancer cell adhesion.

3.2 AIMS

- To use ELISA to measure the concentration of members of the galectin family in the sera of breast cancer patients, colorectal cancer patients, and colorectal cancer patients with liver metastasis and that of healthy individuals.

- To compare the assessments of circulating galectins detection and carcinoembryonic antigen (CEA) detection as cancer markers.

- To assess the binding of members of the galectin family to TF-expressing glycoproteins.

- To investigate the effect of recombinant galectins at concentrations similar to those found in the sera of cancer patients on cancer cell heterotypic adhesion to endothelial cells and ECM (matrigel).

- To assess the effect of chemically modified heparins on galectin binding to TF-expressing glycans.

- To assess the effects of these chemically modified heparins on galectin-mediated cellular adhesion to extracellular matrix components and their adhesion to and transvasion through HMVEC-L endothelial monolayers.

- To assess the effect of core 1 galtransferase suppression on expression of other *O*-linked glycans in human colon cancer HT29 and SW620 cells.

CHAPTER 4

GENERAL METHODS

This chapter contains the details of the major techniques and methods used within this thesis. Specific methods are described within each chapter.

4.1 Materials

All plastics including flasks (T25 cm^2, T75 cm^2 and T150 cm^2), 6 well, 12 well, and 24 well plates were purchased from Sigma-Aldrich Ltd. (Poole, UK). 96 well plates and 96 well half-volume plates were purchased from Appleton Woods Ltd. (Birmingham, UK), White-walled 96 well plates were purchased from Sigma-Aldrich Ltd. (Poole, UK).

All chemicals were of analytical grade and purchased from Sigma-Aldrich Ltd. (Poole, UK) unless otherwise stated.

Recombinant galectins and anti-galectin antibodies were purchased from R&D Systems, Abingdon, UK.

4.2 Cell Lines

Human colon cancer cell line HT29 was obtained from the European Collection of Animal Cell Culture at the Public Health Laboratory Service (Wiltshire, UK). The HT29 cell line was originally established from a colonic adenocarcinoma

from a 44 year old female Caucasian (344). Upon culture under standard conditions, HT29 cells form a multilayer of nonpolarised cells that display an undifferentiated phenotype (345). If grown in a hexose-free medium, they develop into a foetal small intestine-like state with the expression of brush border enzymes and development of micro villi; they are widely used for the study of epithelial malignancy in the colon.

HT29-5F7 cells are subpopulations of HT29 cells that express mainly MUC1 and MUC5B and were isolated as a consequence of their resistance to 5-fluorouracil, the main chemotherapeutic agent for the treatment of colorectal cancer. They are differentiated into enterocyte-like cells, as substantiated by the cell polarity, the presence of an apical brush border, and tight junctions (346). HT29-5F7 cells were kindly provided by Dr. Thecla Lesuffleur (INSERM U560, Lille, France).

SW620 cells were obtained from the European Cell Culture Collections via the Public Health Laboratory Service (Porton Down, Wiltshire, UK). The SW620 cell line was established from a biopsy of a metastastic spread of SW480 from the colon to the abdominal wall of the same patient, a 51-year-old Caucasian male (blood group A, Rh+).

MUC1 positive-transfectants (HCA1.7+) and negative-revertants (HCA1.7-) of HBL-100 human breast epithelial cells were kindly provided by Dr. John Hilkins (Netherlands Cancer Institute, Amsterdam). HCA1.7+ and HCA1.7- cells were created by MUC1 transfection of HBL-100 human breast epithelial cells with full length cDNA encoding MUC1. MUC1 positive HCA1.7+ and revertant HCA1.7- cells were selected through neomycin resistance, and revertant cells lacking MUC1 were bulk selected from the positive transfectants by two to five cycles of cell sorting using FACStar (119).

Human umbilical vein endothelial cells (HUVECs) and human microvascular lung endothelial cells (HMVEC-Ls) were obtained from Lonza (Wokingham, UK).

4.2.1 Cell Culture

All cells were grown as monolayers. All cells except endothelial cells were grown in Dulbecco's modified eagles medium (DMEM) (Sigma-Aldrich LTD., Dorset, UK) supplemented with 10% FCS, 100μg/ml penicillin, 100μg/ml streptomycin and 4mM glutamine (all purchased from Sigma-Aldrich Ltd., UK). HUVECS and HMVEC-Ls were cultured in EGM and EGM-2 endothelial growth media and supplements (EGM Bulletkits and EGM-2 Bulletkits), respectively (Lonza, Wokingham, UK). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air. The cells were released from T25 cm^2 flasks with 2ml trypsin (0.5mg/ml in sterile PBS) and routinely passaged at a 1:6 subculture ratio when they had become 60%-80% confluent. Cells at a ~80% confluence were used for all experiments. The cells were used for a maximum of 12 passages. Endothelial cells were released from T25 cm^2 flasks with 1ml trypsin (0.5mg/ml in sterile PBS) and routinely passaged at a 1:3 subculture ratio when they had become 60%-80% confluent. Cells at a ~80% confluence were used for all experiments. The cells were used for a maximum of 10 passages.

4.2.2 Cell Freezing

80-90% confluent cells were washed twice with PBS and released from the flasks with trypsin. The cell clumps were dispersed by gentle pipetting after the

addition of DMEM routinely used for cell culture. The cell number was counted and the cell suspension was centrifuged at 1000g for 5 mins. Freezing medium (20% (v/v) FCS, 7% (v/v) dimethylsulphoxide (DMSO, Sigma-Aldrich Ltd., UK) and 73% (v/v) DMEM) was pre-warmed to 37°C and was added to the pellet to make a cell density of 10^6 cells/ml. The cell suspension was immediately transferred to 1ml freezing vials (1ml/vial) and put into a box containing dry ice and kept at -80°C. 24hrs later freezing vials were transferred into liquid nitrogen.

4.2.3 Cell Thawing

Cancer cell and endothelial cells reaching 12 and 10 passages respectively were discarded and new cells were taken from stocks frozen in liquid nitrogen. The freezing vials were withdrawn from the liquid nitrogen bank and placed in a 37°C water bath. Once the frozen cells had defrosted, 20ml of fresh 37°C culture medium was added and the mixture was centrifuged at 1000 g for 5 min. The cell pellet was dispersed in 10ml culture medium and seeded in T25 cm^2 culture flasks with 1-2 x 10^5 cells per flask. After 24hrs the culture medium was replaced. The cells were used between 2-12, or 2-10 passages respectively.

4.3 Enzyme-Linked Immunosorbant Assay (ELISA)

ELISA was performed to either assess the comparative binding of a galectin to a target protein, or used with a standard curve, to determine the concentration of a target galectin in human sera.

4.3.1 Generation of a Checkerboard Assay

Recombinant galectin-3 (R&D Systems, UK) underwent doubling dilution from 2.5μg/ml to 0.02μg/ml in coating buffer (Na_2CO_3 1.6g, $NaHCO_3$ 1.46g in 1L H_2O). Wells running vertically on a high-binding 96-well plate (Appleton Woods Ltd., UK) were coated with 50μl of the diluted recombinant galectin-3 and left to incubate overnight at 4°C. The plate was washed twice with 100μl per wash per well with washing buffer (0.05% (v/v) Tween 20 (Sigma-Aldrich) in PBS). 50μl of blocking buffer (1% (w/v) BSA (Sigma-Aldrich Ltd., UK) in PBS) was applied to each well for 1hr at room temperature. The wells were washed once with 100μl per well with washing buffer. Doubling dilution of biotinylated anti-galectin-3 antibody (R&D Systems, UK) was carried out from 5.0μg/ml to 0.001μg/ml in blocking buffer. 50μl of the diluted anti-galectin-3 antibody were applied to wells running horizontally on the plate, and incubated for 1hr at room temperature. Wells were washed with 100μl washing buffer per well. ExtrAvidin (Sigma-Aldrich Ltd., UK) was diluted 1:10,000 in blocking buffer and 50μl was applied to each well for 1hr. The wells were washed twice with 100μl washing buffer per well. SigmaFAST OPD tablets (Sigma-Aldrich Ltd., UK) were dissolved in 20ml distilled H_2O and 50μl applied to each well for approximately 10mins until a yellow colour developed in the standard curve of recombinant galectins. The reaction was stopped with 25μl 4M sulphuric acid and read at 492nm by a plate microreader (Tecan, Männedorf, Switzerland).

4.3.2 Sandwich ELISA for Assessment of Serum Galectins

High-binding 96-well plates (Appleton Woods Ltd., UK) were coated with 50µl of anti-galectin primary antibody diluted (**Table 4.1**) in coating buffer (Na_2CO_3 1.6g, $NaHCO_3$ 1.46g in 1L H_2O) overnight at 4°C. The plate was washed twice with 100µl per wash per well with washing buffer (0.05% (v/v) Tween 20 (Sigma-Aldrich) in PBS). 50µl of blocking buffer (1% (w/v) BSA (Sigma-Aldrich Ltd., UK) in PBS) was applied to each well for 1hr at room temperature. The wells were washed once with 100µl per well with washing buffer. Serum samples were diluted 1:2 with PBS and were applied to the wells for 2hrs at room temperature. The wells were washed twice with 100µl per well of washing buffer. 50µl of biotinylated antibody (R&D Systems, UK) diluted in blocking buffer (**Table 4.1.**) were applied to each well for 1hr at room temperature and washed with 100µl washing buffer per well. ExtrAvidin (Sigma-Aldrich Ltd., UK) was diluted 1:10,000 in blocking buffer and 50µl was applied to each well for 1hr. The wells were washed twice with 100µl washing buffer per well. SigmaFAST OPD tablets (Sigma-Aldrich Ltd., UK) were dissolved in 20ml distilled H_2O and 50µl applied to each well for approximately 10mins until a yellow colour developed in the standard curve of recombinant galectins. The reaction was stopped with 25µl 4M sulphuric acid and read at 492nm by a plate microreader (Tecan, Männedorf, Switzerland).

4.3.3 Optimisation of Galectin Standard Curves

High-binding 96-well plates (Appleton Woods Ltd., UK) were coated with 50µl of anti-galectin primary antibody diluted (**Table 4.1**) in coating buffer (Na_2CO_3

1.6g, $NaHCO_3$ 1.46g in 1L H_2O) overnight at 4°C. The plate was washed twice with 100µl per wash per well with washing buffer (0.05% (v/v) Tween 20 (Sigma-Aldrich) in PBS). 50µl of blocking buffer (1% (w/v) BSA (Sigma-Aldrich Ltd., UK) in PBS) was applied to each well for 1hr at room temperature. The wells were washed once with 100µl per well with washing buffer. Recombinant galectins (R&D Systems, UK) were serially diluted from 500ng/ml to 1ng/ml and 50µl of the solutions were applied to the wells for 2hrs at room temperature. The wells were washed twice with 100µl per well of washing buffer. 50µl of biotinylated antibody (R&D Systems, UK) diluted in blocking buffer (**Table 4.1.**) were applied to each well for 1hr at room temperature and washed with 100µl washing buffer per well. ExtrAvidin (Sigma-Aldrich Ltd., UK) was diluted 1:10,000 in blocking buffer and 50µl was applied to each well for 1hr. The wells were washed twice with 100µl washing buffer per well. SigmaFAST OPD tablets (Sigma-Aldrich Ltd., UK) were dissolved in 20ml distilled H_2O and 50µl applied to each well for approximately 10mins until a yellow colour developed in the standard curve of recombinant galectins. The reaction was stopped with 25µl 4M sulphuric acid and read at 492nm by a plate microreader (Tecan, Männedorf, Switzerland).

4.3.4 Indirect ELISA for Assessing Galectin Binding to TF-glycans

Wells of a high-binding 96-well plate of half volume (Appleton Woods Ltd., UK) were coated with Antarctic fish (*Trematomus borchgrevinki)* Anti-freeze glycoprotein AFGP-I (glycopeptides 1-5) (obtained from Professor Arthur L. DeVries, University of Illinois, Urbana, IL), a highly glycosylated glycoprotein that bears multiple repeats of TF disaccharide), asialofetuin (obtained from Sigma-Aldrich

Ltd., UK) or asialo bovine mucin (obtained from Dr. Shashikala Inamdar, Karnatak University, India) diluted in coating buffer (10μg/ml) (1.6g Na_2CO_3, 1.46 $NaHCO_3$ in 1L H_20, pH 9.6) overnight at 4°C. The plate was washed twice with 100μl per wash per well with washing buffer (0.05% Tween 20 (Sigma-Aldrich Ltd., UK) in PBS). 50μl of blocking buffer (1% Bovine Serum Albumin (Sigma-Aldrich Ltd., UK) in PBS) was applied to each well for 1 hour at room temperature. The wells were washed once with 100μl per well with washing buffer. Recombinant galectins (R&D Systems, UK) were diluted (1μg/ml) and 50μl of the solutions were applied to the wells for 2hrs at room temperature. The wells were washed twice with 100μl per well of washing buffer. 50μl of biotinylated antibody (R&D Systems, Abingdon, UK) diluted in blocking buffer (**Table 4.1.**) were applied to each well for 1hr at room temperature and washed with 100μl washing buffer per well. ExtrAvidin (Sigma-Aldrich Ltd., UK) was diluted 1:10,000 in blocking buffer and 50μl was applied to each well for 1hr. The wells were washed twice with 100μl washing buffer per well. SigmaFAST OPD tablets (Sigma-Aldrich Ltd., UK) were dissolved in 20ml distilled H_2O and 50μl applied to each well for approximately 10min until a yellow colour developed in the standard curve of recombinant galectins. The reaction was stopped with 25μl 4M sulphuric acid and read at 492nm by a plate microreader (Tecan, Männedorf, Switzerland).

Table 4.1. Description of the antibodies used.

Name/ Cat. No.	Usage	Specificity	Ig type	Stock Conc.	Working Conc. For ELISA
Anti-human galectin-1 ab (AF1152)	Primary	Human galectin-1	Goat IgG	0.1mg/ml	2.5μg/ml
Biotinylated anti-human galectin-1 ab (BAF1152)	Secondary	Human galectin-1	Goat IgG	50μg/ml	1.25μg/ml
Anti-human galectin-2 ab (AF1153)	Primary	Human galectin-2	Goat IgG	0.2mg/ml	2.5μg/ml
Biotinylated anti-human galectin-2 ab (BAF1153)	Secondary	Human galectin-2	Goat IgG	50μg/ml	1.25μg/ml
Anti-human galectin-3 ab (AF1154)	Primary	Human galectin-3	Goat IgG	0.2mg/ml	2.5μg/ml
Biotinylated anti-human galectin-3 ab (BAF1154)	Secondary	Human galectin-3	Goat IgG	50μg/ml	1.25μg/ml
Anti-human galectin-4 ab (AF1227)	Primary	Human galectin-4	Goat IgG	0.2mg/ml	2.5μg/ml
Biotinylated anti-human galectin-4 ab (BAF1227)	Secondary	Human galectin-4	Goat IgG	50μg/ml	1.25μg/ml
Anti-human galectin-8 ab (AF1305)	Primary	Human galectin-8	Mouse IgG	500μg/ml	2.5μg/ml
Biotinylated anti-human galectin-8 ab (BAF1305)	Secondary	Human galectin-8	Goat IgG	50μg/ml	1.25μg/ml
Anti-human galectin-9 ab (AF2045)	Primary	Human galectin-9	Goat IgG	0.2mg/ml	2.5μg/ml
Biotinylated anti-human galectin-9 ab (BAF2045)	Secondary	Human galectin-9	Goat IgG	50μg/ml	1.25μg/ml

Table 4.2. Description of target glycoproteins.

Name	Isolated from	Glycan compliment
Anti-freeze protein	Antartic Fish	TF antigen, galactose monosaccharide
Asialofetuin	Asialo derivative of fetuin, usually from fetal bovine serum	TF antigen, *N*-linked glycans.
Asialo bovine mucin	Bovine submaxillary gland	Variety of *O*-glycans

3.4 Cellular Adhesion Assay

Human umbilical vein endothelial cells (HUVEC) or human microvascular lung endothelial cells (HMVEC-Ls) were released from the culture flasks by trypsinization and re-suspended at $1x10^5$/ml in EGM or EGM-2 endothelial culture medium, respectively.

Initial cellular adhesion assays with HUVEC cells were carried out by using sterile glass coverslips placed at the bottom of a 24-well cell culture plates. Following trypsinisation, HUVECs were resuspended into $1x10^5$ cell/ml and 0.5ml of this solution was pipetted into wells containing the sterile coverslips and incubated at 37°C for 2-3 days until monolayers were formed. Cancer cells were detached from T25 cm^2 flasks with 2ml non-enzymatic cell dissociation solution. Cells were then centrifuged at 1000rpm for 5 minutes and resuspended at $5x10^5$ cell/ml before incubation with 5µl/ml DiO cell dye for 30min at 37°C. 0.5ml of cell suspension was incubated with 1µg/ml recombinant galectin for 1hr at 37°C, before applying to the HUVEC monolayers for a further hour at 37°C. Slides were then washed with PBS and mounted on microscope slides before blinding and counting the number of cells in 10 view fields under the fluorescence microscope.

Further experiments were carried out by using the fluorescent cell dye calcein AM. $1x10^4$ endothelial cells were applied to white walled, clear bottomed 96-well plates (Sigma-Aldrich Ltd., UK) at 37°C for 2-3 days until the formation of endothelial cell monolayers. HT29, HT29-5F7 or SW620 cells were detached from culture flasks with non-enzymatic cell dissociation solution (Sigma-Aldrich Ltd., UK) and were washed three times with PBS and re-suspended in 1ml serum-free DMEM to give $1x10^5$ cells/ml. Each ml of cell suspension was incubated with 10μl Calcein AM (Invitrogen, Paisley, UK) at 37°C for 30min on a shaking waterbed at 80rpm. The cells were washed twice with serum free DMEM and re-suspended in fresh serum-free DMEM at $5x10^4$/ml. 500μl of the cell suspension was incubated for 1hr at 37°C with control BSA or recombinant galectin pre-treated without or with 10μg/ml lactose or 20μM asialofetuin (as described in **6.4.1**) for 30min at 37°C. 100μl/well cell suspension was then introduced to the endothelial monolayers for 1hr at 37°C. The endothelial monolayers were washed with 200μl/well PBS before the cell-associated fluorescence was taken with Tecan reader at 490nm excitation filter and 520 nm emission filter.

4.5 Cellular Transvasion Assay

Human microvascular lung endothelial cells (HMVEC-Ls) were released from the culture flasks by trypsinization and re-suspended at $1x10^5$/ml in EGM-2 endothelial culture medium. $1x10^4$ cells were applied to cell culture inserts (BD Biosciences, UK), which were placed in wells of a clear 24-well plate (BD Biosciences, UK), at 37°C for 2-3 days until the formation of endothelial monolayers. To test the integrity of the endothelial cell monolayers, transepithelial electrical

resistance (TEER) readings were taken using Millicell ERS system (Millipore, Watford, UK). The monolayers were used only once stable readings (approximately 300 mV, lowest exceptable proof of integrity being 290mV) were acquired within three days (**Table 4.3**).

Table 4.3. The accumulation of a stable TEER reading from an endothelial monolayer within a transwell insert.

	Day 1	Day 2	Day 3	Day 4
TEER reading	263 mV	280 mV	300 mV	300 mV

HT29-5F7 or SW620 cells were detached from culture flasks with non-enzymatic cell dissociation solution (Sigma-Aldrich Ltd., UK) were washed three times with PBS and re-suspended in 1ml serum-free DMEM to give $1x10^5$ cells/ml. Cell suspensions were incubated with 1μg/ml recombinant galectin at 37°C for 30 mins on a shaking waterbed at 80rpm. 500μl of the cell suspension was added to the transwell and incubated for 16hrs at 37°C. Following incubation, the cell suspension within the cell insert was removed carefully with a pipette and 10μl/ml of Calcein AM (Invitrogen, Paisley, UK) added to the media outside of the insert for 30mins. The insert was then removed from the plate and the bottom side washed twice with 0.5ml PBS per wash. Using a cell scraper (Sigma-Aldrich Ltd., UK), the cells that translocated to the outside of the cell insert were scraped into a well of a 6-well plate (BD Biosciences, UK) containing 1ml of serum-free DMEM. The cell suspension was transferred to eppindorff tube and centrifuged at 1000rpm for 5mins. The pellet was

resuspended in 300µl, and 100µl of the suspension transferred to wells of a white-walled 96 well plate (Sigma-Aldrich Ltd., UK) before the cell-associated fluorescence was taken with Tecan reader at 490nm excitation filter and 520 nm emission filter.

4.6 SDS Polyacrylamide Gel Electrophoresis (PAGE)

SDS-Page was performed to separate cellular proteins according to size following the Laemmli method (347). 1.5ml thick polyacrylamide gels were cast using a self assembly system (Biorad, Hemel Hempstead, UK). Solubilised proteins were separated by SDS-PAGE on either 12% running gel with a 4% stacking gel, or 4% running gel with a 3.75% stacking gel.

The preparation and composition of the reagents was as follows:

Resolving buffer 4X: Tris-base 36.3g, pH 8.8 (with 2M HCl) then make up to 200ml with H_2O.

Stacking buffer 4X: Tris-base 12.0g, pH 6.8 (with 2M HCl) then make up to 200ml with H_2O.

Running buffer: Tris-base 30.67g, Glycine 64.04g, 2.2g SDS (Sigma-Aldrich Ltd., UK) and H_2O up to 4000ml

Ammonium persulphate: 10% Ammonium persulphate (w/v) (Sigma-Aldrich Ltd., UK)

SDS: 10% (w/v) (Sigma-Aldrich Ltd., UK)

Acrylamide: 30% (w/v) (Sigma-Aldrich Ltd., UK)

Sample buffer: 4X stacking buffer 2.5ml, glycerol 1.0ml (Sigma-Aldrich Ltd., UK), mercaptoethanol 0.5ml (Sigma-Aldrich Ltd., UK), 20% SDS in H_2O 1.0ml, 1% bromothymol blue 25µl (Sigma-Aldrich Ltd., UK).

Table 4.4. Composition of resolving gels.

Reagent	**4% Resolving gel**	**12% Resolving gel**
Acrylamide (30%)	1.33ml	4.0ml
Resolving buffer	2.5ml	2.5ml
Deionised H_2O	6.0ml	2.75ml
10% SDS	100µl	100ul
TEMED	5µl	5µl
10% Ammonium persulphate	60µl	50µl

Table 4.5. Composition of stacking gel.

Reagent	3.75% Stacking gel	4% Stacking gel
Acrylamide (30%)	1.25ml	1.33ml
Stacking buffer	2.5ml	2.5ml
Deionised H_2O	6.1ml	5.5ml
10% SDS	100µl	100µl
TEMED	10µl	10µl
10% Ammonium persulphate	50µl	50µl

The sample (70-80% confluent cells lysed with 0.8ml sample buffer per T25 cm^2 flask) was heated at 100°C for 5min in an Eppendorf heater. Between 10µl and 40µl were loaded on to each lane on a 4% or 12% resolving polyacrylamide gel which were run at 200V in running buffer (25mM Tris, 192mM glycine, 10% SDS) until the bromophenol blue dye front reached the bottom of the stacking gel, and then ran at 100V until the dye front reached 0.5 cm from the bottom of the resolving gel. After electrophoresis, the gels were electrotransferred to a nitrocellulose membrane.

A prestained molecular weight standard marker (Biorad Ltd., Hemel Hempstead, UK.) was used throughout the experiments.

Table 4.6. Composition of prestained marker.

Prestained marker	kDa
Myosin	216
β-galactosidase	132
Bovine serum albumin	78
Carbonic anhydrase	45.7
Soybean trypsin inhibitor	32.5
Lysozyme	18.4
Aprotinin	7.6

4.7 Western Blotting and Lectin Blotting

To transfer the proteins resolved by electrophoresis to a nitrocellulose membrane, the gel was first soaked in transfer buffer (Tris 6.06g, glycine 28.83g, methanol 400ml and H_2O to 2000ml). The gel was then sandwiched in a transfer cassette between a nitrocellulose membrane and 3mm filter paper either side. On each outer most side a transfer sponge was placed and the cassette placed into the transfer apparatus (Biorad, Hemel Hempstead, UK) with an ice pack for 1 hr at 100V in transfer buffer.

After transfer, the membranes were blocked with PBS-buffered saline (PBS) containing 1% (w/v) BSA (Sigma-Aldrich Ltd., UK) for 1hr in a sealed container on a rolling platform. Membranes were then probed with appropriate primary antibodies

(**Table 4.7**) or biotin-lectin for 1hr followed by 5 washes of 10mins each in a sealed container placed on a rolling platform in PBS with 0.1% (v/v) Tween-20 (PBS-T). The membranes were then incubated with peroxidase-conjugated secondary antibodies or HRP-avidin (**Table 4.7**), followed by 12 washes in PBS-T of 5mins each in a sealed container placed on a rolling platform. Signal detection was achieved using a chemiluminescence Super-signal immunoblotting detection kit (Pierce; Rockford IL, USA), which was detected with a Fluor-S multi-imager (Biorad; Hemel Hempstead, UK). Equal loading was confirmed using an anti-actin antibody. Densitometric analysis of immunoreactive protein bands was performed using Quantity One software (Biorad; Hemel Hempstead, UK).

4.8 Slot Blotting

Nitrocellulose membrane was soaked in PBS for 10mins before being sandwiched within the slot blot rig (PR600 SlotBlot; Hoeffer Scientific Instruments, California, USA). Cells were directly lysed in slot blot buffer (4X stacking buffer 2.5ml, glycerol 1.0ml (Sigma-Aldrich Ltd.), mercaptoethanol 0.5ml (Sigma-Aldrich Ltd.), 20% SDS on H_2O 1.0ml). Cell lysate was then diluted 1:1 with Tris buffer. 50µl of the diluted sample was loaded on to the respective well after further dilution with 100µl of Tris buffer to ensure even distribution of proteins. The vacuum pump was then allowed to run for 10mins before removal of the membrane and washing three times in PBS, followed by blocking with 5% (w/v) BSA in PBS in a sealed container placed on a rolling platform. The nitrocellulose membrane could then be used for western/lectin blotting.

Table 4.7. Description of antibodies.

Antibody (Source)	Raised in/purified from	Monoclonal/ polyclonal	Stock conc.	Dilution	Buffer
anti-MUC1 B27.29 (Dr. Mark Reddish; Biomira Inc, Canada)	Mouse	Monoclonal	5mg/ml	1:10,000	PBS with 1% (w/v) BSA
Anti-TF TF5 (Dr. Bo Jansson; BioInvent Therapeutic, Sweden	Human	Monoclonal	0.8mg/ml	1:3000	PBS with 1% (w/v) BSA
Anti-Tn clone HB-Tn1 (Dako, Ely, UK)	Mouse	Monoclonal	0.2mg/ml	1:3000	PBS with 1% (w/v) BSA
Anti-Sialyl Tn clone HB-STn1 (Dako,Ely, UK)	Mouse	Monoclonal	0.2mg/ml	1:3000	PBS with 1% (w/v) BSA
Anti-Actin (Dako,Ely, UK)	Rabbit	Monoclonal	0.2mg/ml	1:2000	PBS with 1% (w/v) BSA

Table 4.8. Description of lectins.

Lectin (Cat number)	Purified from	Ligand	Stock Conc	Dilution	Buffer
PNA-Biotinylated (Vector, B-1075)	*Arachis hypogaea* (Peanut)	TF	1mg/ml	1:3000	PBS with 1% (w/v) BSA
GSL-II-Biotinylated (Vector, B1205)	*Griffonia simplicifolia*	Core 3	2mg/ml	1:3000	PBS with 1% (w/v) BSA

4.9 Statistical Analysis

Serum sample data was analysed using Kruskall-Wallis non-parametric ANOVA test. Other sample groups were analysed using one-way analysis of variance (ANOVA), specifically using the Dunnett's comparison with a control (StatsDirect v2.3.1; StatsDirect Ltd; Sale, UK). Differences were considered significant when $P<0.05$.

CHAPTER 5

ASSESSMENT OF THE EXPRESSION OF CIRCULATING GALECTINS IN THE SERUM OF HEALTHY PEOPLE AND CANCER PATIENTS

5.1 HYPOTHESES

The concentrations of members of the galectin family in the bloodstream of cancer patients may be different from that in healthy individuals and such changes may be useful as cancer diagnostic markers.

5.2 AIMS

- To use ELISA to measure the concentration of members of the galectin family in the sera of breast cancer patients, colorectal cancer patients, and colorectal cancer patients with liver metastasis and that of healthy individuals.

- To compare the assessments of circulating galectin detection and carcinoembryonic antigen (CEA) detection as cancer markers.

5.3 INTRODUCTION

Assay of proteins and glycoproteins in human serum has been utilised clinically for a wide range of reasons, including disease identification and monitoring. The search for an ideal tumour marker has produced many tests to be used for the diagnosis and management of cancer. The uses of tumour markers have a variety of potential uses, which include screening, diagnosis, establishing prognosis, monitoring treatment, and detecting relapse. The value of a marker is defined by both sensitivity and specificity, where sensitivity measures the proportion of actual positives which are correctly identified as such, while specificity measures the proportion of negatives which are correctly identified.

5.3.1 Circulating Galectin-3

Galectin-3 has been widely studied, and has been implicated in the development and subsequent spread of colorectal cancer. The majority of research has focused on galectin-3 that is expressed either intracellularly, or extracellularly but is still associated with the cell membrane. Iurisci *et al.* (287) have investigated the level of galectin-3 expression in the sera of cancer patients when compared to healthy individuals. There was no correlation between circulating galectin-3 levels and age or blood group. Overall, in all types of cancer patients (breast, gastrointestinal, lung, or ovarian cancer, melanoma, and non-Hodgkin's lymphoma), there was a significant increase in serum galectin-3 levels, and specifically, there was up to a 5-fold increase of circulating galectin-3 concentration in the sera of colorectal cancer patients. Furthermore, serum galectin-3 levels were significantly higher in patients with metastatic disease when compared to patients with localized primary tumours, and in

four out of five colorectal carcinoma patients, there was a significant decrease in serum galectin-3 concentrations two days after tumor resection when compared to before surgery (287). This source of increased serum galectin-3 in cancer patients still remains unclear. It was suggested that the tumour tissues themselves were likely to produce and secrete galectin-3 in the sera. Galectin-3 immunohistochemistry showed that galectin-3 was expressed not only on malignant cells but on macrophages and stromal cells (mostly fibroblasts) which were located near the lesion site (287). It could be suggested that the increased amount of galectin-3-secreting cells through cancer-associated proliferation causes the significantly elevated levels of the circulating galectin, and could explain why the level is reduced upon tumour resection.

To compare the efficacy of members of the galectin family as potential serum cancer markers, their specificity and sensitivity must be compared to already established markers.

5.3.2 CEA and Colorectal Cancer

Carcinoembryonic antigen (CEA) was first described in 1965 (348, 349). CEA is a glycoprotein that has a molecular mass of 180-200 kDa, 60% of which is carbohydrate. CEA demonstrates considerable heterogeneity, which is due to variation in its carbohydrate side chains. The majority of the carbohydrate is composed of mannose, galactose, *N*-acetylglucosamine, fucose and sialic acid (350). CEA is a member of the immunoglobulin superfamily, and has structural similarity to that of ICAM-1 and ICAM-2, which initially led to suggestions that CEA might act as an adhesion molecule. *In vitro* experiments confirmed that CEA was capable of both

homophilic (CEA binding to CEA) and heterophilic (CEA binding to non-CEA molecules) interactions (351-353).

CEA is an antigen that was identified as being present in both foetal colon tissue and colon adenocarcinoma in adults, but was initially thought to be absent from healthy adult colon tissue. CEA is also known as CEACAM5 and is present in the glycoclayx of some healthy epithelial cells. In cancer its expression rises, the distribution changes with increased cytoplasmic expression and CEA is then secreted into the circulation. Further work showed that CEA concentrations in tumours were on average 60-fold higher than in the non-malignant gastrointestinal epithelial tissues (354). In one of the earliest reports on serum CEA, it was shown that CEA concentration was increased in 35 of 36 patients with colorectal cancer (355). In contrast, high CEA values were not detected in healthy individuals, pregnant women, patients with non-gastrointestinal cancer, or in patients with miscellaneous benign gastrointestinal diseases (355). Thirty years after its initial detection in serum, CEA is one of the most widely used markers worldwide, and is the most frequently used marker in colorectal cancer. Despite its widespread use it is clear that it does not have sufficient specificity for use in screening of asymptomatic people and is appropriately restricted to monitoring after surgical resection.

The concentration of CEA in colon cancer patients is shown to increase with increasing disease stage. It has been shown that the proportion of patients with increased CEA concentrations (2.5μg/L) were as follows: Dukes' A, 28%; Dukes' B, 45%; Dukes' C, 75%; and Dukes' D. 84% (356). Furthermore, several studies have shown that well-differentiated colorectal cancers produce more CEA per gram of total protein than poorly differentiated samples (357, 358). Similarly, serum concentrations of CEA tend to be higher in patients with well-differentiated tumours compared with

those with poorly differentiated tumours (359). Therefore, a lack of differentiation or poor differentiation may explain why some patients with advanced colorectal cancer do not have increased serum CEA values.

A number of studies have shown that patients with high preoperative levels of CEA have a worse prognosis than those with low concentrations of the marker (360, 361). At least seven different reports have investigated the prognostic impact of CEA in either node-negative (cancer not present in the lymph nodes), or Dukes' B patients (356, 362-367). In five of these studies, high CEA concentrations predicted adverse prognosis. In the remaining two (363, 365) however, no significant relationship was found between marker concentrations and patient outcome. It is therefore reasonable to conclude that the majority of studies suggest that preoperative CEA can provide prognostic data in patients with Dukes' B colorectal cancer. CEA may therefore be able to aid the identification of the subset of patients with aggressive disease that might benefit from adjuvant chemotherapy.

5.3.3 Other Serum Markers in Colorectal Cancer

A major limitation of CEA as a marker is that 20-30% of patients with colorectal cancer fail to produce elevated serum levels, despite the presence of advanced disease (368). Other markers are therefore an important follow-up of patients. Following CEA, CA 19-9 is the most widely investigated gastrointestinal tumour marker. The CA 19-9 assay detects a mucin containing the sialylated Lewis^a epitope. Although CA 19-9 is considered the best marker for pancreatic adenocarcinona, CA 19-9 is less sensitive than CEA for the detection of colorectal cancer (CRC) (369). Nevertheless, elevated preoperative levels of CA 19-9 have been

found to correlate with adverse patient outcome (369-372). CA 242 has also been investigated, but it's sensitivity as a marker is most effective when combined with CEA. When comparing CEA and CA 242 in the surveillance of 149 patients for recurrent disease, CEA alone had a sensitivity of 76%, and a specificity of 86%. The corresponding sensitivity and specificity for CA 242 were 60% and 87%, respectively. The combination of the two markers increased the sensitivity to 88%, but reduced specificity to 78%. This study concluded that although CA 242 alone is inferior to CEA, it may complement CEA in the follow-up after curative resection for colorectal cancer (373). It was also shown that CA 242 was superior to CEA in detecting lung metastases, but that CEA was more sensitive than CA 242 in diagnosing liver metastases (374).

TPA (tissue polypeptide antigen) measures fragments of cytokeratin 8, 18 and 19, and TPS (tissue polypeptide specific antigen), Tests which detect fragments of cytokeratin 18, have been subjected to some evaluation in colorectal cancer. However, these methods demonstrate a lack of sensitivity and specifity. Further studies have suggested that TPA may act to complement CEA in the detection of CRC (375).

TIMP-1 is a multifunctional glycoprotein that has been shown to inhibit metalloproteinase activity, stimulate cell growth and inhibit apoptosis (376). By using ELISA, it was shown that total levels of TIMP-1 were significantly higher in patients with colonic or rectal cancer than healthy individuals or patients with inflammatory bowel disease (377). With 95% specificity, TIMP-1 detected in colonic cancer has a sensitivity of 65% and rectal cancer with a sensitivity of 42%. Combining CEA with TIMP-1 increased sensitivity for colonic cancer from 65% to 75% and rectal cancer from 42% to 54%, at 95% specificity. Other studies have shown that high

preoperative plasma levels of TIMP-1 independently predict an adverse outcome in patients with colorectal cancer (378).

5.4 METHODS

5.4.1 Materials

HRP- conjugated anti-mouse antibodies were obtained from Dako UK Ltd., Cambridgeshire, UK. Both Sigma*FAST* tablets and ExtrAvidin were purchased from Sigma-Aldrich Ltd., UK. Carcinoembryonic Antigen (CEA) Enzyme Immunoassay Test Kit was purchased from MP Biomedicals, Oakbank, UK.

Serum Samples:

Forty serum samples from colorectal cancer patients without metastasis (26 males and 14 females) and 11 with liver metastasis (7 males and 4 females) and 40 samples from breast cancer patients were obtained from the CTBRC cancer tissue bank (Liverpool, UK). Thirty-one serum samples from healthy people were obtained from Sera Laboratories International (Haywards Heath, UK). The patients' ages were from 25 to 91 (mean age 63) and healthy people from 20 to 51 (mean age 37, 12 males and 19 females) (**Table 5.1**).

Table 5.1. Patient data for serum obtained for ELISA.

Sample Type	n	Age		Gender	Clinical Diagnosis	Stage
		Range	Median			
Healthy	31	20 - 53	39	M: 12 F: 19		
Breast Cancer	40	25 - 91	57	F: 40	IDC: 40	I: 4 II: 18 III: 178
Colorectal Cancer	40	41 - 90	66	M: 26 F: 14	Adenocarcinoma: 40	A: 2 B: 23 C1: 8 C2:7
Colorectal Cancer with Liver Mets	11	46 - 70	51	M: 7 F: 4	Liver mets	C2: 1 B:2

5.4.2 ELISA

Enzyme-linked immunosorbant assays (ELISAs) were carried out as described in **4.3**. OD readings were read at 492nm by a plate microreader (Tecan, Männedorf, Switzerland).

Combined Galectin-3 and -4 ELISA:

Anti-galectin-3 primary antibody and anti-galectin-4 primary antibody were diluted to 2.5μg/ml in coating buffer (Na_2CO_3 1.6g, $NaHCO_3$ 1.46g in 1L H_2O). 25μl of each diluted antibody solution were mixed together to form 50μl combined anti-galectin-3 and -4 solution, which coated the bottom of the well. High-binding 96-well plates were coated with the combined antibody solution overnight at 4°C. Recombinant galectin-3 and -4 were serially diluted from 500ng/ml to 1ng/ml and combined in equal volume. 50μl of the solutions were applied to the wells for 2hr at room temperature. The rest of the protocol was carried out as described in **4.3**.

CEA Test Kit:

Prior to the assay, the reagents were allowed to reach room temperature, and all reagents were gently mixed before use. The desired number of coated-well strips were placed into the holder, and 50μl of CEA standards or 50μl human serum were placed into the wells, followed by 100μl enzyme conjugate, and left to incubate for 1hr at room temperature. Wells were then washed three times with 300μl of X1 wash buffer, and the plate blotted on absorbent paper towels. 100μl of TMB substrate was applied to all wells and left to incubate for 10 minutes at room temperature. 50μl of stop solution was added to all wells, and the plate was gently shaken to mix the solution. The absorbance was read on a plate reader as described in **4.3**, after 15min incubation.

5.5 RESULTS

5.5.1 Preliminary design of ELISA protocol for future assessments of galectin concentrations in human serum samples.

The following experiments aimed to optimise the concentration of the secondary biotinylated anti-galectin antibody, with set concentrations of primary antibody and ExtrAvidin.

Table 5.2. ELISA used to perform a checkerboard analysis to determine a functional concentration of secondary antibody.

	Recombinant galectin (ug/ml)									
	5.00	**2.50**	**1.25**	**0.63**	**0.31**	**0.16**	**0.08**	**0.04**	**0.02**	**0.01**
2.50	3.88	OVER	3.93	3.73	3.73	3.46	3.15	2.75	2.04	0.19
1.25	3.43	3.59	3.55	3.19	3.14	2.65	2.12	1.50	1.06	0.21
0.63	2.57	2.55	2.53	2.42	2.10	1.93	1.48	1.07	0.78	0.25
0.31	1.75	1.70	1.63	1.73	1.41	1.11	1.05	0.75	0.55	0.21
0.16	1.17	1.00	1.04	0.99	0.78	0.72	0.55	0.42	0.29	0.16
0.08	1.06	0.68	0.69	0.59	0.46	0.45	0.34	0.34	0.35	0.18
0.04	0.59	0.61	0.49	0.45	0.41	0.36	0.26	0.24	0.22	0.21
0.02	0.58	0.61	0.61	0.42	0.32	0.38	0.24	0.23	0.26	0.23

A checkerboard analysis was carried out to provide an appropriate concentration of biotinylated anti-galectin-3. ELISA was carried out as described in **3.3.1**. A concentration of biotinylated anti-galectin-3 antibody that was deemed the most appropriate was 1.25μg/ml. This concentration ensured an OD value well within the detection range of the plate reader throughout a wide range of galectin-3 concentrations, without becoming saturated (**Table 5.2**).

In order to confirm the suitability of the components of the ELISA protocol, a standard curve was set up to analyse the detection of recombinant galectin-3. Biotinylated anti-galectin-3 was used at a concentration of 1.25μg/ml. ExtrAvidin was

used at 1:10,000 dilution. Recombinant galectin-3 under went doubling dilution with a starting concentration of 2μg/ml.

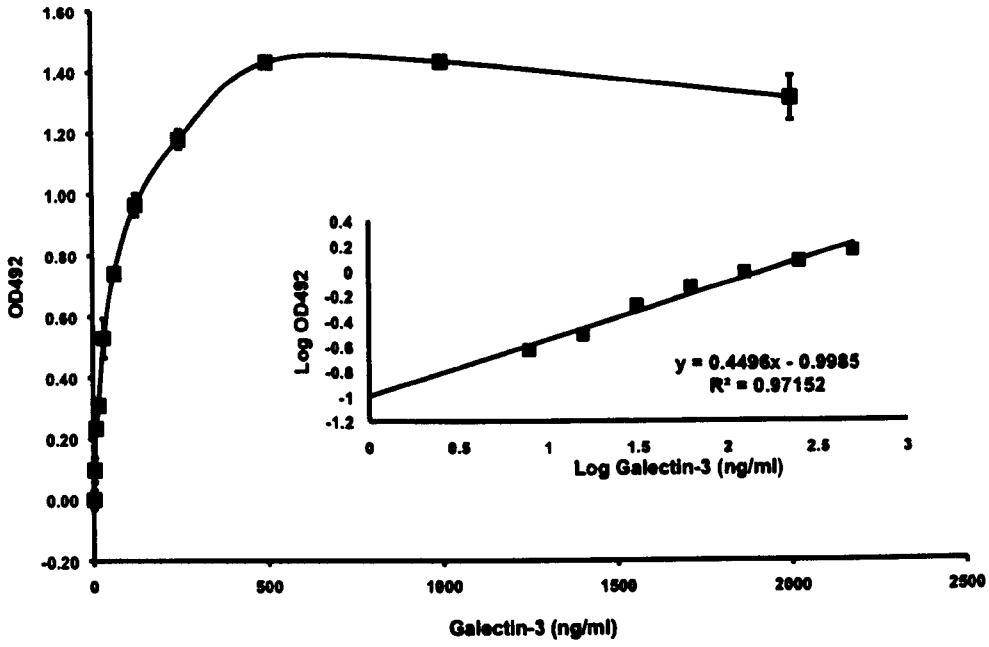


Figure 5.1. Standard curve of recombinant galectin-3 at a range of concentrations (OD492).

Anti-galectin-3 was diluted to 2.5μg/ml in coating buffer, and incubated in wells overnight at 4°C. 100μl 1.25μg/ml of biotinylated anti-galectin-3 antibody was applied to the plates for 1hr at room temperature. 100μl ExtrAvidin per well was diluted 1:10,000 and applied for 1 hr at room temperature in separate wells. Plates were washed twice with 100μl washing buffer per well before applying 100μl Sigma*FAST* OPD solution until a yellow colour appeared (7mins). The reaction was then stopped with 50μl 4M sulphuric acid. The experiment was performed in duplicate.

A saturated standard curve was achieved, which would be appropriate for determining the concentration of galectins within serum samples by using standard regression. In order to calculate the galectin concentrations in the serum, the non-saturated area of the curve was logged (**Figure 5.1**).

5.5.2 Detection of galectin levels in human serum samples.

Initial experiments were run to determine a standard curve for each recombinant galectin. The concentration of the primary antibody and secondary antibodies were as stated in **Chapter 4, Table 4.1**.

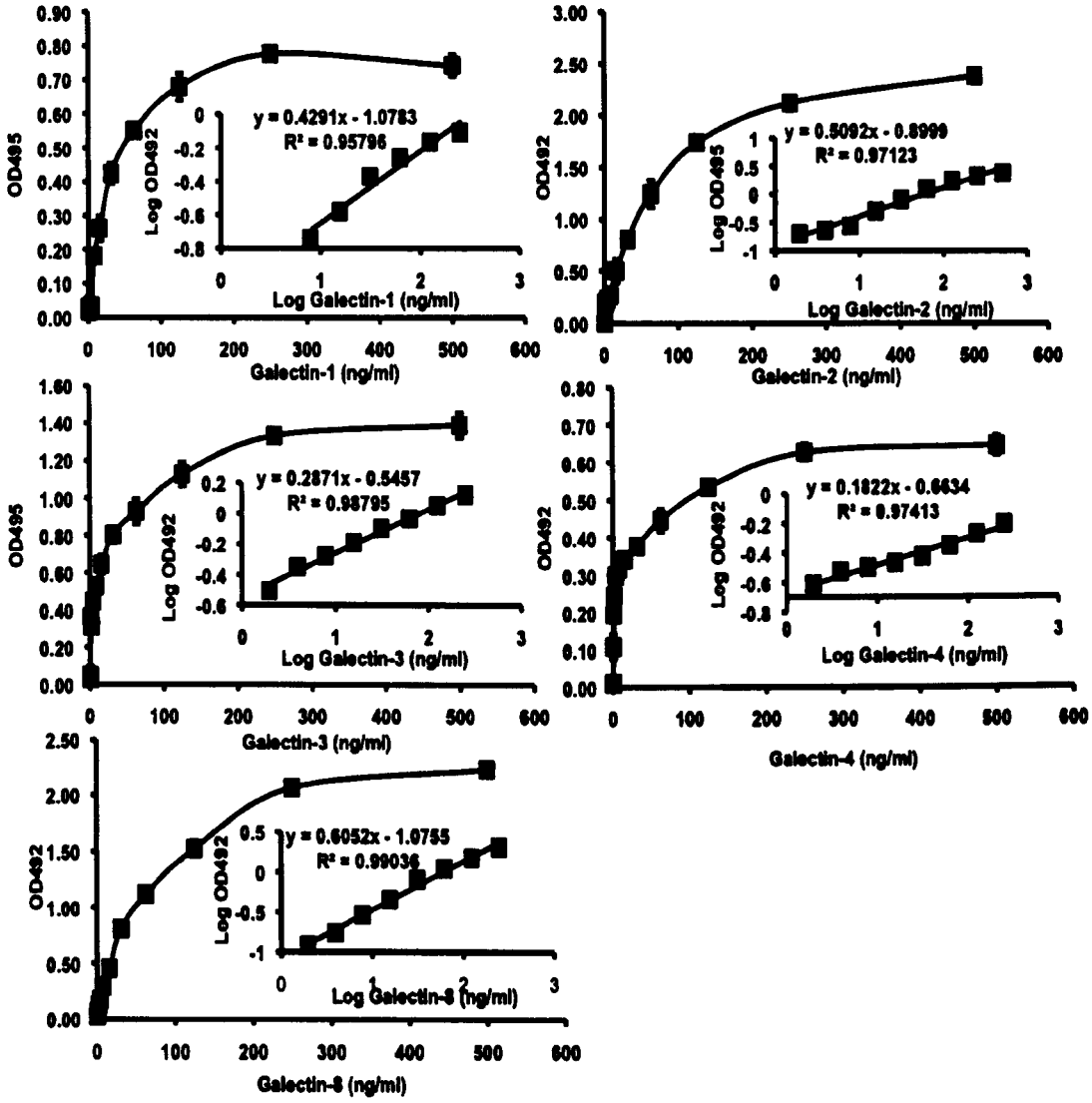


Figure 5.2. ELISA defining the standard curve of recombinant galectin-1, -2, -3, -4, and -8 at a range of concentrations (OD492).

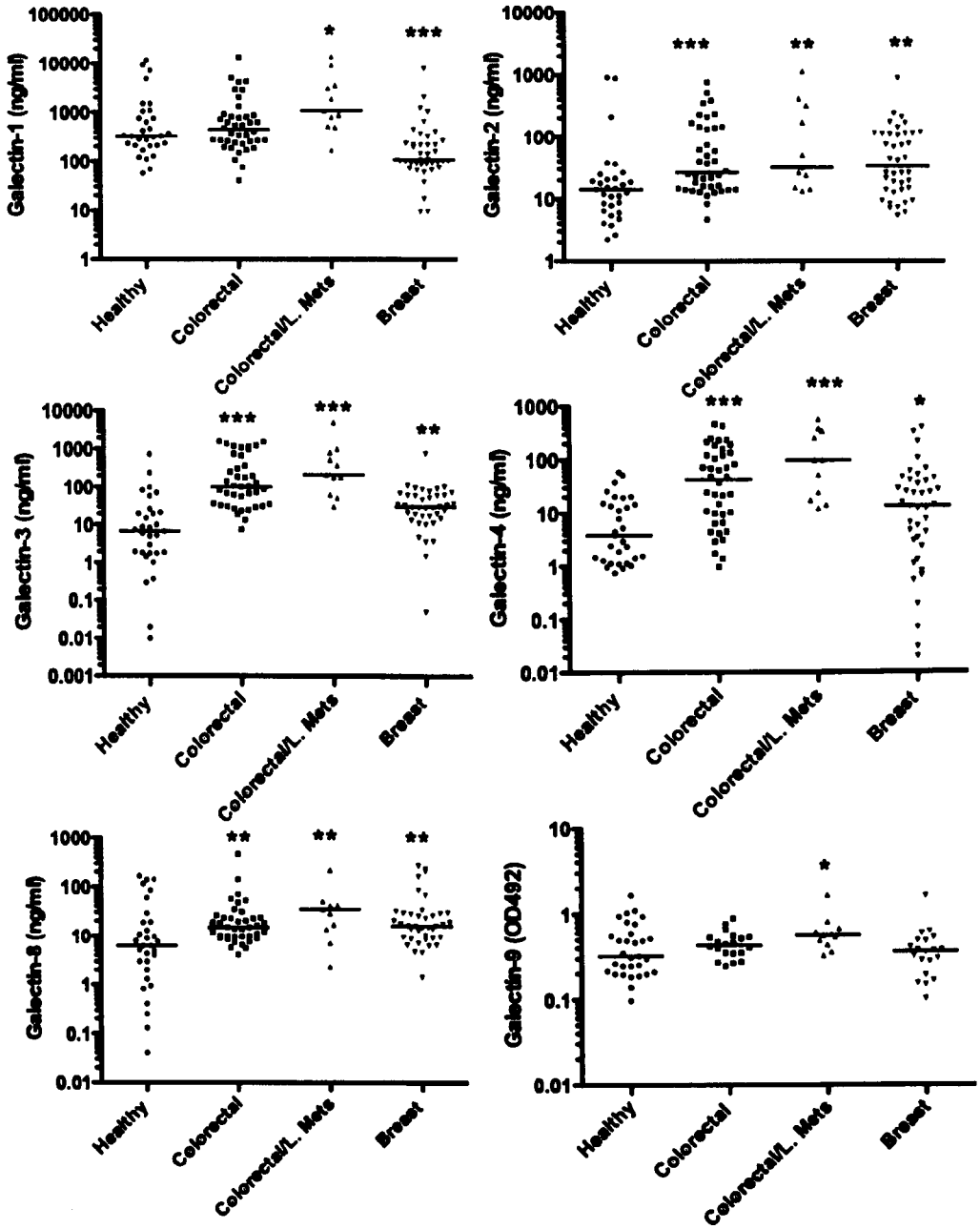


Figure 5.3. ELISA detecting the level of expression of galectin-1, -2, -3, -4, and -8 in the sera of cancer patients and healthy individuals. At the time of these experiments, recombinant galectin-9 was not available and a standard curve could not be generated to calculate concentrations. Therefore galectin-9 is shown as an OD value. (* P<0.001, ** P<0.01, * P<0.05).**

Lines within the sample data represent the median values of the sample group. The experiment was performed in duplicate, and statistically analysed using Kruskall Wallis non-parametric ANOVA.

By using standard regression analysis utilising the standard curves (**Figure 5.2**), the concentration (ng/ml) of the galectin within the serum samples was calculated from ELISA. At the time of these experiments, recombinant galectin-9 was not available and a standard curve could therefore not be generated to calculate serum galectin-9 concentration. Therefore, the expression of serum galectin-9 is shown as an OD value within this thesis (**Figure 5.3**).

Table 5.3. Circulating galectins in the sera of colorectal and breast cancer patients and healthy people.

		Median (ng/ml)	Range (ng/ml)	5th Percentile	95th Percentile	p* value
Gal-1	Healthy	328.8	56.6 - 11670.2	88.8	8315.0	
	Colorectal Cancer	442.7	40.3 - 13263.2	103.8	4353.5	
	Colorectal cancer with Liver Metastasis	1064.2	169.2 - 13834.1	331.1	11499.1	<0.05
	Breast Cancer	106.2	9.3 - 7757.0	16.6	1229.3	<0.0001
Gal-2	Healthy	14.1	2.2 - 914.8	3.2	547.2	
	Colorectal Cancer	26.5	4.7 - 764.8	11.2	261.9	<0.001
	Colorectal cancer with Liver Metastasis	32.1	13.3 - 1155.8	13.8	786.1	<0.01
	Breast Cancer	34.1	5.4 - 898.6	7.0	211.5	<0.01
Gal-3	Healthy	6.8	0.0 - 736.5	0.2	167.2	
	Colorectal Cancer	101.0	7.5- 1603.9	13.0	1258.4	<0.0001
	Colorectal cancer with Liver Metastasis	215.0	31.0 - 5106.4	41.7	3064.7	<0.0001
	Breast Cancer	30.6	0.1 - 749.2	1.5	125.2	<0.01
Gal-4	Healthy	3.9	0.8 – 59.6	0.9	45.1	
	Colorectal Cancer	43.2	1.0 – 474.9	1.7	262.9	<0.0001
	Colorectal cancer with Liver Metastasis	98.7	12.3 - 584.2	13.3	488.7	<0.0001
	Breast Cancer	13.7	0.0 - 409.7	0.1	224.6	<0.05
Gal-8	Healthy	6.3	0.0 - 166.3	0.2	141.7	
	Colorectal Cancer	14.9	4.1 - 488.8	5.7	57.1	<0.01
	Colorectal cancer with Liver Metastasis	35.1	2.4 - 223.0	4.8	136.9	<0.01
	Breast Cancer	16.2	1.4 - 272.4	4.8	210.1	<0.01

*Compared with that in healthy people

Galectin-1: Serum samples obtained from patients with colorectal cancer and liver metastasis demonstrated a significant increase (P=0.0132, 3.2-fold increase) in the expression of circulating galectin-1, when compared to serum samples from healthy individuals. However, serum samples obtained from patients with breast cancer demonstrated a significant decrease (P<0.0001, 3.1-fold decrease) in the expression of circulating galectin-1, when compared to serum samples from healthy individuals.

Galectin-2: Levels of galectin-2 within the serum of patients with colorectal cancer, and colorectal cancer and liver metastasis demonstrated a significant increase (P=0.0007, P=0.002, respectively, with a 1.9-fold and 2.3-fold increase, respectively) when compared to serum samples from healthy individuals. Galectin-2 levels in the serum samples from patients with breast cancer also demonstrated a significant increase (P=0.0021, 2.4-fold increase) when compared to serum samples from healthy individuals.

Galectin-3: Levels of galectin-3 within serum samples of patients with colorectal cancer, and colorectal cancer and liver metastasis demonstrated a significant increase (P<0.0001, P<0.0001, respectively, with a 14.9-fold and 31.6-fold increase respectively) in the expression of circulating galectin-3, when compared to serum samples from healthy individuals. Galectin-3 levels in the serum samples from patients with breast cancer also demonstrated a significant increase (P=0.0018, 4.5-fold increase) in the expression of circulating galectin-3, when compared to serum samples from healthy individuals.

Galectin-4: Levels of galectin-4 within serum samples of patients with colorectal cancer, and colorectal cancer and liver metastasis demonstrated a significant increase (P<0.0001, P<0.0001, respectively, with a 11.0-fold and 25.3-fold increase respectively) in the expression of circulating galectin-4, when compared to serum samples from healthy individuals. Galectin-4 levels in the serum samples from patients with breast cancer also demonstrated a significant increase (P=0.0462, 3.5-

fold increase) in the expression of circulating galectin-4, when compared to serum samples from healthy individuals.

Galectin-8: Levels of galectin-8 within serum samples of patients with colorectal cancer, and colorectal cancer and liver metastasis demonstrated a significant increase (P=0.0085, P=0.0023, respectively with a 2.4-fold and 5.6-fold increase, respectively) in the expression of circulating galectin-8, when compared to serum samples from healthy individuals. Galectin-8 levels in the serum samples obtained from patients with breast cancer also demonstrated a significant increase (P=0.0033, 2.6-fold increase) in the expression of circulating galectin-8, when compared to serum samples from healthy individuals.

Galectin-9: At the time of these experiments, recombinant galectin-9 was not available to be used as standard for the calculation of galectin-9 concentrations. Therefore, the ELISA OD readings were presented here. Serum samples obtained from patients with colorectal cancer and liver metastasis demonstrated a significant increase (P=0.0148, 2.0-fold increase) in the expression of circulating galectin-9, when compared to serum samples from healthy individuals. The experiment was performed in duplicate, and statistically analysed using Kruskall-Wallis non-parametric ANOVA test.

5.5.3 Comparison of Galectin and CEA detection as serum markers for cancer.

As galectin-3 and -4 showed the most significant increase in serum concentration when comparing both colorectal cancer groups and healthy individuals, further assessments were performed to compare the specificity and sensitivity of a combined galectin-3/-4 ELISA, combining the detection of galectin-3 and -4 in a single ELISA, and CEA ELISA when testing serum samples. The combined galectin-3 and -4 ELISA was carried out as stated in **5.4.2**. CEA ELISA was conducted with a CEA Enzyme Immunoassay test kit from MP Biomedicals and carried out as stated in **5.4.2**.

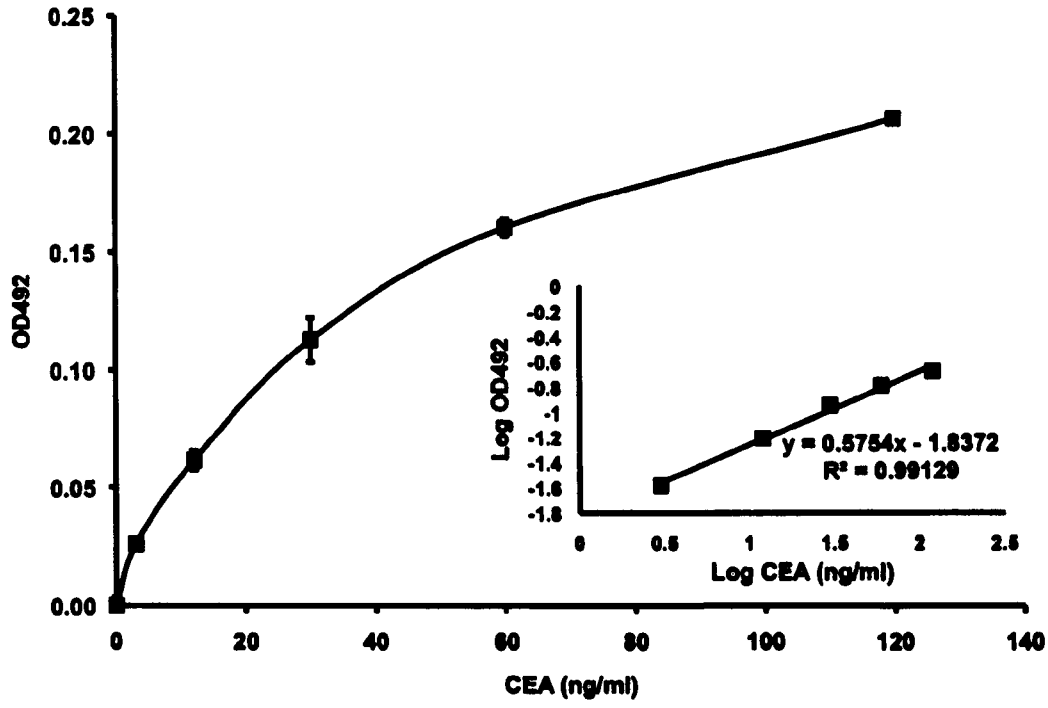


Figure 5.4. ELISA defining the standard curve of CEA at a range of concentrations (OD492).

A saturated standard curve was achieved for CEA, which would be appropriate for determining the concentration of CEA within serum samples by using standard regression. In order to perform this, the non-saturated area of the curve was logged. The experiment was performed in duplicate (**Figure 5.4**).

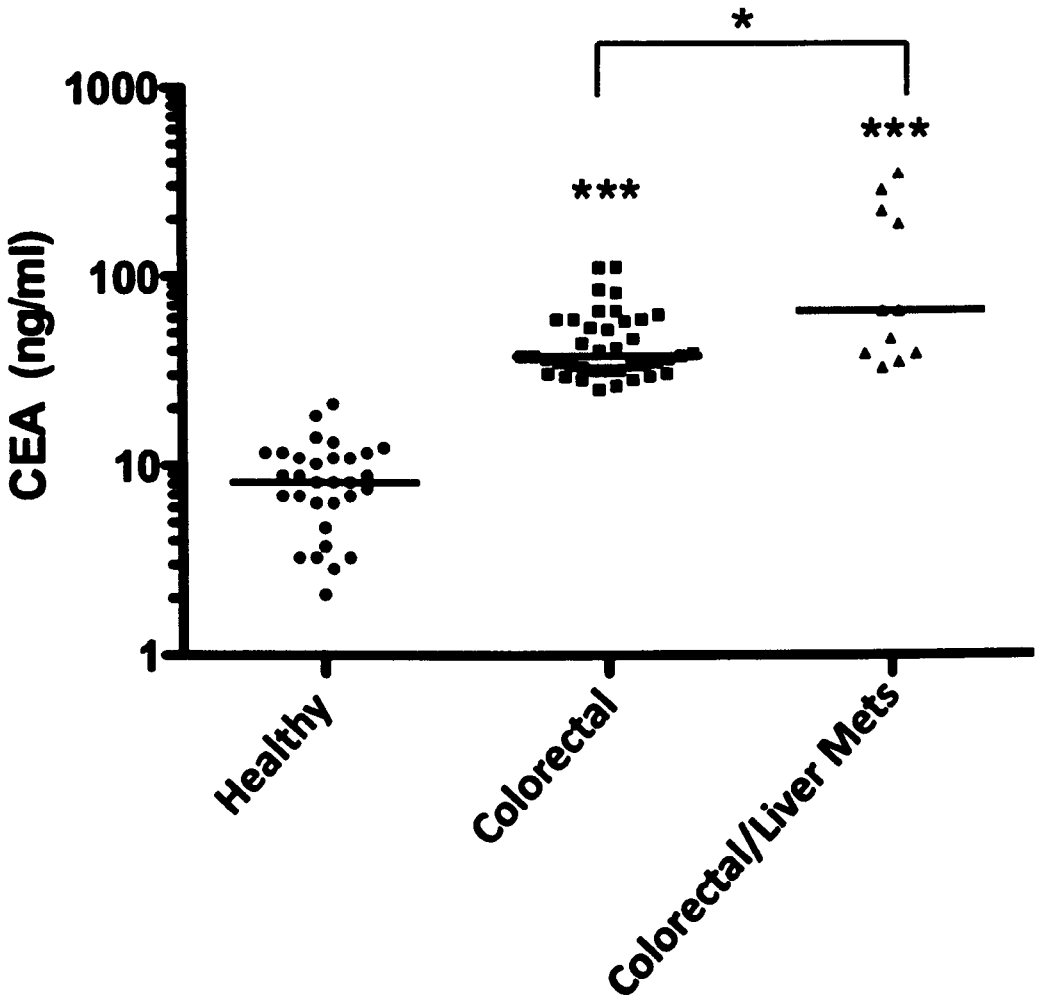


Figure 5.5. ELISA detecting the level of expression of CEA in the sera of cancer patients and healthy individuals (OD492). (* P<0.001, ** P<0.01, * P<0.05).**

Serum samples obtained from patients with colorectal cancer, and colorectal cancer with liver metastasis demonstrated a significant increase (P<0.0001 and

P<0.0001, respectively, with a 4.6-fold and 8.0-fold increase respectively) in the expression of circulating CEA, when compared to serum samples from healthy individuals. There was a significant increase between the level of circulating CEA in samples from patients with colorectal cancer and those with colorectal cancer and liver metastasis (P=0.0037) The experiment was performed in duplicate, and statistically analysed using Kruskall-Wallis non-parametric ANOVA test (**Figure 5.5**).

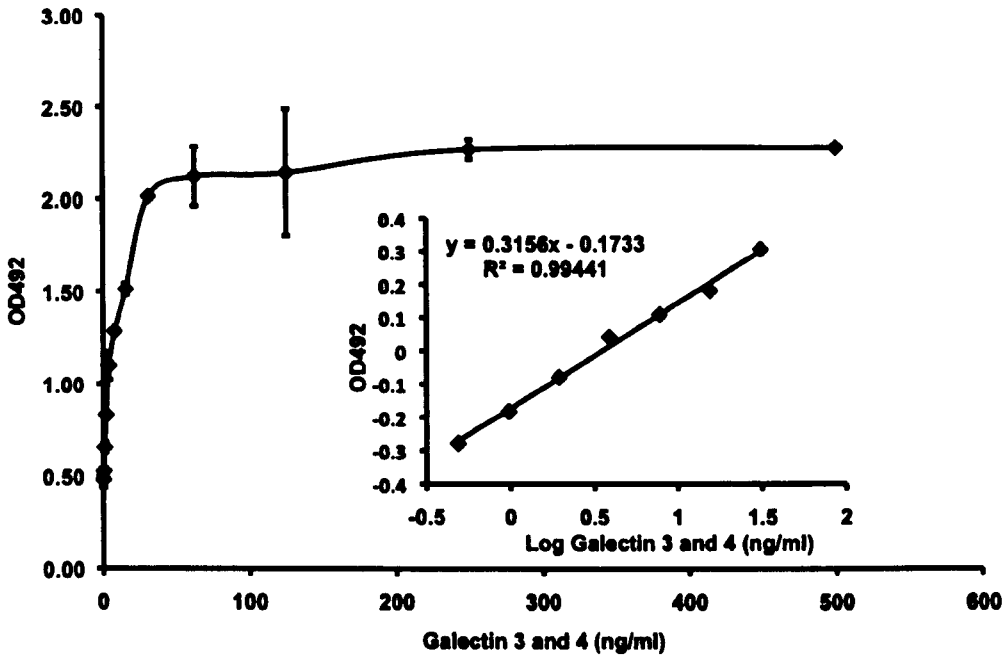


Figure 5.6. ELISA defining the standard curve of combined galectin-3 and -4 at a range of concentrations (OD492).

Galectin-3 and –4 were combined in a single ELISA, with the wells coated with a combinantion of anti-galectin-3 and anti-galectin-4 antibodies. A saturated

standard curve was achieved for galectin-3 and -4. The experiment was performed in duplicate (**Figure 5.6**).

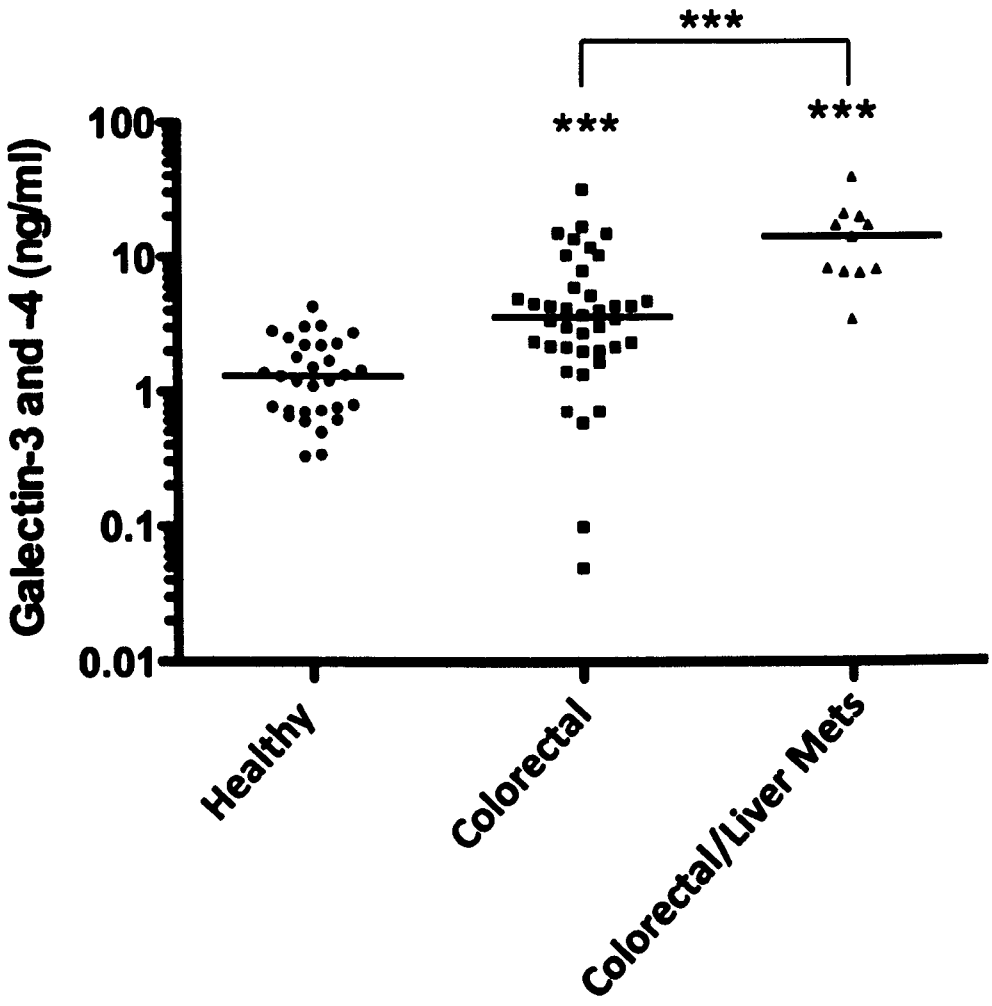


Figure 5.7. ELISA detecting the level of expression of galectin-3 and -4 in the sera of cancer patients and healthy individuals (OD492). (* P<0.001, ** P<0.01, * P<0.05).**

Serum samples obtained from patients with colorectal cancer, and colorectal cancer and liver metastasis demonstrated a significant increase (P<0.0001 and

P<0.0001, respectively, with a 2.8-fold and 11.0-fold increase respectively) in the expression of circulating galectin-3 and -4, when compared to serum samples from healthy individuals. There was a significant increase in the combined level of circulating galectin-3 and -4 in samples from patients with colorectal cancer and those with colorectal cancer and liver metastasis (P=0.0002) The experiment was performed in duplicate, and statistically analysed using Kruskall-Wallis non-parametric ANOVA test (**Figure 5.7**).

Sensitivity and specificity of the ELISA-based serum assays were analysed using the following equations:

$$\text{Sensitivity} = \frac{\text{Number of True Positives}}{\text{Number of True Positives} + \text{Number of False Negatives}}$$

$$\text{Specificity} = \frac{\text{Number of True Negatives}}{\text{Number of True Negatives} + \text{Number of False Positives}}$$

True positives referred to a CEA or galectin-3 and -4 concentration within serum from a patient from a cancer group that was above the designated threshold, whereas false positives referred to a CEA or galectin-3 and -4 concentration within serum from an individual within the healthy group that was above the designated threshold. True negatives referred to a CEA or galectin-3 and -4 concentration within serum from a healthy individual that was below the designated threshold, whereas false negatives referred to a CEA or galectin-3 and -4 concentration within serum from a patient within a cancer group that was below the designated threshold.

The CEA threshold for cancer detection has already been established for different groups of individuals. For non-smokers at 50 years of age it has been defined as 3.4-3.6 μg/L, for non-smokers at 70 years of age it has been defined as 4.1 μg/L, and for smokers at any age, the upper threshold has been defined as 5μg/L (379, 380). For the purpose of comparison within this thesis, all galectin-3 and -4 ELISA thresholds will be compared to the standard CEA threshold of 5μg/L (5ng/ml) (**Table 5.4, Table 5.5**).

Table 5.4. The positive and negative values of CEA from sera from patients with cancer, and healthy individuals at of the standard reference concentration threshold.

CEA				
		Positive	**Negative**	**Total samples**
5ng/ml	Healthy	24	7	31
	Colorectal cancer	40	0	40
	Liver metastasis	11	0	11

Table 5.5. The positive and negative values of the combined galectin-3/-4 assessment from sera from patients with cancer, and healthy individuals at a range of concentration thresholds (ng/ml).

Galectin-3/ -4				
		Positive	**Negative**	**Total samples**
0.5ng/ml	Healthy	28	3	31
	Colorectal cancer	38	2	40
	Liver metastasis	11	0	11
1ng/ml	Healthy	19	12	31
	Colorectal cancer	35	5	40
	Liver metastasis	11	0	11
5ng/ml	Healthy	0	31	31
	Colorectal cancer	11	29	40
	Liver metastasis	10	1	11
10ng/ml	Healthy	0	31	31
	Colorectal cancer	8	32	40
	Liver metastasis	6	5	11
20ng/ml	Healthy	0	31	31
	Colorectal cancer	1	39	40
	Liver metastasis	2	9	11

Using the specified calculations, a CEA ELISA was compared to an ELISA combining galectin-3 and -4.

Table 5.6. The positive and negative values of CEA from sera from patients with cancer, and healthy individuals at a threshold of 5ng/ml.

	Healthy vs Colorectal		Healthy vs Liver mets		Colorectal vs Liver mets	
CEA	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
5ng/ml	100	22	100	22	100	0

Table 5.7. The positive and negative values of combined galectin-3 and -4 assessment from sera from patients with cancer, and healthy individuals at a range of concentration thresholds (ng/ml).

	Healthy vs Colorectal		Healthy vs Liver mets		Colorectal vs Liver mets	
Galectin-3/-4	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
0.5ng/ml	95	10	100	10	100	5
1ng/ml	88	39	100	39	100	13
5ng/ml	28	100	91	100	91	73
10ng/ml	20	100	55	100	55	80
20ng/ml	3	100	18	100	18	98

When comparing the concentration of the target proteins within the CEA-based ELISA and the galectin-3/ -4 based ELISA, a variety of concentration thresholds were analysed (**Table 5.7**).

Colorectal Cancer vs Healthy Controls:

CEA-based ELISA demonstrated a high level of sensitivity when analysed at a threshold of 5ng/ml (100%), but with a low level of specificity (22%). Within the galectin-based ELISA, at concentrations of 0.5ng/ml and 1ng/ml, there were high levels of sensitivity (95%, 88%, respectively) but low levels of specificity (10%, 39%, respectively). At thresholds of a higher concentration, 5ng/ml to 20ng/ml, the galectin-based ELISA achieved higher levels of specificity (100%, 100% and 100%, respectively) at the expense of sensitivity (28%, 20%, and 3%, respectively). The galectin-based ELISA did not reach the same level of sensitivity obtained by the CEA-based ELISA when comparing serum samples from colorectal cancer patients and samples from healthy individuals, which indicated that CEA-based ELISA represents a more accurate method of serum marker detection in this case.

Colorectal Cancer with Liver Metastasis vs Healthy Controls:

As previously seen when analysing colorectal cancer serum samples against healthy samples, CEA-based ELISA produced a high level of sensitivity at the 5ng/ml threshold (100%) but a lower level of specificity (22%) when comparing healthy samples to samples from patients with colorectal cancer and liver metastasis. Galectin-based ELISA demonstrated a maximum level of sensitivity at thresholds set at 0.5ng/ml and 1ng/ml (100% and 100%, respectively), and a maximum level of specificity at thresholds set at 5ng/ml to 20ng/ml (100%, 100%, and 100%, respectively), again at the expense of sensitivity (91%, 55%, and 18%, respectively). The best balance between sensitivity and specificity when using galectin-based ELISA was obtained at a threshold of 5ng/ml (91% and 100%, respectively). When

comparing samples from patients with colorectal cancer with liver metastasis and those from healthy individuals, galectin-based ELISA was more comparative to CEA-based ELISA than when comparing colorectal cancer-only serum samples and healthy samples. Thresholds of 0.5ng/ml and 1ng/ml with the galectin-based ELISA demonstrated the same level of sensitivity as seen within the CEA-based ELISA. However, galectin-based ELISA only exceeded CEA-based ELISA in specificity at 1ng/ml. 5ng/ml up to 20ng/ml galectin provided less sensitivity than CEA-based ELISA, but a much greater level of specificity. When comparing the ELISA methods, 1ng/ml threshold of galectin-based ELISA exceeded CEA ELISA in both aspects.

Colorectal Cancer vs Colorectal Cancer with Liver Metastasis:

CEA-based ELISA provided a maximum sensitivity at the threshold of 5ng/ml (100%), but with very limited specificity (0%). Galectin-based ELISA demonstrated the highest level of sensitivity at 0.5ng/ml and 1ng/ml thresholds (100% and 100%, respectively). 5ng/ml galectin threshold represented the best balance between sensitivity and specificity (91% and 73%, respectively). Furthermore, at a range of threshold levels, 0.5ng/ml to 10ng/ml, galectin-based ELISA provided a better level of specificity and sensitivity that that provided by CEA-based ELISA.

5.6 DISCUSSION

An ELISA protocol was developed to optimise the assessment of the levels of galectins in the serum of cancer patients and healthy people. The levels of galectin-2, -3, -4, and -8, demonstrated a significant increase in the sera of individuals with colorectal cancer, colorectal cancer with liver metastasis, when compared to healthy individuals. Galectin-2, -3 and -8 increased in the sera of breast cancer patients. However, the expression of circulating galectin-1 only increased significantly within samples from patients with colorectal cancer with liver metastasis, and decreased significantly in samples from individuals with breast cancer. None of the serum galectin assays show sufficient predictive value to be useable clinically in diagnosis. However, there is a limited amount of serum samples to allow adequate investigation into the clinical usefulness of galectins for cancer staging, although it seems unlikely at this time.

Iurisci *et al.* (287) had previously investigated the level of galectin-3 expression in the sera of cancer patients when compared to healthy individuals. It was demonstrated that low serum levels of galectin-3 were detected in healthy individuals (median, 62 ng/ml; range, 20–313 ng/ml) when compared to cancer patients (287). Within the serum samples analysed in this thesis, a lower level was also seen in sera from healthy individuals (median, 6.8ng/ml; range, 0.1ng/ml-736.5ng/ml). Iurisci *et al.* also stated that when compared with healthy individuals, galectin-3 serum levels in patients with breast cancer were increased significantly (287). The levels of circulating galectin-3 within the sera analysed within this thesis demonstrated the same pattern. Serum obtained from patients with breast and colorectal cancer demonstrating a significant increase of galectin-3 expression when compared to healthy individuals. Further analysis by Iurisci *et al.* demonstrated that galectin-3

concentrations in sera from patients with metastatic disease were higher than in sera from patients with localized tumors. A similar pattern was demonstrated within the serum samples from patients with colorectal cancer that were analysed within this thesis.

Watanabe *et al.*(381) have demonstrated very recently that circulating levels of galectin-3 and -4 in colorectal cancer patients were significantly higher compared to those in controls, which complemented the data demonstrated within this thesis, and that galectin-4 levels significantly decreased after surgery.(381)

Due to the higher level of significance demonstrated within the increase of galectin-3 and -4 expression within sera from cancer patients, work within this thesis investigated their potential as serum biomarkers for colorectal cancer. Although in the case of colorectal cancer, and colorectal cancer with liver metastasis, galectin-3 and -4 are equally as significant, CEA data points provide less of a spread, suggesting a more significant and appropriate candidate for a serum cancer marker. This was further investigated by analysising the specificity and sensitivity of each ELISA. CEA-based ELISA provided the most useful marker for comparing serum samples obtained from patients with colorectal cancer and those from healthy individuals. However, galectin-based ELISA generally provided a more sensitive and specific method when comparing serum samples from patients with colorectal cancer with liver metastasis and healthy individuals, as well as when comparing samples from patients with colorectal cancer, and those from patients with colorectal cancer with liver metastasis. Further work is required to expand this data set and determine any correlations between circulating galectin expression and disease states.

The increased levels of circulating galecin-3 in cancer patients have been shown earlier in our group to act as an important promoter in cancer cell haematogenous dissemination to secondary tumour sites in metastasis (315, 330). Galectin-3 was shown to interact with the oncofetal Thomsen-Friedenreich antigens on the transmembrane mucin protein MUC1 expressed by the cancer cells (316). The galectin-3-TF/MUC1 interaction induces polarisation of MUC1 cell surface localization, which results in exposure of the smaller cell-surface adhesion molecules, that are otherwise concealed by the large, heavily-glycosylated MUC1. Incubation of MUC1-expressing cancer cells with galectin-3 demonstrated an increased heterotypic adhesion of the cancer cells to blood vascular endothelium (315) and homotypic aggregation of the cancer cells to form micro-tumour emboli which prolongs the survival of tumour cells in the circulation (316).

As galectin-1, -2, -3, -4 and -8 increase in the sera of colorectal cancer patients with liver metastasis, it could suggest the possibility that these galectins, in a way similar to galectin-3, play a role in the regulation of cancer cell haematogenous spread in metastasis. Further experiments within this thesis aim to explore this hypothesis.

CHAPTER 6

EFFECT OF CIRCULATING GALECTINS ON CANCER CELL ADHESION AND TRANS-ENDOTHELIAL MIGRATION

6.1 HYPOTHESES

Other galectin members that show increased circulation in the bloodstream of cancer patients may, like galectin-3, influence adhesion of disseminating cancer cells to vascular endothelial cells and thus also promote metastasis by interaction with cancer associated MUC1.

6.2 AIMS

- To assess the binding of members of the galectin family to TF-expressing glycoproteins.

- To investigate the effect of recombinant galectins at concentrations similar to those found in the sera of cancer patients on cancer cell heterotypic adhesion to endothelial cells and ECM (Matrigel).

6.3 INTRODUCTION

6.3.1 Mechanism of galectin-3 induced homotypic adhesion of cancer cells and adhesion of cancer cells to endothelium.

Earlier work carried out by our group (315, 316, 382) revealed that circulating galectin-3 promotes metastasis by increasing cancer cell-cell homotypic aggregation and cancer cell heterotypic adhesion to vascular endothelium. These effects of galectin-3 were seen to occur partly as a consequence of galectin-3 interaction with cancer-associated MUC1, which breaks the 'protective shield' of the cell-surface MUC1 through polarisation, leading to the exposure of smaller cell-surface adhesion molecules/ligands, which includes CD44 and ligands for E-selectin (315). Galectin-3, at pathological concentrations, was shown to increase cancer cell adhesion to macrovascular (HUVEC) and microvascular (HMVEC-L) endothelial cells under static and flow conditions, to increase trans-endothelial migration, and to decrease the latency of experimental metastasis in athymic mice (315). The galectin-3-MUC1 interaction also enhances cancer cell-cell homotypic aggregation (382). The formation of cancer-cell emboli has important implications for the survival of tumour cells in the blood/lymphatic circulation, which leads to metastatic spread (14, 383). Cells in aggregated form have been shown to have a much higher survival rate in the circulation than single cells, resisting anoikis (384) and with a resulting prolongation of survival of disseminating tumour cells in the circulation leading to increased metastasis (385).

As members of the galectin family all recognize galactose-terminated carbohydrate structures, and are often co-expressed in same tissue types and cells, we hypothesised that the increase circulation of the other galectin members seen in cancer patients may also have a similar influence as shown for galectin-3 on cancer

cell-cell interactions by binding to TF on cancer associated MUC1 and subsequent clustering of MUC1.

6.3.2 Carbohydrate Targets of Galectins

The galectin family of β-galactoside-binding proteins has over a dozen human members, and each galectin recognises different galactose-terminated glycans, and has different biological consequences (386-388). Galectin-1 has been shown to inhibit mast cell degranulation (389), whereas galectin-3 induces degranulation in mast cells (390). Furthermore, galectin-1 blocks leukocyte chemotaxis (391), while galectin-3 has the opposite effect, inducing leukocyte chemotaxis (392). Galectin-1, -2 and -3 have all been reported to signal T cells through different receptors (317, 393, 394).

There is, however, evidence that different galectins may recognise related receptors. Galectin-3 attenuates galectin-1 inhibition of growth in neuroblastoma cells at the receptor level, and both galectin-1 and galectin-3 induce superoxide production in human neutrophils (395-397). Although some differences have been reported in glycan recognition by these galectins, there are still questions remaining about glycan recognition and subsequent effects on galectin binding, and further investigations may provide a new functional map of the CRD for each galectin.

By using glycan microarray systems, it has been shown that galectin-2 and galectin-3 exhibited higher binding than galectin-1 to fucose-containing A and B blood group antigens (398). Galectin-2 exhibited significantly reduced binding to all sialylated glycans, whereas galectin-1 bound α2-3- but not α2-6-sialylated glycans, and galectin-3 bound to some glycans terminating in either α2-3- or α2-6-sialic acid (398). Each galectin exhibits higher binding for glycans with poly-N-

acetyllactosamine sequences (Galβ1-4GlcNAc)$_n$ when compared with N-acetyllactosamine (LacNAc) glycans (Galβ1-4GlcNAc). However, only galectin-3 bound internal LacNAc within poly(LacNAc) (398).

6.3.3 TF-Expressing Glycoproteins

Antifreeze Glycoprotein:

Antifreeze glycoproteins (AFGPs) constitute the major fraction of protein in the blood serum of Antarctic notothenioids (icefish) and Arctic cod. These compounds enable the fish to survive in subzero, ice-laden polar oceans. These glycoproteins consist of a varying number of repeating units of (Ala-Ala-Thr)$_n$, with minor sequence variations, such as the first Ala in some of the repeats are replaced by Pro. There are eight distinct classes of this glycopeptide, which range in relative molecular mass from 33.7 kDa to 2.6 kDa (399, 400). Antifreeze proteins express multiple TF antigen on the Thr residues, although the amount of TF is not fully characterised (401, 402).

Bovine (Asialo) Fetuin:

Bovine fetuin is a globular protein of 341 amino acids. Bovine fetuin homologs have been identified in several species, which include rat, sheep, pig, mouse and human with 60-70% homology at the amino acid level and 80-90% homology at the cDNA level (403-405). Fetuin was originally identified as a fetal plasma protein in fetal and newborn calf serum (406). Mammalian fetuins are a family of related glycoproteins that are members of the cystatin superfamily, and are characterized by two tandemly arranged cystatin-like domains (403). Fetuin appears

to be a multifunctional protein with various biological roles, including inhibiting phase separation in serum, and modulating apatite formation during mineralization (407). Furthermore, bovine fetuin has been suggested to be lipid (408) and calcium binding (409).

Fetuin is well-characterized as a glycoprotein, and is shown to have three sites of N-linked glycosylation (410). There are also three *O*-linked Core 1 (Galβ1-3GalNAc) structures on fetuin, two of which carry sialic acid (one carrying Neu5Acβ2-3Gal, and the other carrying both Neu5Acβ2-3Gal and Neu5Acα6-2GalNAc) and one which carries no sialic acid (411). The *O*-glycosylation sites within fetuin are particularly proline-rich, and the sequence Gly-Pro-Ser-Pro-Thr-Ala has been proposed as a site of *O*-glycosylation (412). Another possible candidate for this site in the hexapeptide Gly-Pro-Thr-Pro-Ser-Ala, found at 260-265 in the fetuin sequence. The experiments in this thesis have used asialofetuin, ie bovine fetuin treated with sialidase to remove the most terminal sialic acid, thus revealing subterminal galactose and potentially increasing the level of binding by galectins.

(Asialo) Bovine Submaxillary Mucin:

As previously described, the epithelial linings of the cavities and tracts of higher vertebrates are lined with mucus secretions produced by the epithelial glands (413). These viscoelastic secretions act as a lubricant and as a protective layer against the environment for the underlying epithelium. The bovine submaxillary mucin (BSM) is a component of saliva, which coats the food bolus and acts as a lubricant to reduce friction during swallowing and contributes significantly to the gastric mucus pool (414, 415).

BSM is a glycoprotein with a molecular weight of about $4x10^5$ and contains about 70% carbohydrate (416). Through cDNA cloning, it has been demonstrated that the composite sequence contains 1589 amino acid residues, and consists of five distinct protein domains, which are numbered from the C-terminus. Domains III and V consist of similar repeated peptide sequences with an average of 47 residues, which are rich in serine and threonine. Domains II and IV do not contain these tandem repeat sequences, but do have a high levels of serine and threonine, which are potential *O*-glycosylation sites (417). The amount of carbohydrate chains per molecule has not been characterized, but BSM carries a variety of *O*-glycans, which are sialic acid-rich. BSM can carry any of five *O*-glycan trisaccharides that are based on the core 1, core 2 and core 5 structures (418). As with bovine fetuin, the experiments in this thesis have used asialo bovine (submaxillary) mucin ie bovine submaxillary mucin treated with sialidase to remove the most terminal sialic acids.

The work described in this chapter first examined the interaction of members of the galectin family with TF-expressing glycans and then moved on to assess effect of these galectins on cancer cell-cell interactions.

6.4 METHODS

6.4.1 Materials

Recombinant galectins and anti-galectin antibodies were obtained from R&D Systems, Abingdon, UK. 96-well and 24-well plates were obtained from Sigma-Aldrich Ltd., UK. Lactose was obtained from Sigma-Aldrich Ltd., UK. Non-enzymatic cell dissociation solution was obtained from Sigma-Aldrich Ltd., UK. Matrigel was obtained from BD Biosciences, UK. *O*-glycanase was obtained from Glyko Inc., (Oxford, UK). The Vybrant DIO cell labeling solution was from Molecular Probes (Eugene, OR). Paraformaldehyde was obtained from TAAB Ltd., Berks, England. Peanut agglutinin (PNA) and anti-mouse-texas-red-conjugated antibody were obtained from Dako UK Ltd., Cambridgeshire, UK. MUC1-targeted siRNA, scrambled control siRNA and siRNA transfectant reagents were obtained from Thermo Scientific (Dharmacon), CO, USA. Anti-MUC1 (B27.29) antibodies were kindly provided by Dr. Mark Reddish (Biomira Inc., Edmonton, Canada). Human anti-TF antibody (TF5) was kindly provided by Dr. Bo Jansson (BioInvent Therapeutic, Lund, Sweden).

6.4.2. ELISA

ELISA was performed as described in **4.3.**

6.4.3 *O*-glycanase Treatments

O-glycanase treatments were carried out, as described below, on both ASM and surface proteins on HT29-5F7 colon cancer cells to remove the terminal TF antigen as an intact disaccharide unit from serine or threonine within proteins carrying it. The TF antigen is a potential binding partner of the galectins under investigation, and it has been shown that, through this structure, galectin-3 interacts with cancer-associated MUC1.

_O_-glycanase Treatment of ASM:

The procedure for de-*O*-glycosylation of ASM was as follows:

15μl of 200μg/ml ASM was incubated with 6μl reaction buffer and 4μl of *O*-glycanase (0.003U/ml) for 2hrs at 37°C in a rotating water bath at 100rpm. Following *O*-glycanase treatment, ASM was diluted to 10μg/ml with ELISA coating buffer and 50μl/well of this solution was used to coat the bottom of wells within a half-volume 96-well high binding plate overnight at 4°C.

Treated ASM and control ASM were used in two ELISAs, one using PNA to detect the level of TF after incubation to determine successful treatment, and another to determine the level of galectin binding.

_O_-glycanase treatment of HT29-5F7 Colon Cancer Cells:

The procedure for de-_O_-glycosylation of the cell-surface proteins of HT29-5F7 cells was as follows:

HT29-5F7 cells were grown as a monolayer in complete DMEM at 37°C in a humidified atmosphere of 5% CO_2, 95% air. Once they reached 70% confluence HT29-5F7 cells were released from T25 cm^2 flasks with 2ml non-enzymatic cell dissociation solution. Once released, the cells were centrifuged at 1000rpm for 5mins and resuspended into an aliquot of 1 x 10^5 in an eppindorff and centrifuged again at 3000rpm. The pellet was resuspended with 100µl of _O_-glycanase incubation buffer. This aliquot was divided into two, and one used as an untreated control. The aliquot to undergo _O_-glycanase treatment was incubated with 0.02U/ml (0.8µl) _O_-glycanase for 2hrs at 37°C in a shaking water bath at 100rpm before being used for adhesion assays, and for FACS analysis of TF expressions.

FACS Assay:

Sub-confluent HT29-5F7 cells were released from the flask by incubation with 2ml non-enzymatic cell dissociation solution for 5–10mins at 37°C, and washed twice with PBS 10ml each time. HT29-5F7 cells (1×10^6/ml in _O_-Glycanase 1×buffer) were incubated with 0.02U/ml _O_-glycanase in _O_-Glycanase 1×buffer for 3 hrs at 37°C. Cells were then centrifuged at 1500rpm for 5mins. After removal of the supernatant, 5ml 2% paraformaldehyde was added to fix the cells for 15mins at room temperature. After washing the cells twice with 10ml PBS and centrifugation at 1500rpm to remove the supernatant, the cells (10^6/ml) were incubated with 5% goat serum for

30mins at room temperature on a rolling platform. After removal of the supernatant following centrifugation at 1500rpm famines, the cells were re-suspended into 10^6/ml in 1% goat serum in PBS and divided 1ml/tube in 1.5ml tubes. Fluorescent-conjugated peanut lectin was purchased from Vector Laboratories Ltd (Peterborough, UK), and 1:400 working concentration was applied to the cells solution for 1hr at room temperature on the rolling platform. After three washes with PBS, the cells were re-suspended in PBS in 0.5ml/tube. The cells was analysed by flow cytometry.

6.4.4 siRNA MUC1

Cell Plating:

HT29-5F7 colon cancer cells were diluted in antibiotic-free DMEM with 5% FCS to a plating density of 5 x 10^4 cells/ml. 100µl of cells were placed in to each well of a 96 well cell culture plate and incubated for 24hrs at 37°C.

Transfection:

The following protocol was followed:

A 2µM siRNA solution in siRNA buffer (Dharmacon) was prepared, from this 17.5µl was added to 17.5µl serum-free/antibiotic-free medium. In a separate tube, 1.4µl of DharmaFECT4 (Dharmacon) was added to 33.6µl of serum-free/antibiotic-free DMEM. These tubes were pipetted up and down gently and left for 5mins before mixing them together and leaving them to stand for a further 20mins. To this solution 280µl antibiotic-free DMEM was added and to each well 100µl was added. The plate

was incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air for 72hrs. The experiment was performed in triplicate.

Western Blotting:

Wells containing cells were washed twice and treated with 80µl lysis buffer for 15mins at room temperature. Cellular lysate was transferred to eppindorffs and diluted with lysis buffer 1:1, and either frozen at -18°C for short-term storage or loaded on 4% or 12% gels for SDS-PAGE (as described in **4.6**) following boiling at 100°C for 5mins. Immunoblotting was carried out as described in **4.7**.

6.4.5 Immunocytochemistry

MUC1-transfected breast cancer cells, HCA1.7+, were grown as a monolayer in complete DMEM at 37°C in a humidified atmosphere of 5% CO_2, 95% air. Once they reached 70% confluence HCA1.7+ cells were released from T25 cm^2 flasks with 2ml non-enzymatic cell dissociation solution. Once cells were detached into a single cell solution by pipetting up and down, cells were centrifuged in serum-free DMEM, and resuspended into 5 x 10^5 cells/ml. 1ml of cell suspension was incubated in the presence or absence of 1µg/ml or recombinant galectins or 4µg/ml PNA in a shaking water bath at 100rpm at 37°C for 1hr. Two 1cm circles were drawn onto a poly lysine slide with a thin black marker pen, within which 100µl cell suspension was pipetted and incubated for 30mins at room temperature. Following the gentle removal of the medium on the slides, 150µl 2% paraformaldehyde was pipetted onto the cells for 15mins before washing twice with PBS. 100µl 5% goat serum in PBS as a blocking

buffer was pipetted onto the cells for 30mins at room temperature. Anti-MUC1 B27.29 antibody was diluted 1:2000 with 1% goat serum and 100μl of the antibody was applied to each circle for 1hr at room temperature. The slides were then washed three times with PBS before the application of Texas red-conjugated secondary anti-mouse antibody, diluted 1:400 in 1% goat serum for 1hr at room temperature. Slides were then washed three times with PBS, and a drop of mounting medium applied to each circle before covering with cover slides. The slides were then blinded and analysed under the fluorescent microscope. Cells were counted for MUC1 in a continuous ring around the cell surface, and for MUC1 in a discontinuous ring around the cell surface. 500 cells were counted for each sample set, which were randomly selected under the lower power field 15X. Images were taken at the higher power field, 25X.

6.5 RESULTS

6.5.1 Galectins bind to O-glycan expressing glycoproteins.

Initial experiments were carried out to compare the carbohydrate recognition features of other galectins in comparison to galectin-3, which has already been well characterised by previous work in this group (316). The level of galectin binding was analysed using three distinct glycoproteins, antifreeze glycoprotein, expressing only TF antigen, asialofetuin, expressing TF as its only *O*-glycan structure but bearing a variety of *N*-linked structures, and asialo bovine mucin (ASM), which expresses a variety of *O*-glycan structures.

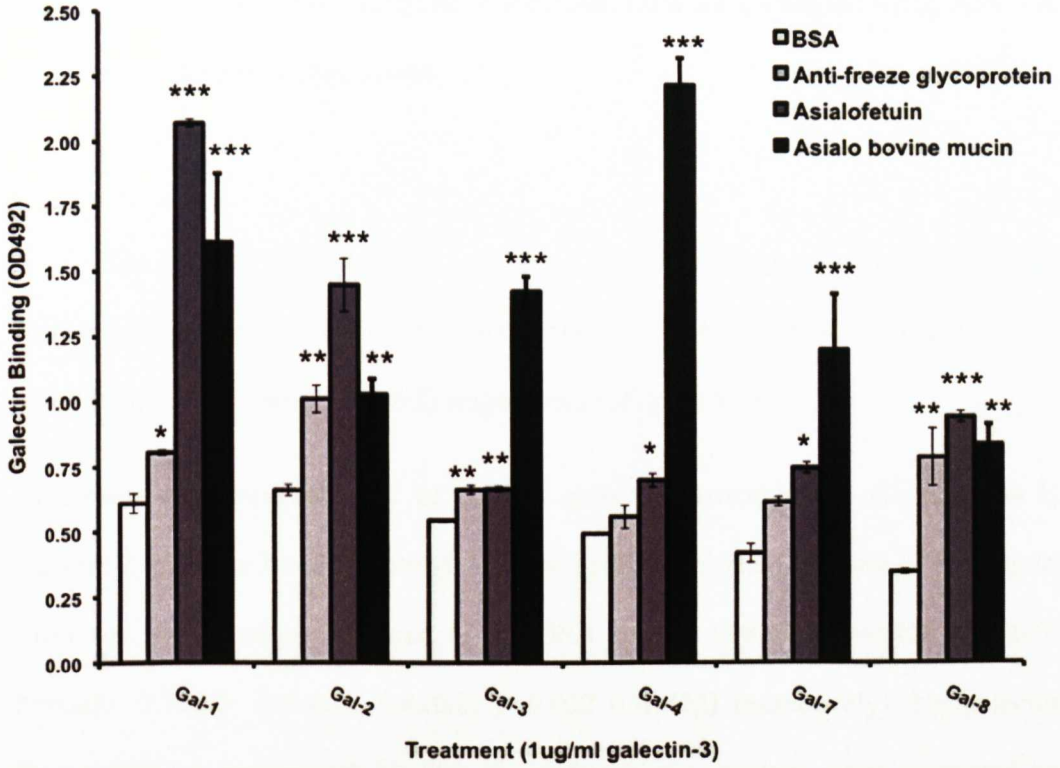


Figure 6.1. Level of galectin binding to TF-expressing glycoproteins. (* P<0.001, ** P<0.01, * P<0.05).**

ELISA was used to detect the level of galectin binding to target glycoproteins. ELISA was performed in a half-volume 96-well plate. Each well was coated with 50μl of 10μg/ml ASM, anti-freeze glycoprotein or asialofetuin. **BSA:** Bovine serum albumin, a control well with BSA as a coating substance. Galectins do not bind to BSA as it is not a glycoprotein, and therefore serves well to identify any abnormal background readings within the experiment. Galectin-3 alone was applied to this well. **ASM:** Asialo bovine mucin, bovine submaxillary mucin that has had all sialic acid residues removed. **Antifreeze glycoprotein:** A glycoprotein bearing only TF antigen. **Asialofetuin:** Fetuin that has had all sialic acid residues removed. Asialofetuin expresses TF antigen and *N*-linked moieties. Experiments were performed in

duplicate with each sample analysed in triplicate. Data were analysed using ANOVA followed by Dunnett's comparison.

The level of galectin-1, -2, -3, -4, -7 and -8 binding was compared to the corresponding galectin binding in wells containing BSA (0.6±0.1, 0.7±0.0, 0.5±0.0, 0.5±0.0, 0.4±0.1, 0.3±0.0 (OD492) respectively) (**Figure 6.1**).

Antifreeze glycoprotein: Out of the six galectins investigated, only galectin-1, galectin-2, galectin-3 and galectin-8 demonstrated a significant increase of binding to antifreeze glycoprotein compared to the BSA control (0.8±0.0; P=0.008, 1.0±0.0; P=0.009, 0.7±0.0; P=0.005, 0.8±0.1; P=0.002 (OD492) respectively). No galectin demonstrated a preferential binding to antifreeze glycoprotein when compared to asialofetuin or ASM.

Asialofetuin: All galectins demonstrated binding to asialofetuin, but with a stronger recognition than that seen in wells coated with antifreeze glycoprotein. Galectin-1 and -2 demonstrated the most preferential binding to asialofetuin out of all the glycoproteins analysed (2.1±0.0; P<0.0001, 1.5±0.2; P<0.0001 (OD492) respectively). Galectin-3, however, demonstrated the same level of binding to asialofetuin as to antifreeze glycoprotein (0.7±0.0; (OD492) P=0.004). Galectin-8 bound significantly to asialofetuin (0.9±0.0; (OD492) P=0.0003), less so than galectin-1, -2, and -3, but more so than galectin-4 (0.7±0.0; (OD492) P=0.016).

Asialo Bovine Mucin: All galectins demonstrated binding to ASM. Galectin-1 demonstrated a significant level of binding to ASM (1.6±0.3; (OD492) P<0.0001), when compared to BSA control wells however this level of binding was less

significant that that seen with asialofetuin. Furthermore, galectin-2 also demonstrated a significant level of binding to ASM (1.0±0.1; (OD492) P=0.007), but less significant that that seen with asialofetuin. Galectin-3, -4 and -7 bound to ASM with the most significance when compared to the other glycoproteins (1.4±0.1; (OD492) P<0.0001, 2.2±0.1; (OD492) P<0.0001, 1.2±0.2; (OD492) P<0.0001 respectively). Galectin-8 demonstrated a significant level of binding when compared to BSA and antifreeze glycoprotein (0.8±0.1; (OD492) P=0.001), but less so than asialofetuin.

Considering the preferential binding towards asialofetuin and asialo bovine mucin over that of antifreeze glycoprotein, it is apparent that galectins develop a stronger affinity for targets with a more complex complement of carbohydrates on their surface. Whereas antifreeze protein only expresses the TF antigen, the non-complex core 1 structure, asialofetuin and asialobovine structure either possess a variety of *O*-glycan structures, or TF antigen with expression of *N*-glycan moieties. This result would indicate that different galectins show different binding affinities to TF-expressing glycans in solid phase. Further experiments were carried out to assess the importance of the TF structure on these glycans on their interaction with galectins. Experiments were carried out with ASM, which demonstrated a significant level of galectin-binding across all members of the family assessed.

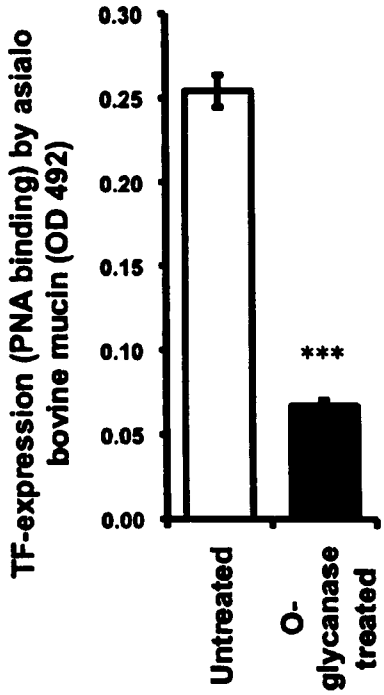


Figure 6.2a. TF-expression on ASM with or without pre-treatment with *O*-glycanase. (* P<0.001, ** P<0.01, * P<0.05).**

Following the treatment of ASM with 4μl Streptococcal *O*-glycanase, specific for removal of unsubstituted TF, ELISA was used to detect the level of TF on the surface of the glycoprotein. Biotinylated PNA (diluted 1:3000) was used to detect the TF antigen. ELISA was performed in a high binding 96-well plate. Each well was coated with 100μl of 10μg/ml treated or untreated ASM. Samples were analysed in triplicate. Data waw analysed by ANOVA followed by Dunnett's comparison.

ELISA confirmed that ASM treatment with *O*-glycanase markedly reduced the quantity of TF on ASM. The level of PNA binding was significantly reduced in *O*-glycanase treated ASM samples (73.8±1.4% reduction), when compared to the untreated controls. (0.07±0.0, 0.2±0.0, respectively, (OD492) P<0.0001) **(Figure 6.2a)**.

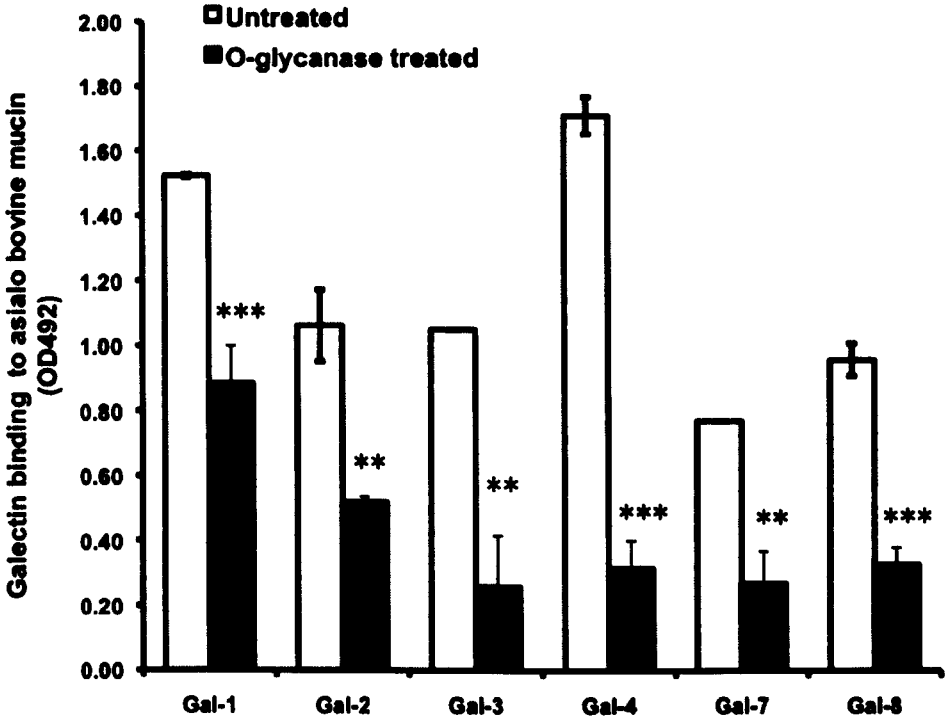


Figure 6.2b. Level of galectin binding to asialo bovine mucin with or without pre-treatment with TF-specific Streptococcal *O*-glycanase. (* P<0.001, ** P<0.01, * P<0.05).**

ELISA was used to detect the level of galectin binding to ASM or ASM treated with *O*-glycanase. ELISA was performed in a half-volume 96-well plate. Each well was coated with 50μl of 10μg/ml ASM or *O*-glycanase treatment. 1μg/ml of recombinant galectin was incubated within each well, and the level of galectin binding measured by OD492, which corresponded to the amount of biotinylated anti-galectin antibody. Experiments were performed in duplicate, with each sample analysed in triplicate. Data were analysed by ANOVA followed by Dunnett's comparison.

O-glycanase treatment of ASM significantly reduced the binding of all galectins (0.9±0.1; P=0.0007, 0.5±0.0; P=0.001, 0.3±0.2; P=0.001, 0.3±0.1; P<0.0001, 0.3±0.0; P=0.001, 0.3±0.1; P=0.0001 (OD492) for galectin-1, -2, -3, -4, -7 and -8 respectively) when compared to controls (those not treated with *O*-glycanase) (1.5±0.0, 1.1±0.1, 1.1±0.0, 1.7±0.1, 0.8±0.0, 1.0±0.1 (OD492) for galectin-1, -2, -3, -4, -7 and -8 respectively) (**Figure 6.2b**). Therefore, *O*-glycanase treatment of ASM provided a reduction in binding of 42.0%, 51.3%, 75.5%, 81.5%, 65.11% and 65.8% for galectin-1, -2, -3, -4, -7 and -8 respectively. This suggests that members of the galectin family recognize the TF structure, and that it acts as a major binding target for these glycans. Previous work done within this group has shown that through binding to TF antigen on cancer-associated MUC1, galectin-3 can induce a variety of cellular mechanisms, including heterotypic adhesion and homotypic aggregation (316, 382).

6.5.2 Galectins induce an increase in MUC1-expressing cancer cell adhesion to endothelial cell monolayers and extracellular matrix components (Matrigel).

Considering that all the galectin members under investigation within this thesis recognise TF antigen, experiments were undertaken to see if these galectin family members could also affect cancer cell-cell interactions in the same way as galectin-3.

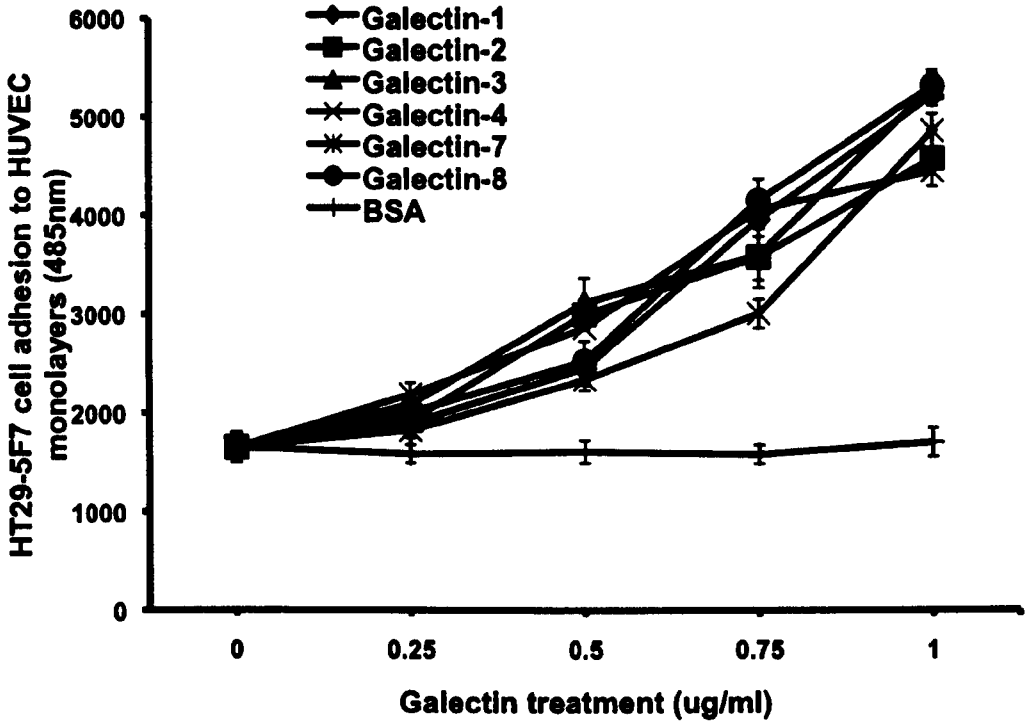


Figure 6.3. Level of adhesion of HT29-5F7 cells to HUVEC monolayers with or without pre-incubation with galectins. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays were performed in a 96-well cell culture plate, with each well containing a continuous HUVEC monolayer. **BSA**: Bovine serum albumin represents the negative control wells, with no recombinant galectin pre-incubation, to give a basal level of HT29-5F7 cell binding to HUVEC monolayers. **Galectin treatments:** HT29-5F7 colon cancer cells were pre-incubated with a variety of recombinant galectins, at a range of concentrations for 1hr at 37°C, before incubating with HUVEC monolayers for another 1hr at 37°C. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis used was ANOVA followed by Dunnett's comparison.

All galectins were used at a concentration of 1μg/ml. For galectin-1, -2 and -3, this concentration fell within the range of serum concentration in patients with

colorectal cancer and liver metastasis. For galectin-4 and -8, this concentration exceeded the maximum concentration in the sera of the same patients, but was used for comparison throughout all experiments within this thesis, unless otherwise stated. All galectins induced an increase in HT29-5F7 colon cancer cell adhesion to HUVEC monolayers. Furthermore, all galectins induced an increase in cellular adhesion in a dose response way. 1ug/ml galectin-1, -2, -3, -4, -7 and -8 were shown to produce adhesion levels of 5213.2±106.2; P<0.0001, 4577.3±287.5; P<0.0001, 5295.8±148.3; P<0.0001, 4446.5±152.5; P<0.0001, 4857.8±168.7; P<0.0001, 5310.8±165.7; P<0.0001 (485nm) respectively, which corresponded to a 315.5±6.4%, 277.0±17.3%, 320.5±9%, 269.1±9.2%, 294.0±10.2% and 321.4±10.0% increase in adhesion when compared to the untreated control (**Figure 6.3**).

6.5.3 Inhibition of galectin-mediated adhesion by lactose and ASM.

In order to assess whether the galectin-mediated adhesion is mediated by the carbohydrate recognition ability of the galectins, an inhibition assay was performed.

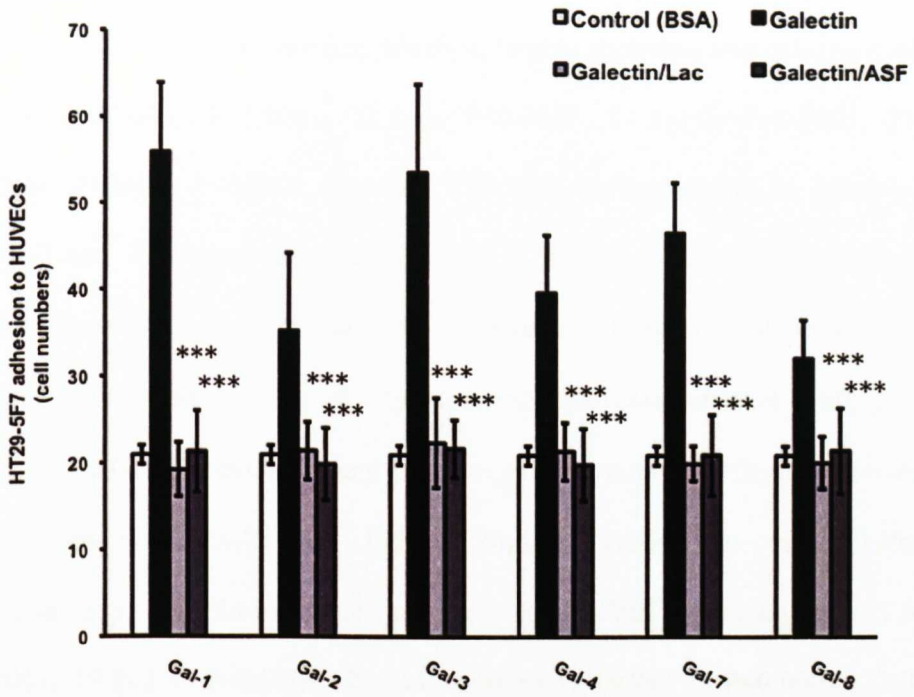


Figure 6.4. Level of adhesion of HT29-5F7 cells to HUVEC monolayers with or without treatment of galectins that have been pre-incubated with lactose or asialo bovine mucin. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 24-well cell culture plate, with each well containing a sterile slide on which a continuous HUVEC monolayer was grown. **Lac:** 10mM Lactose, **ASF:** 20μg/ml asialofetuin. Experiments were performed in duplicate with each sample analysed in duplicate. Analysis used was ANOVA followed by Dunnett's comparison.

As previously demonstrated, recombinant galectins-1, -2, -3, -4, -7 and -8 induced a significant increase in HT29-5F7 adhesion to HUVEC monolayers (55.9±2.5; P<0.0001, 35.2±4.8; P<0.0001, 53.4±3.2; P<0.0001, 49.6±2.0; P<0.0001, 46.5±1.8; P<0.0001, 32.1±1.4; P<0.0001 (cell numbers), respectively). To assess the effect of galectin inhibition on cellular adhesion, recombinant galectins were pre-

incubated with either 10mM lactose, or 20μg/ml asialofetuin for 1hr at 37°C. Lactose, a well-established galectin inhibitor, largely abolished any galectin-mediated adhesion (19.2±0.9; P<0.0001, 21.4±1; P<0.0001, 22.3±1.6; P<0.0001, 21.4±1; P<0.0001, 20.0±0.6; P<0.0001, 20.1±0.9; P<0.0001, (cell numbers) for galectin-1, -2, -3, -4, -7 and -8 respectively (**Figure 6.4**). In previous experiments, recombinant galectins demonstrated a significant level of binding to 10μg/ml asialofetuin, although some galectins bound with less affinity, so an increased concentration of 20μg/ml was utilised to analyse the extent of any possible galectin inhibition from pre-incubating the galectins with asialofetuin. Indeed, 20μg/ml asialofetuin was effective in abolishing any galectin-mediated adhesion to HUVEC monolayers (21.3±1.5; P<0.0001, 19.9±1.3; P<0.0001, 22.3±1; P<0.0001, 19.9±1.3; P<0.0001, 21.0±1.5; P<0.0001, 21.5±1.6; P<0.0001 (cell numbers) for galectin-1, -2, -3, -4, -7 and -8 respectively).

Having revealed that galectins increased the adhesion of MUC1-bearing HT29-5F7 colorectal cancer cells to endothelial monolayers, further experiments were undertaken to determine if they also affect cancer cell adhesion to extracellular matrix components. A thin layer of Matrigel was utilised to form an ECM-like binding surface containing the necessary components similar to those seen *in vivo*.

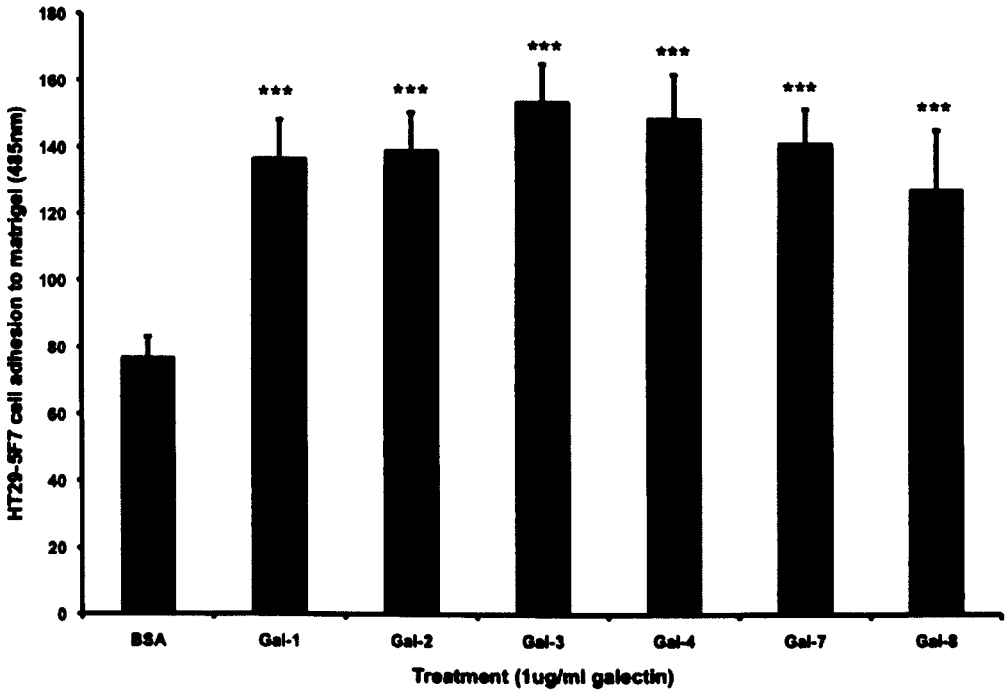


Figure 6.5. Level of adhesion of HT29-5F7 cells to Matrigel with or without pre-incubation with galectins. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a thin layer of Matrigel. 1μg/ml recombinant galectins were incubated with calcein AM-labelled HT29-5F7 cells for 1hr at 37°C, before incubation with Matrigel layers for a further hour at 37°C. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis used was ANOVA followed by Dunnett's comparison.

All galectins, as with endothelial cell monolayers, induced a significant increase in adhesion of MUC1-expressing HT29-5F7 cancer cells to Matrigel layers (136.7±11.6; P<0.0001, 139.7±11.2; P<0.0001, 153.7±11.3; P<0.0001, 148.7±13.1; P<0.0001, 141.7±10.1; P<0.0001, 127.7±18.0; P<0.0001 (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively) (**Figure 6.5**). This corresponded with an increase of

177.2±15.0%, 180.4±14.6%, 199.2±14.7%, 192.8±17.0%, 183.3±13.2% and 165.2±23.4% for galectin-1, -2, -3, -4, -7 and -8, respectively, with no galectin showing a significantly higher ability to induce adhesion more than another when compared to untreated controls (77.7±6.3).

The effect of galectins on cancer cell adhesion to Matrigel was also investigated with another human colon cancer cell line, SW620, which has also been shown to express MUC1.

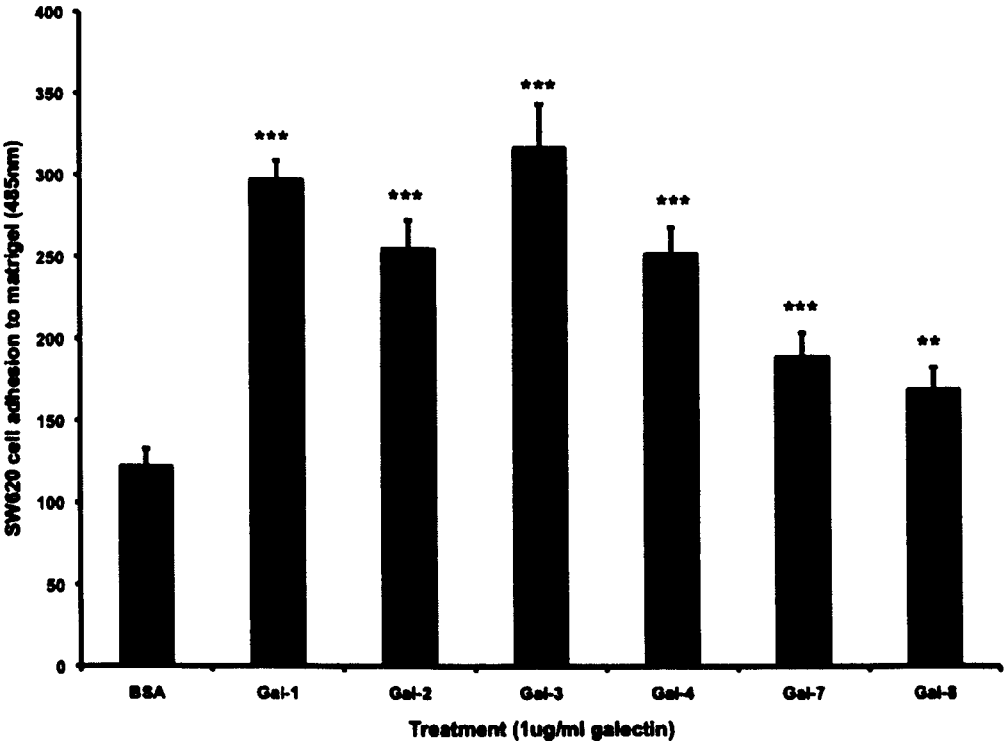


Figure 6.6. Level of adhesion of SW620 cells to Matrigel with or without pre-incubation with galectins. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a thin layer of Matrigel. 1μg/ml recombinant galectins were incubated with calcein AM-labelled SW620 cells for 1hr at 37°C, before incubation with Matrigel

layers for a further hour at 37°C. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis was by ANOVA followed by Dunnett's comparison.

While all galectins induced a significant increase of SW620 colorectal cancer cell adhesion to Matrigel layers, galectin-1, -2, -3, -4 and -7 demonstrated the most significant increase (297.7±11.6; $P<0.0001$, 255.7±17.1; $P<0.0001$, 317.7±26.3; $P<0.0001$, 252.7±16.6; $P<0.0001$, 189.7±14; $P<0.0001$ (485nm) respectively). Galectin-8 also induced an increase in SW620 adhesion, but at a comparatively lower significant effect (169.7±13.2; $P=0.003$, respectively) when compared to untreated controls (122±10.6) (**Figure 6.6**). This corresponded with an increase of 243.6±9.5%, 209.2±14.0%, 260.0±21.6%, 206.5±13.2%, 155.1±11.5% and 138.5±10.8% for galectin-1, -2, -3, -4, -7 and -8, respectively.

The lectin peanut agglutinin (PNA) has been shown to bind specifically to TF antigen (419, 420). Further experiments were undertaken to see if the presence of PNA also inhibit cancer cell adhesion as the galectins.

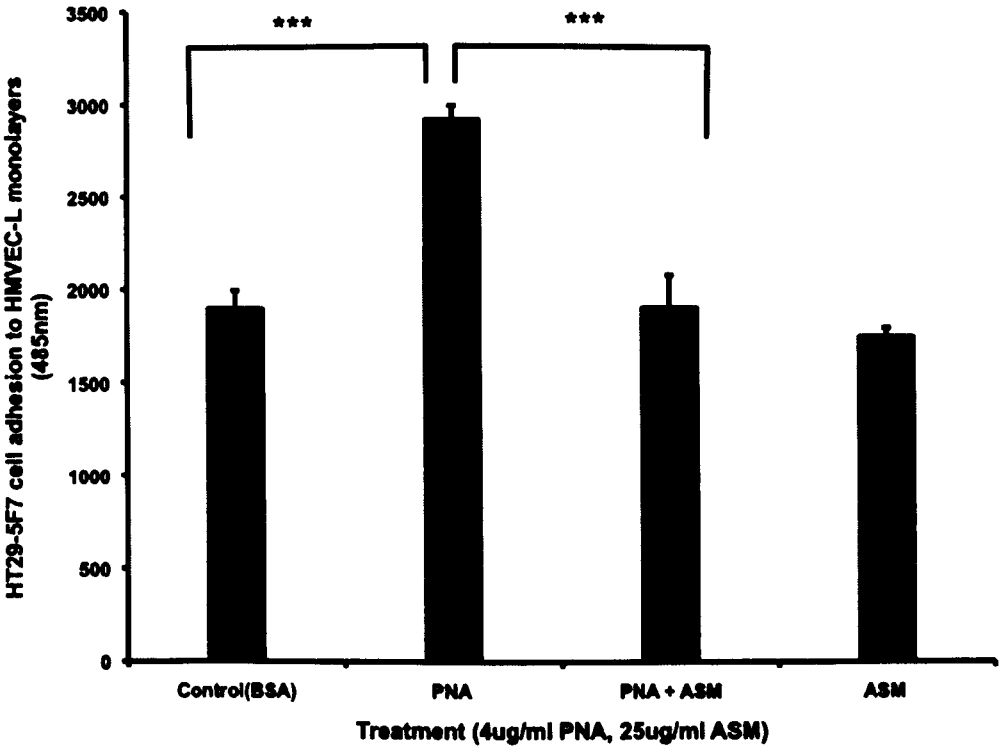


Figure 6.7. Effect of the presence of PNA on HT29-5F7 cell adhesion to HMVEC-L monolayers. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a HMVEC-L monolayer. 4μg/ml PNA were incubated with HT29-5F7 cells pre-labelled with calcein AM in the presence or absence of 25ug/ml ASM- for 1hr at 37°C, before incubation with HMVEC-L monolayers for a further hour at 37°C. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis used was ANOVA followed by Dunnett's comparison.

The presence of 4μg/ml PNA induced a significant increase in HT29-5F7 adhesion to HMVEC-L monolayers (2918.0±76.6; (485nm) P<0.0001) when compared to untreated controls (1897.3±95.0 (485nm)), which corresponded to an

increase of 153.8±4.0%. This PNA-mediated effect was abolished by the pre-incubation of PNA with ASM (1904.2±173.0; (485nm) P=0.999) (**Figure 6.7**).

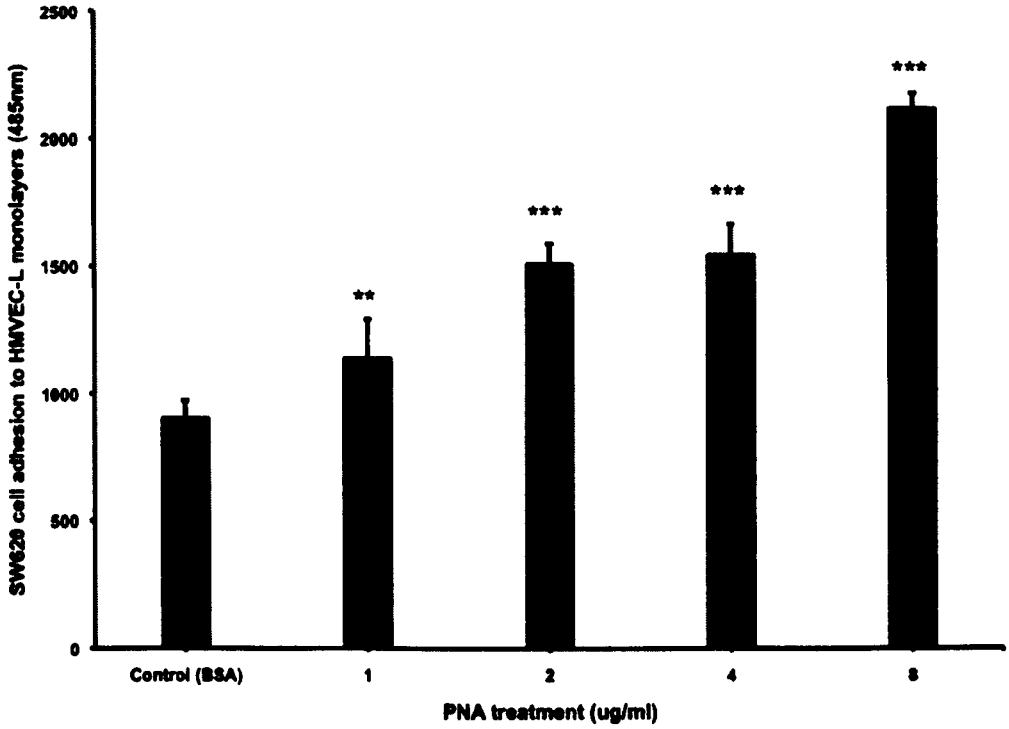


Figure 6.8. Effect of PNA on SW620 cell adhesion to HMVEC-L monolayers. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a HMVEC-L monolayer. Calcein AM-labelled SW620 colon cancer cells were pre-incubated with PNA at a range of concentrations for 1hr at 37°C, before incubating with HUVEC monolayers for another 1hr at 37°C. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis was by ANOVA followed by Dunnett's comparison.

PNA induced a dose-dependent increase in SW620 colon cancer cell adhesion to HUVEC monolayers. At 8ug/ml it produced 235.6±6.8% increase of the cell adhesion to HMVEC-L monolayers; $P<0.0001$) when compared to untreated controls (899.8±73.5 (485nm) (**Figure 6.8**).

6.5.4 Pre-fixation of the cells negates galectin-mediated cellular adhesion.

Previous work carried out within the group has shown that the mobility of the cancer-associated MUC1 is of pivotal importance in galectin-3-mediated cancer cell adhesion (315). It was shown that galectin-3 interacts with the TF antigens on the transmembrane MUC1, and that the galectin-3-TF/MUC1 interaction induces polarisation of MUC1 cell surface localization and exposure of the smaller cell adhesion molecules, which otherwise are concealed by MUC1. Having shown here that several galectin members all increased adhesion of MUC1 expressing HT29-5F7 cancer cells to HUVEC monolayers, further experiments were carried out to investigate whether the mobility of the MUC1 cell surface localization is also involved in galectin-mediated cell adhesion. Following cell labelling with the fluorescent label DiO, cellular proteins were fixed with 2% paraformaldehyde for 15mins, before treatment with 1μg/ml recombinant galectin for 1hr and subsequent application to the HUVEC monolayers

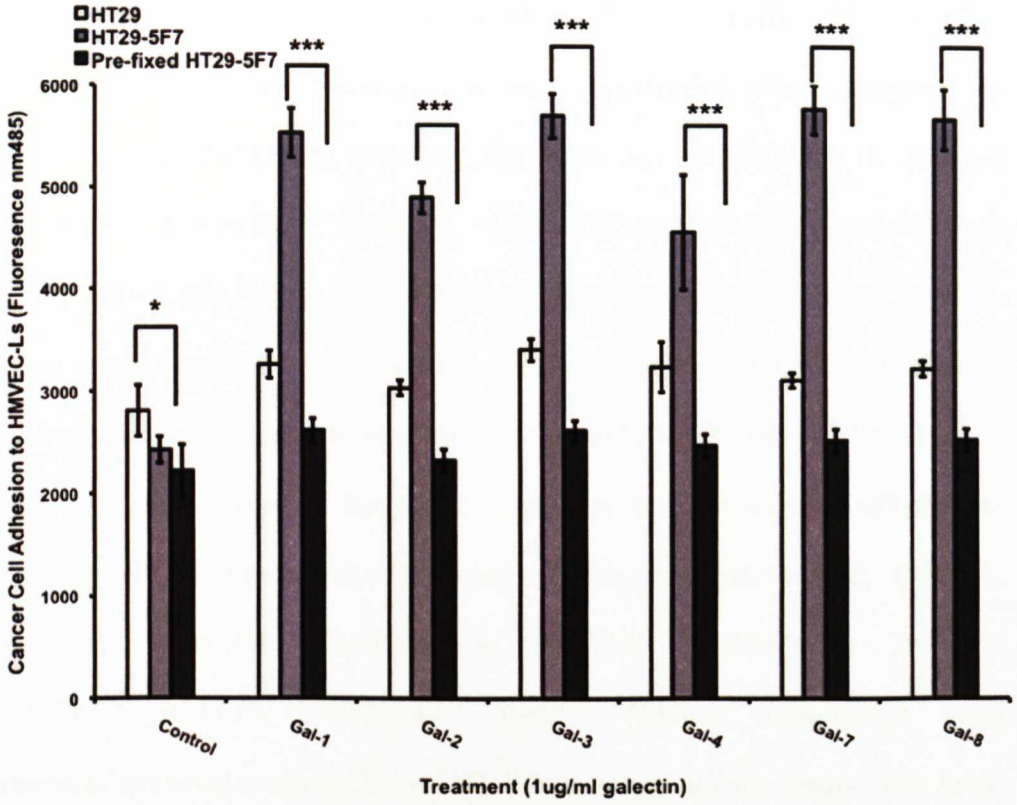


Figure 6.9. Level of adhesion of HT29, HT29-5F7 and pre-fixed HT29-5F7 cells to HMVEC-L monolayers with or without pre-incubation with galectins. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a HMVEC-L monolayer. Calcein AM-labelled colon cancer cells were pre-incubated with recombinant galectins at a range of concentrations for 1hr at 37°C, before incubating with HMVEC-L monolayers for another 1hr at 37°C. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis used was ANOVA followed by Dunnett's comparison.

It was found that treatment of the cells with 1μg/ml recombinant galectin-1, -2, -3. -4, -7 and -8 induced a significant increase in HT29-5F7 colon cancer cell adhesion to HMVEC-L monolayers (5524.5±586.6; P<0.0001, 4887.0±370.6;

P<0.0001, 5690.8±524.6; P<0.0001, 4554.0±1376; P<0.0001, 5755.8±587.8; P<0.0001, 5659.0±718.1; P<0.0001 (485nm) respectively) when compared to untreated controls (2425±307). However, this effect was not seen with the parental HT29 cells (3256.8±326.4; P=0.195, 3028.3±183.6; P=0.810, 3399.0±264.6; P=0.049, 3234.2±604.3; P=0.234, 3104.8±178.0; P=0.576, 3218.2±181.5; P=0.266 (485nm) respectively) when compared to untreated controls (2805±616 (485nm)), which express a much lower level of MUC1 than HT29-5F7 cells or SW620 colon cancer cells. Furthermore, all the galectin members failed to increase adhesion of HT29-5F7 cells prefixed with 2% paraformaldehyde (2616.5±281.2; P=0.051, 2324.8±259.1; P=0.956; 2613.2±241.9; P=0.055; 2470.0±265.0; P=0.365, 2516.5±263.3; P=0.213, 2528.2±249.7; P=0.184 (485nm) respectively) when compared to untreated controls (2223±616). When compared with control cells, those that did not undergo galectin treatment, the basal level of HT29 cells was the highest, followed by HT29-5F7 cells, and pre-fixed HT29-5F7 cells demonstrated the lowest basal level of adhesion to HMVEC-L monolayers (**Figure 6.9**).

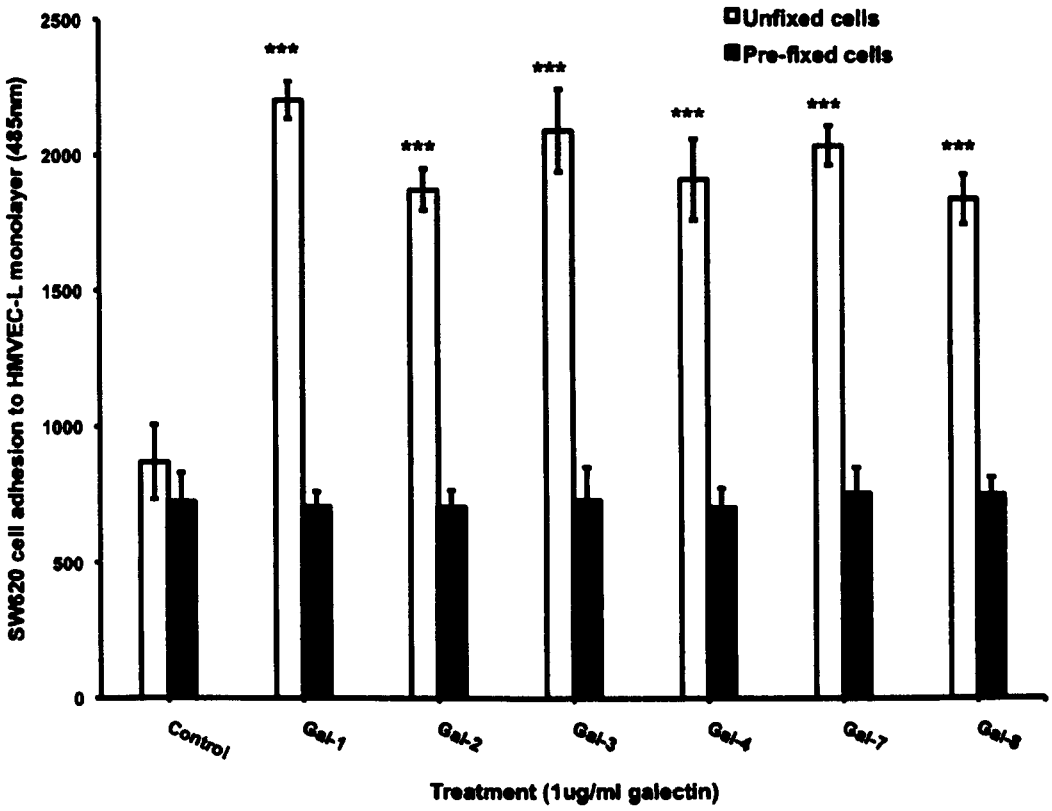


Figure 6.10. Adhesion of untreated or pre-fixed SW620 cells to HMVEC-L monolayers with or without pre-incubation with galectins. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a HMVEC-L monolayer. Calcein AM-labelled SW620 cancer cells were pre-incubated with 1μg/ml recombinant galectin for 1hr at 37°C, before incubating with HMVEC-L monolayers for another 1hr at 37°C. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis was by ANOVA followed by Dunnett's comparison.

Similar experiments were also carried out with SW620 cells. It was found that treatment of the cells with 1μg/ml recombinant galectin induced a significant increase in SW620 colon cancer cell adhesion to HMVEC-L monolayers (Galectin-1,

2201.3±69.1; P<0.0001. Galectin-2, 1869.8±76.6; P<0.0001. Galectin-3, 2086.7±152.2; P<0.0001. Galectin-4, 1907.7±149.7; P<0.0001. Galectin-7, 2032.8±72.6; P<0.0001. Galectin-8, 1834.7±91.6; P<0.0001 (485nm) respectively) when compared to untreated controls (870.1±137.0 (485nm)). As seen with HT29-5F7 cells, pre-fixation of SW620 cells with 2% paraformaldehyde, negated any galectin-mediated adhesion to HMVEC-L monolayers (705.5±54.0; P=0.998, 705.7±59.9; P=0.998, 729.7±122.0; P>0.999, 702.7±69.7; P=0.995, 751.8±97.0; P=0.985, 748.8±62.2; P=0.992 (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively) when compared to untreated controls (724.5±106.6 (485nm)) (**Figure 6.10**).

These results suggest that movement of the cell surface molecules is of critical importance in galectin-mediated cell adhesion. HT29 cells have a low level of expression of MUC1, so any cell-surface adhesion molecules are already exposed, allowing for cell-cell or cell-matrix adhesion. This explains why HT29 has a significantly higher basal level of adhesion to HMVEC-Ls when compared to pre-fixed HT29-5F7 cells. However, due to the lack of MUC1, galectin are unable to mediate their effects, as there is no target mucin to polarise. Essentially, their mechanism of action has been removed. HT29-5F7 and SW620 cells express a high level of MUC1, which, as previously explained, conceals the cell-surface adhesion molecules preventing a basal level of adhesion as high as that seen in HT29 cells. However, MUC1 has a natural fluidity to its movement, and *in vivo* naturally moves and transiently exposes adhesion molecules. However, when the MUC1 molecule is fixed in place, as seen in this experiment, all movement is negated, and adhesion molecules remain concealed behind the protective shield of the MUC1, preventing any galectin-mediated adhesion.

6.5.5 Interference with MUC1 and MUC1-associated TF negate galectin-mediated cellular adhesion.

Previous experiments have enforced the role of MUC1 and its repolarisation in the galectin-mediated adhesion mechanism. Previous work within this group has demonstrated that galectin-3 binds to MUC1 through the cancer-associated TF antigen (316). Further experiments were undertaken to analyse the importance of the oncofetal TF in the galectin recognition to the MUC1 mucin protein.

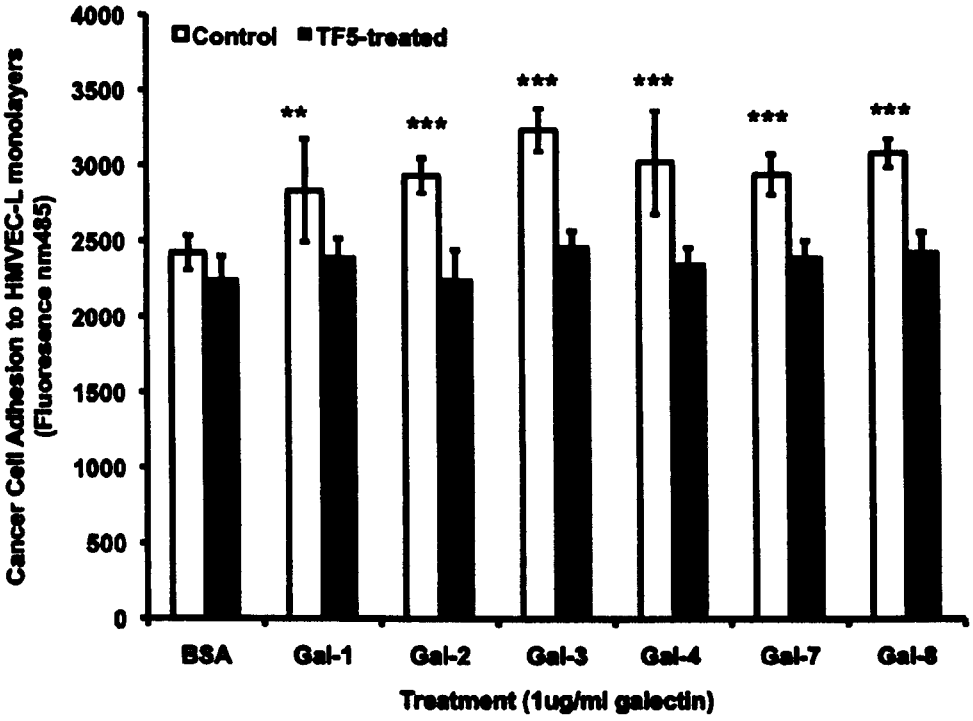


Figure 6.11. Level of adhesion of HT29-5F7 cells to HMVEC-L monolayers following pre-treatment with anti-TF TF5 antibody. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a HMVEC-L monolayer. Calcein AM-labelled HT29-5F7 cancer cells were pre-incubated with 10μg/ml anti-TF TF5 antibody for 1hr at 37°C to conceal any TF structures that are available for galectin-binding. HT29-5F7 cancer cells were pre-incubated with 1μg/ml recombinant galectin for 1hr at 37°C, before incubating with HMVEC-L monolayers for another 1hr at 37°C. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis was by ANOVA followed by Dunnett's comparison.

As previously demonstrated, 1μg/ml recombinant galectin induced a significant increase in HT29-5F7 adhesion to HMVEC-Ls (2835.2±341.5; P=0.0082, 2934.7±114.5; P=0.0008, 3240.2±144.3; P<0.0001, 3024.8±342.4; P=0.0001, 2941.2±134.0; P=0.0007, 3080.7±93.2; P<0.0001 (485nm) respectively, for galectin-1, -2, -3, -4, -7 and -8) when compared to an untreated control (2420.8±112.5). However, the galectin-mediated adhesion was abolished by pre-incubating the cancer cells with anti-TF TF5 antibody (2400.7±117.1; P=0.999, 2253.0±190.8; P=0.109, 2475.0±93.9; P=0.938, 2356.3±102.2; P=0.876, 2401.0±102.0; P=0.999, 2433.3±122.9; P>0.999 (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively) (**Figure 6.11**).

The MUC1 expressing HT29-5F7 colon cancer cells, bear considerable amounts of TF-expressing MUCI. Upon pre-incubation with the anti-TF TF5 antibody, the TF5 binds and conceals the TF antigen, preventing binding of galectins to this ligand.

The following experiments were performed to remove the galectin targets, either by enzymatically cleaving off the TF antigen with *O*-glycanase as used

previously on ASM, or by transiently knocking down MUC1 in its entirety with siRNA. Successful reduction in TF expression was assessed by FACS assay, which was performed by Dr. Qicheng Zhao, University of Liverpool.

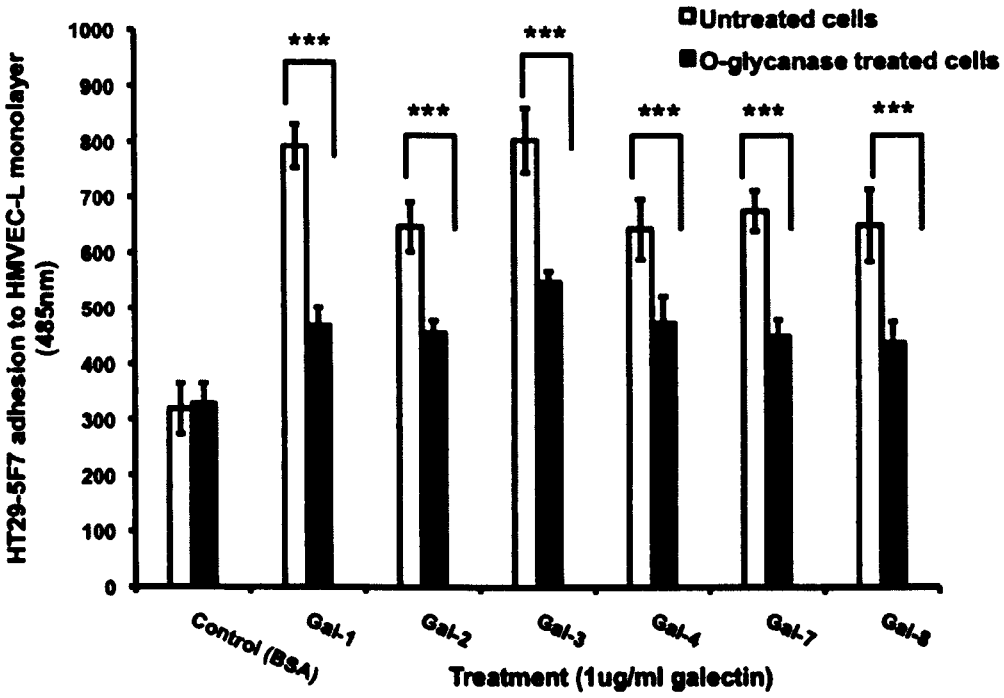


Figure 6.12. HT29-5F7 cell adhesion to HMVEC-L monolayers before and after *O*-glycanase treatment. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a HMVEC-L monolayer. HT29-5F7 colon cancer cells were treated with 0.02U/ml *O*-glycanase. *O*-glycanase treated cells and control cells were labelled with Calcein AM and pre-incubated with 1μg/ml recombinant galectin for 1hr at 37°C, before incubating the cells with HMVEC-L monolayer for a further hour at 37°C. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis used was ANOVA followed by Dunnett's comparison.

As previously demonstrated, 1μg/ml recombinant galectin significantly increases the level of HT29-5F7 colon cancer cell adhesion to HMVEC-L monolayers (791.5±38.9; P<0.0001, 647.0±44.4; P<0.0001, 802.7±57.3; P<0.0001, 643.4±53.5; P<0.0001, 675.9±36.0; P<0.0001, 649.2±64.7; P<0.0001 (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively) when compared to untreated controls (318.7±46.0 (485nm)). The galectin-mediated adhesion seen in untreated cells is of a far higher level than that of the *O*-glycanase treated cells (468.7±32.1; P<0.0001, 456.5±21.7; P<0.0001, 548.5±18.6; P<0.0001, 474.4±48.4; P=0.0002, 450.5±29.2; P<0.0001, 439.0±37.0; P<0.0001 (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively) (**Figure 6.12**). This implies that the TF antigen on cancer cells, specifically the *O*-glycans, is essential in galectin-mediated cancer cell adhesion.

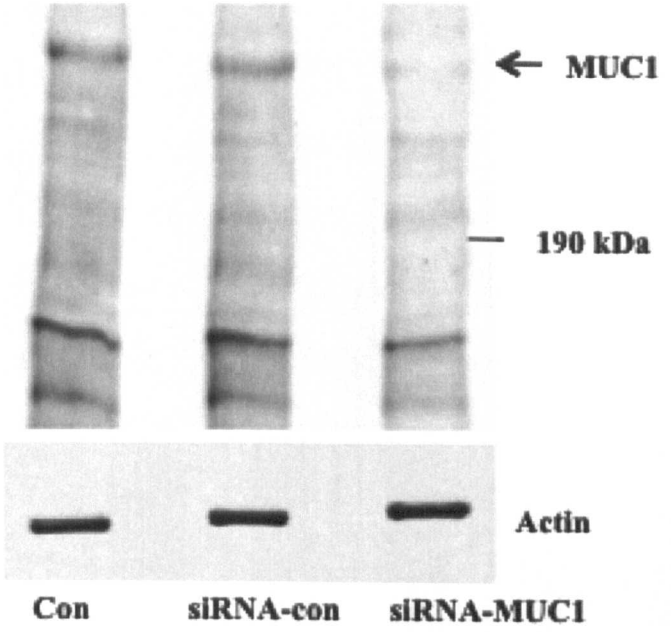


Figure 6.13a. Expression of MUC1 in HT29-5F7 cells with or without MUC1 knockdown with targeted siRNA.

Con: control sample of untreated HT29-5F7 cells. **siRNA-con:** HT29-5F7 cells treated with a scrambled siRNA for 72hrs at 37°C. **siRNA-MUC1:** HT29-5F7 cells treated with siRNA MUC1 for 72hrs at 37°C.

Western blot confirmed that treatment with siRNA MUC1 successfully suppressed MUC1 expression in HT29-5F7 cells. MUC1 was, on average expressed 80% more in untreated HT29-5F7 colon cancer cells than the HT29-5F7 cells treated with siRNA (**Figure 6.13a**).

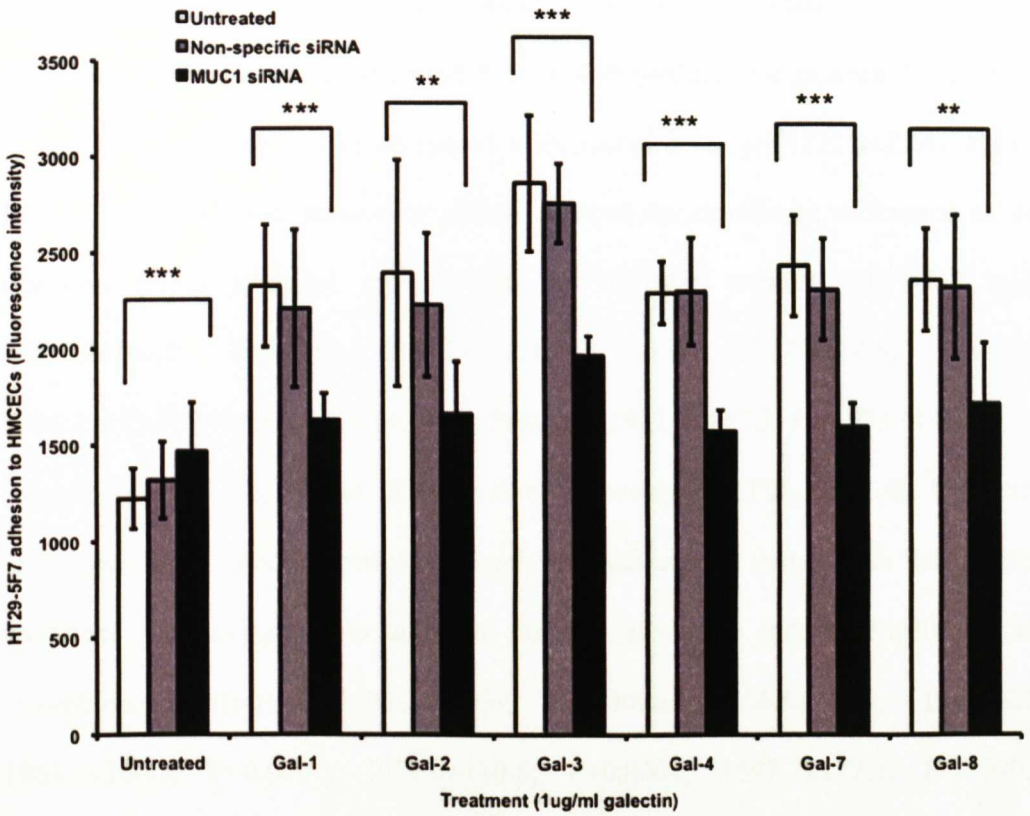


Figure 6.13b. HT29-5F7 cell adhesion to HMVEC-L monolayers with or without MUC1 siRNA suppression. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays were performed in a 96-well cell culture plate, with each well containing a HMVEC-L monolayer. HT29-5F7 colon cancer cells were treated with control or MUC1 siRNA for 72hrs. The cells were labelled with calcein AM, and incubated with 1μg/ml recombinant galectin for 1hr at 37°C, before application to the HMVEC-L monolayer for a further hour at 37°C. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis was by ANOVA followed by Dunnett's comparison.

Again, 1μg/ml recombinant galectin induced a significant increase in HT29-5F7 colon cancer cell adhesion to HMVEC-L monolayers (2324.8±129.9; P<0.0001,

2392.3±239.7; P<0.0001, 2857.0±144.0; P<0.0001, 2290.0±65.7; P=0.0001, 2431.3±107.3; P<0.0001, 2358.2±108.4; P<0.0001 (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively) when compared to untreated controls (1222.0±27.8). HT29-5F7 cells treated with scrambled siRNA showed no significant difference to the adhesion levels mediated by galectins in untreated control HT29-5F7 cells (2207.7±166.5; P=0.739, 2228.8±151.1; P=0.739, 2754.5±84.8; P=0.692, 2298.2±113.7; P=0.996, 2309.0±107.6; P=0.557, 2322.7±152.3; P=0.973 (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively). However, HT29-5F7 cells that have undergone MUC1 knockdown show much less adhesion in response to the galectin treatment when compared to untreated control cells when incubated with 1μg/ml recombinant galectin (1632.2±135.6; P=0.0006, 1664.5±269.9; P=0.0202, 1964.6±100.3; P=0.0001, 1574.0±110.5; P<0.0001, 1597.7±122.2; P<0.0001, 1719.2±127.7; P=0.0034 (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively). Furthermore, the basal level of untreated HT29-5F7 cell adhesion is significantly lower than that of HT29-5F7 cells that have undergone MUC1 knockdown (1521±45; P<0.0001) (**Figure 6.13b**). This is similar to the behaviour of HT29 cells, where the lower levels of MUC1 already expose the adhesion molecules, bestowing an increase in the basal level of cancer cell heterotypic adhesion to endothelial cell monolayers.

6.5.6 Galectins induce an increase in MUC1-expressing colon cancer cell migration through endothelial cells and extracellular matrix components (Matrigel).

Cancer cell adhesion to vascular endothelium is a major step in the multistep pathway of metastasis. After adherence to the endothelium, cancer cells migrate though the blood vessel walls (extravasion) into the surrounding tissue of the distal

site. Further experiments were carried out to assess the effect of galectins on cancer cell migration through trans- endothelial cells and extracellular matrix, both of which are important steps in metastasis.

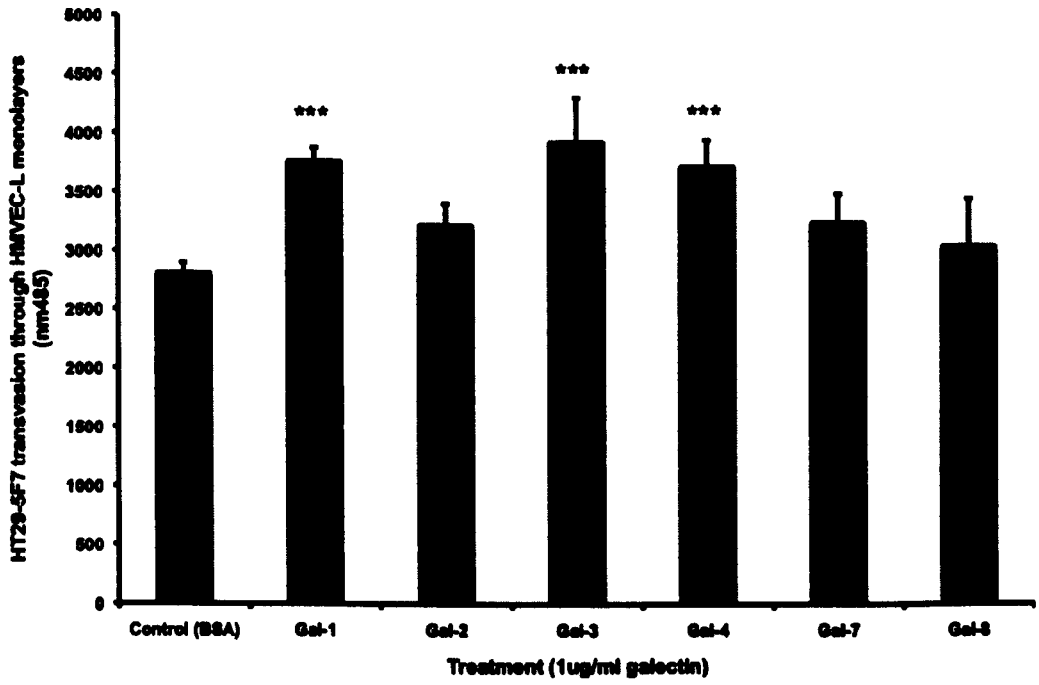


Figure 6.14. Effect of galectins on HT29-5F7 cell trans-endothelial migration through HMVEC-Ls. (* P<0.001, ** P<0.01, * P<0.05).**

Trans-endothelial migration of HT29-5F7 cells through HMVEC-Ls was assessed with and without galectin treatment. Calcein AM-labelled HT29-5F7 cells were pre-incubated with 1μg/ml recombinant galectin for 1hr at 37°C, before application to HMVEC-L monolayers cultured in transwells. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis used was ANOVA followed by Dunnett's comparison.

When compared to an untreated control (2802.5±90.4), only galectin-1, galectin-3 and galectin-4 induced a significant level of trans-endothelial migration through the HMVEC-L monolayers (3755.3±110.0; P=0.0002, 3914.8±375.4; P<0.0001, 3711.0±224.1; P=0.0004 (485nm) respectively). This corresponded to an increase of 135.9±6.5%, 146.1±15.6%, and 145.3±20.7%, in trans-endothelial migration for galectin-1, -3, and -4, repectively. Galectin-2, -7 and -8 showed no significant effect on trans-endothelial migration (3209.8±186.9; P=0.155, 3229.0±250.2; P=0.127, 3034.8±404.4; P=0.466 (485nm) respectively) (**Figure 6.14**). HT29-5F7 cells generally possess a low metastatic potential, and it was unknown as to whether this selectivity of the galectins to induce trans-endothelial migration was true to the action of the galectins, or more a representation of the nature of the cells. Further invasion experiments were carried out with SW620 cells, which are MUC1 possessing colon cancer cells, and have a higher metastatic potential than HT29-5F7 cells (421).

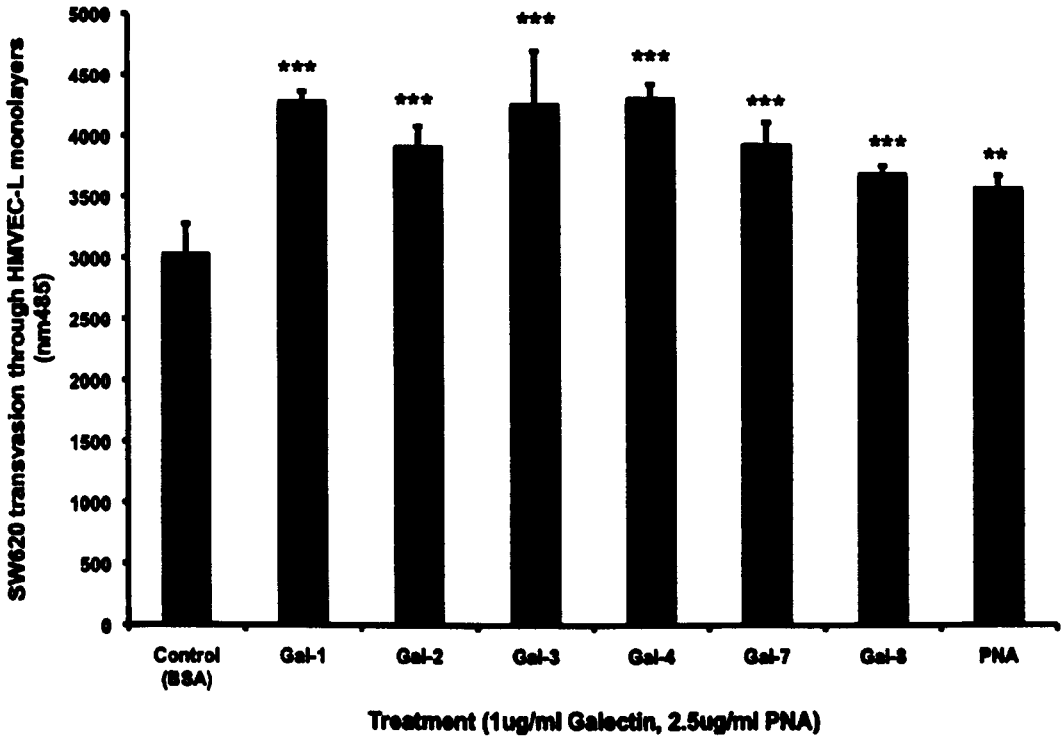


Figure 6.15. SW620 cell trans-endothelial migration through HMVEC-Ls in response to galectin treatment. (* P<0.001, ** P<0.01, * P<0.05).**

Trans-endothelial migration assays were performed in a 24-well insert within a cell culture plate, with each insert containing a HMVEC-L monolayer. Calcein AM-labelled SW620 cells were pre-incubated with 1μg/ml recombinant galectin for 1hr at 37°C, before application for a further hour to the HMVEC-L monolayers in transwells. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis used was ANOVA followed by Dunnett's comparison.

When compared to untreated controls (3030.1±246.5 (485nm)), all galectins induced a significant level of trans-endothelial migration through the HMVEC-L monolayers (4281.3±75.3; P<0.0001, 3909.0±163.8; P<0.0001, 4255.2±433.5; P<0.0001, 4305.2±114.1; P<0.0001, 3930.3±177.2; P<0.0001, 3691.3±61.4; P=0.0008 (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively. This corresponded

to an increase of 141.3±2.5%, 129.0±5.4%, 140.4±14.3%, 142.1±3.8%, 129.7±5.8%, and 121.8±2.0% of trans-endothelial migration for galectin-1, -2, -3, -4, -7, and -8, repectively. Pre-incubation of HT29-5F7 cells with PNA also increased the cells trans-endothelial migration through HMVEC-L monolayers (3568.3±106.9; P=0.0067 (485nm)), which corresponded to an increase of 117.8±3.5% (**Figure 6.15**).

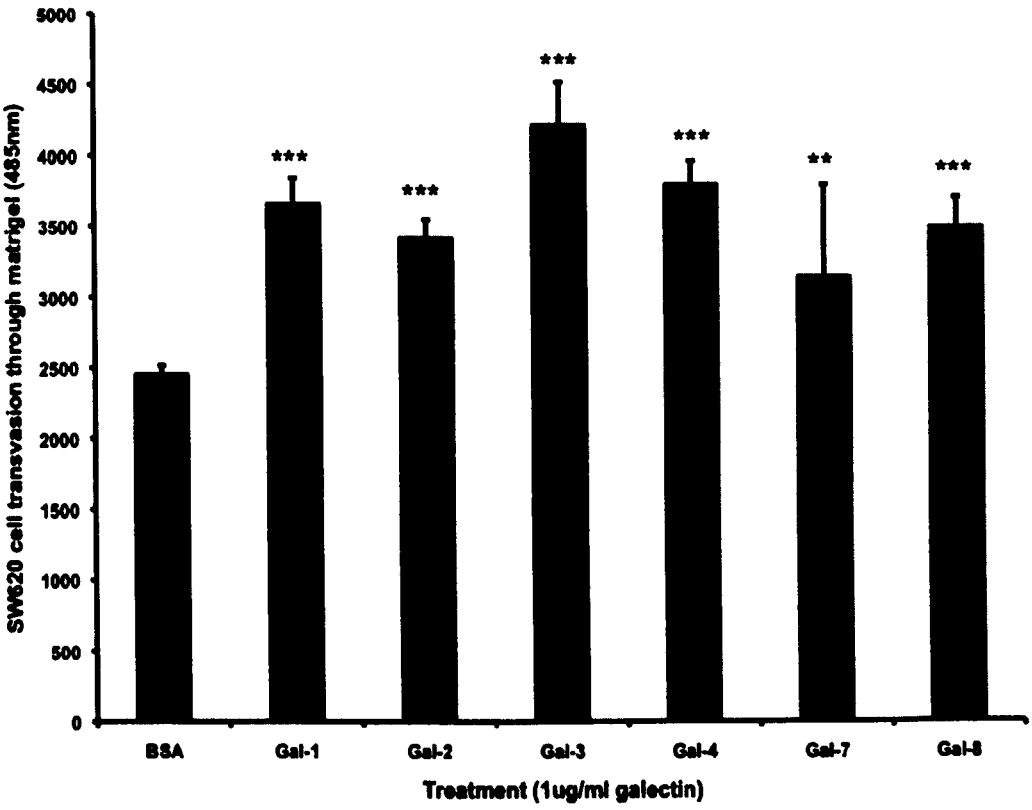


Figure 6.16. SW620 cell trans-endothelial migration through Matrigel with or without pre-incubation with galectins. (* P<0.001, ** P<0.01, * P<0.05).**

Trans-endothelial migration assays were performed in a 24-well insert within a cell culture plate, with each insert containing a gelled Matrigel layer. Calcein AM-labelled SW620 cells were pre-incubated with 1μg/ml recombinant galectin for 1hr at 37°C, before application for a further hour to the HMVEC-L monolayers within the

cell culture inserts. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis used was ANOVA followed by Dunnett's comparison.

When compared to untreated controls (2457.0±57), all galectins induced a significant increase in the level of migration through Matrigel (3658.0±172.9; $P<0.0001$, 3418.0±124.1; $P=0.0009$, 4215.0±296.2; $P<0.0001$, 3791.0±163.2; $P<0.0001$, 3138.0±644.1; $P=0.019$, 3486.0±205.8; $P=0.0004$ (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively). This corresponded to an increase of cellular migration of 148.9±7.0%, 139.1±5.1%, 171.5±12.1%, 154.3±6.6%, 127.7±26.2%, and 141.9±8.4% for galectin-1, -2, -3, -4, -7, and -8, repectively (**Figure 6.16**). These results have demonstrated that galectins can mediate cellular adhesion and migration through both ECM and endothelial cell monolayers.

6.5.7 Galectins induce polarisation of MUC1 on the surface of HCA1.7+ cells.

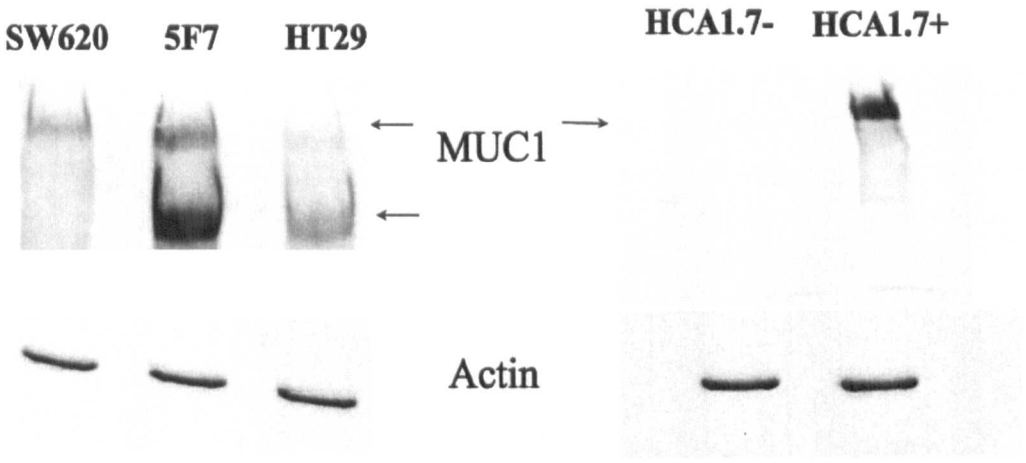


Figure 6.18. MUC1 expression in various cells.

SW620, 5F7, HT29: Colon cancer cells. **HCA1.7+:** Breast cancer cells transfected with MUC1. **HCA1.7-:** The negative revertants from MUC1 transfection.

MUC1 within the SW620, HT29 and HT29-5F7 is identifiable as two distinct bands. This is due to difference in tandem repeat number between the two alleles that encode MUC1, where the higher band represents the higher molecular weight MUC1 allele, and the lower band represents the smaller MUC1 allele (**Figure 6.18**). Previous experiments have demonstrated that galectin-3 binds more strongly to the higher weight MUC1 band, such as the one transfected into the HCA1.7+ cells (316). This presumably reflects the longer high-threonine tandem repeats and consequently greater expression of TF.

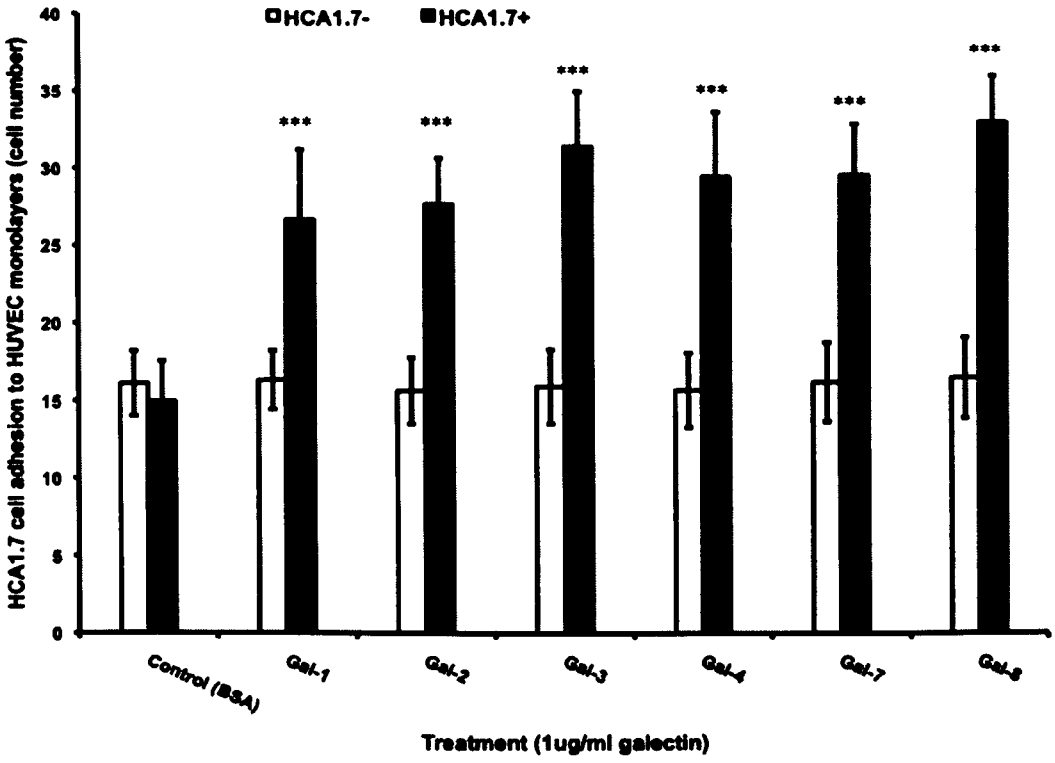


Figure 6.19. Adhesion of HCA1.7+ and HCA1.7- cells to HUVEC monolayers in response to galectins. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays were performed in a 24-well cell culture plate, with each well containing a sterile slide on which a continuous HUVEC monolayer was grown. Experiments were performed in duplicate with each sample analysed in triplicate. Analysis used was ANOVA followed by Dunnett's comparison.

The presence of each recombinant galectin at 1μg/ml, significantly increased the adhesion of MUC1 positive transfected HCA1.7+ breast cancer cells to HUVEC monolayers (26.6±4.5; P<0.0001, 27.6±3.0; P<0.0001, 31.3±3.5; P<0.0001, 29.4±4.2; P<0.0001, 29.5±3.3, P<0.0001, 32.9±3.0; P<0.0001 (485nm) for galectin-1, -2, -3, -4, -7 and -8 respectively). This corresponded to an increase in cellular adhesion of 177.9±30.4%, 184.8±19.9%, 209.7±23.6%, 196.7±28.2%, 197.3±22.3%, and

220.6±20.0% for galectin-1, -2, -3, -4, -7, and -8, repectively. However, the presence of each galectin had no significant effect on adhesion of the MUC1-negative transfectants HCA1.7- cells, (16.2±1.8; P=0.997, 15.6±2.1; P=0.867, 15.8±2.4, P=0.9979, 15.6±2.4; P=0.914, 16.2±2.5; P>0.999, 16.4±2.6; P=0.963 (485nm) for galectin-1, -2, -3, -4, -7 and -8, respectively) (**Figure 6.19**). This indicates that the galectin-MUC1 interactions also affect adhesion of human breast cancer cells.

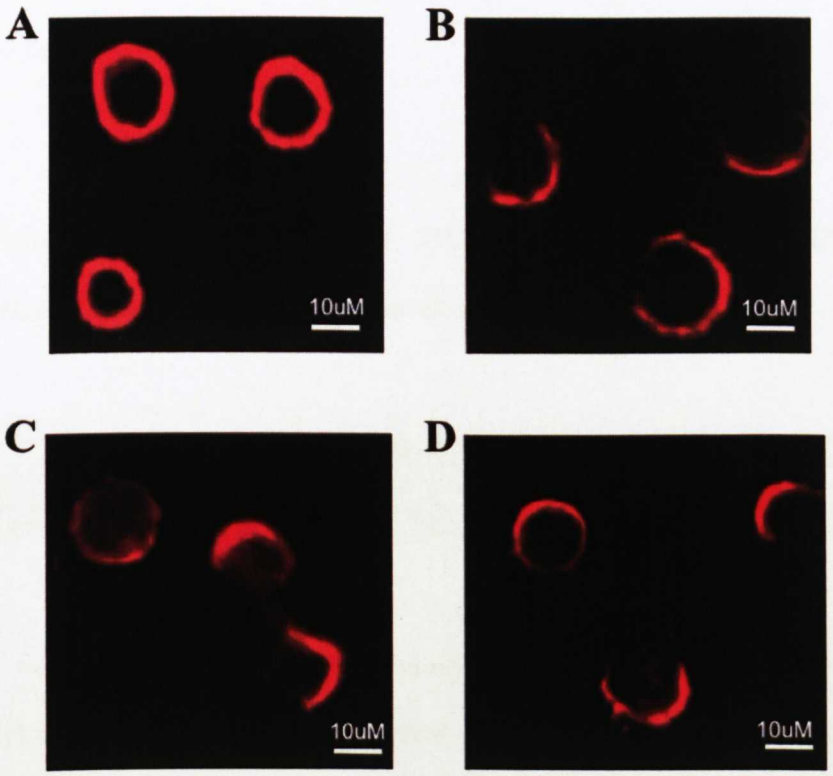


Figure 6.20. MUC1 Immunocytochemistry shows polarisation of MUC1 in MUC1 transfected HCA1.7+ cells in response to galectin or PNA treatment.

MUC1 was visualised by using an anti-MUC1 B27.29 antibody, followed by a Texas red-conjugated anti-mouse antibody. **A: Untreated cells.** MUC1 over the

surface of the HCA1.7+ cells. This image is a representation of what was considered in this assessment as continuous MUC1 cell surface localisation. **B: Cells treated with 1μg/ml galectin-3. C: Treated with 1μg/ml galectin-4. D: Treated with 4μg/ml PNA.** Following treatment with galectin for 1hr at 37°C, many cells show discontinuous MUC1 cell surface localization. These images show typical MUC1 localisation after treatment (**Figure 6.20**).

Quantitative analysis was undertaken on blinded slides to directly compare the ratio between cells that demonstrated discontinuous and continuous MUC1 cell surface localisation.

Table 6.1. Quantification of MUC1 repolarisation in MUC1 transfected HCA1.7+ with or without pre-incubation with galectins.

	Control (BSA)	Gal-1	Gal-2	Gal-3	Gal-4	Gal-7	Gal-8
Continuous MUC1 Localisation	457	164	181	161	189	173	156
Discontinuous MUC1 Localisation	43	336	319	339	311	327	344
Percentage of Discontinuous Localisation	8.6	67.2	63.8	67.8	62.2	65.4	68.8

Immunohistochemistry was undertaken, and blinded slides were analysed for MUC1 repolarisation. 500 individual cells were analysed per sample. Analysis used was Fishers Exact test.

Untreated cells showed a much higher level of cells demonstrating a continuous MUC1 cell surface localization, those cells that appeared as a uninterrupted circle (43) compared to cells that demonstrated discontinuous localisation (457; $P<0.0001$). However, upon incubation of the HCA1.7+ cells with 1μg/ml recombinant galectins, there is a notable shift towards a significant increase in

cells that demonstrated discontinuous localisation (336; P<0.0001, 319; P<0.0001, 339; P<0.0001, 311; P<0.0001, 327; P<0.0001, 344; P<0.0001 (cell number) for galectin-1, -2, -3, -4, -7 and -8 respectively) than those that demonstrated continuous MUC1 localisation (164, 181, 161, 189, 173, 156, (cell number) for galectin-1, -2, -3, -4, -7 and -8 respectively) (**Table 6.1**).

Table 6.2. MUC1 repolarisation in MUC1 transfected HCA1.7+ with or without pre-incubation with PNA.

	Control (BSA)	Galectin-3	PNA
Continuous MUC1 Localisation	451	152	181
Discontinuous MUC1 Localisation	49	348	319
Percentage of Discontinuous Localisation	9.8	69.6	63.8

Immunohistochemistry was undertaken, and blinded slides were analysed for MUC1 repolarisation. Treatment with 1μg/ml recombinant galectin-3 was used as a positive control to compare PNA against, as well as the untreated control. 500 individual cells were analysed per sample. Analysis used was Fishers Exact test.

As previously demonstrated, untreated cells showed a much higher level of cells demonstrating a continuous localisation of MUC1 around the complete surface of the cell (451) compared to cells that demonstrated discontinuous localisation (49; (cell number) P<0.0001). Again, upon incubating the HCA1.7+ cells with 1μg/ml recombinant galectin-3, there is a notable shift towards a significant increase in cells that demonstrated discontinuous localisation than those that demonstrated continuous MUC1 localisation. Furthermore, treatment of the cells with 4μg/ml PNA increased cells that demonstrated discontinuous localisation (319 vs. 181, (cell number) P<0.0001) (**Table 2.6**).

These results support the proposed model that galectin-MUC1 interaction induces change of MUC1 cell surface localization. The interaction between PNA and cancer-associated TF also results in polarization of MUC1 on the cell surface

6.6 DISCUSSION

Previous work within our group has investigated the role of circulating galectin-3 on cancer cell-cell interactions in metastasis (315, 316, 382). Those studies have shown that galectin-3 binds to the Thomsen-Friedenreich antigen (Galβ1-3GalNAcα1-Ser/Thr), on cancer-associated MUC1 and induces MUC1 cell surface relocalisation (polarization). The change of MUC1 cell surface polarization breaks the continuous 'shield' of MUC1 on the cell surface and subsequently exposes the cell surface adhesion molecules, which leads to increase cell-cell interactions between cancer cells and between cancer and endothelial cells.

Galectins, as a family, are defined by their ability to recognise β-galactosides. It is shown in this study that members of the galectin family recognise the TF antigen, but when comparing their binding to TF-expressing glycoproteins, all the galectins bound less favourably, or with a weaker association to antifreeze proteins which only express the TF structure, than to ASM and asialofetuin which express TF along side other *O*- or *N*-glycans. Furthermore, while some galectins favoured binding to ASM, a glycoprotein containing TF as its only *O*-glycan, other galectins favoured binding to asialofetuin, which expresses a complement of *N*-glycans. This indicates the subtle differences of binding among the galectin members to their potential targets. The discovery that reduction of the TF structure by *O*-glycanase dramatically reduced the

binding of the galectin members to ASM suggests an important role of the TF antigen in galectin binding to target proteins.

As other members of the galectin family, including galectin-1, -2, -4, -7 and -8, all showed similar binding to TF antigen as galectin-3, experiments were undertaken to see if these galectins also have similar influence on cancer cell adhesion as that of galectin-3. It was found that following treatment with recombinant galectins, the MUC1 expressing HT29-5F7 cells demonstrated a dose-dependent increase in adhesion to HUVEC monolayers. This galectin-mediated cell adhesion was abolished be pre-incubating the galectins with the galectin inhibitors lactose or asialofetuin. This indicated that the galectin-mediated cell adhesion response was based on the carbohydrate recognition abilities of the galectins. The same increase in adhesion of HT29-5F7 cells was seen on HMVEC-L monolayers and extracellular matrix components. Similar influences of the galectins were also seen in SW620 colon cancer cells, and HCA1.7+ breast cancer cells.

The galectin-mediated cancer cell adhesion was shown to be negated by pre-fixation of the cells with paraformaldehyde, suggesting the importance of cell surface molecular mobility. Galectins also demonstrated the ability to induce a significant increase of cell adhesion of the MUC1 positively transfected but not the MUC1 negative revertants of transfected human breast cancer cells. This supports the importance of MUC1 expression in galectin-mediated cell-cell interactions. Concealing TF structures with the anti-TF antibody TF5, or by removing the TF structure specifically with *O*-glycanase, significantly reduced the level of galectin-mediated adhesion, indicating the importance of the cell surface TF structure in galectin-mediated cell adhesion. Together these results suggest that the TF structure on MUC1 is critical in galectin-mediated cell adhesion to endothelium.

Using immunocytochemistry, it was demonstrated that all galectins tested induced change of MUC1 relocalisation. This change of MUC1 cell surface localization was also observed followed treatment of the cells with the TF-binding peanut lectin (PNA), This further support the importance of the lectin-TF interaction on cell adhesion, and it is likely that such a striking repolarisation of MUC1 protein in response to galectin or PNA is related to the multivalency of the lectins. All galectins have been identified as multivalent by either possessing two CRD, like the tandem repeat type, or by associating into dimers, like the chimeric type and prototype. PNA is also a multivalent protein with two carbohydrate binding sites within its molecule (271). The multivalent property of these molecules allows them to form lattice structures with their binding targets on the cell surface (422).

Following disseminating cancer cell adhesion to the endothelium at a distant site, the next step in the process of metastasis is invasion of the cancer cells through the endothelium and into the stroma beyond it. Treatment of HT29-5F7 or SW620 cells with galectin-1, -3 and -4 induced a significant increase of cell migration through HMVEC-L monolayers as well as gelled Matrigel layers.

These experiment have indicated that the presence of circulating galectins in the blood stream of cancer patients is likely to enhance circulating tumour cell adhesion, and trans-endothelial migration leading to metastasis.

CHAPTER 7

CHEMICALLY MODIFIED HEPARINS AS GALECTIN-3 INHIBITORS

7.1 HYPOTHESES

Chemically modified heparins may inhibit galectin binding, thus may be used as potential metastasis inhibitors.

7.2 AIMS

- To assess the effect of chemically modified heparins on galectin binding to TF-expressing glycans.

- To assess the effects of these chemically modified heparins on galectin-mediated cellular adhesion to extracellular matrix components and their adhesion to and trans-endothelial migration through HMVEC-L endothelial monolayers.

7.3 INTRODUCTION

7.3.1 Heparin

Heparin is a mixture of polymers ranging from 3 kDa – 30 kDa, and when extracted as native heparin, consists of a wide range of different sized and varyingly sulphated molecules (423). Heparin molecules are glycosaminoglycans, long unbranched structures, and consist of variably sulphated and acetylated repeating disaccharide units (424). The most common disaccharide unit that occurs within heparin is composed of a 2-*O*-sulfated iduronic acid and 6-*O*-sulfated, N-sulfated glucosamine. This dimer has been shown to make up 85% of heparins from beef lung and approximately 75% of those from porcine intestinal mucosa (425). Oligosaccharide mapping and nuclear magnetic resonance spectroscopy have demonstrated that human heparin is structurally similar to porcine intestinal mucosal heparin but distinctly different from bovine lung heparin (426). Human heparin also has substantially more *in vitro* anticoagulant activity than either of these pharmaceutical heparins, and has been obtained for experimental work from a hemangioma, which is rich in mast cells (426).

Heparin was discovered in 1916, but did not enter clinical trials until 1935 (427, 428). As demonstrated by its name (hepar, Greek for 'liver') it was originally isolated from canine liver cells. By 1937 heparin was generally considered a safe, readily available, and effective blood anticoagulant (429). Heparin is produced by basophils, and within granules in mast cells that line blood vessels and in mucosal tissues. Current commercial heparin is obtained mainly from porcine intestine. Heparin prevents the formation of clots and the extension of existing clots within the blood, as well as accelerating thombolysis when used with standard thrombolytics

(430, 431). Heparin's major anticoagulant effect is accounted for by a unique pentasaccharide, AGA*IA, where G is β-D-glucuronic acid, I is 2-*O*-sulphated α-L-iduronic acid and A* a 3-*O*-sulphated (usually also 6-*O*-sulphated) *N*-sulphated glucosamine (432) (433), with a high affinity binding sequence to antithrombin III (ATIII) (434-438). This pentasaccharide is present in only one third of mammalian heparin molecules (439-444). The anticoagulant effect is largely mediated through interaction of this pentasaccharide with ATIII, which produces a conformational change in ATIII (445-447) and markedly accelerates its ability to inactivate the coagulation enzymes thrombin (factor IIa), factor Xa, and factor IXa. Of these three enzymes, thrombin is the most sensitive to inhibition by heparin/ATIII (448-450). Heparin catalyses the inactivation of thrombin via ATIII by acting as a template to which both the enzyme and inhibitor bind to form a ternary complex (430, 451, 452). In contrast, the inactivation of factor Xa by ATIII/heparin complex does not require ternary complex formation and is achieved by binding of the enzyme to ATIII (436-438).

Heparin molecules that contain fewer than 18 saccharides are unable to bind thrombin and ATIII simultaneously and, therefore, are unable to accelerate the inactivation of thrombin by ATIII, but retain their ability to catalyse the inhibition of factor Xa through ATIII (452-454). Heparin also catalyses the inactivation of thrombin by a plasma cofactor, heparin cofactor II (455). This cofactor is specific for thrombin, and does not require the unique ATIII-binding pentasaccharide, and it requires much higher does of heparin (456-459) than those needed to catalyse the activity of ATIII.

The clearance of heparin is influenced by its chain length, with the higher molecular weight species being cleared from the circulation more rapidly than the

lower molecular weight species. This results in an in *vivo* accumulation of the lower molecular weight species that have a reduced ratio of antithrombin to antifactor Xa activity (460).

7.3.2 Low Molecular Weight Heparins

In recent years a range of low molecular weight fractionated heparins (LMWH) has been marketed for human use. These have a more predictable effect and are associated with lower risk of bleeding than standard heparins and consequently do not require montoring. They are also assoicated with a lower risk for the rare but often fatal complication of heparin-associated thrombocytopaenia in which antibodies develop against heparin-platelet complexes resulting in aggregation and thrombosis of major vessels. The partially depolymerised and fractionated LMWH consists of fragments with an average size of 5 kDa. Although LMWH preparations differ from each other based on the preparation method, chemical or enzymatical depolymerisation, their pharmacokinetical parameters are comparable (461).

LMWHs are currently approved for the prophylaxis and treatment of deep vein thrombosis (DVT), but their indications are likely to be expanded.

7.3.3 Heparin and Inflammation

Successful host response to tissue injury and pathogen invasion is facilitated by the recruitment of leukocytes from the blood and lymphatic systems to the target tissues. Members of the selectin family of adhesion receptors, including E-selectin, P-selectin and L-selectin, mediate the initial adhesive events that direct the movement of

leukocytes across the endothelium in target tissues. P-selectin is stored within resting platelets and endothelial cells, and translocated to the cell surface upon activation. L-selectin is constitutively expressed on most leukocyte types and mediates their interactions with endothelial ligands. Both of these selectins promote the initial tethering of leukocytes during extravasation at sites of inflammation. Endogenous ligands for P-selectin and L-selectin (such as PSGL-1) are expressed on leukocytes and endothelial cells (462-465). Selectins recognize and bind sialylated, fucosylated carbohydrate antigens such as sialyl Le^x (Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ-) (466, 467). In fact, the expression of sialylated, fucosylated glycans like sialyl Le^x correlate with a poor prognosis because of tumor progression and metastatic spread (468-471). Furthermore, it was noted that carcinoma cell-surface mucins carrying sialyl Le^x can be ligands for all three members of the selectin family of cell adhesion molecules (472, 473), and is associated with a poor prognosis (474). In mice models, this metastasis was shown to attenuated by P-selectin deficiency or by treatment with heparin (474).

Heparin and heparan sulfate can be recognized by L-selectin and P-selectin (475), and heparin and heparin-like oligosaccharides can inhibit L-selectin or P-selectin binding to Sialyl Le^x-related structures on HL-60 (Human promyelocytic leukemia) (476-478). Injected heparin also affects the inflammatory response and causes leukocytosis (479-481), and attenuates tumor metastasis in mice by inhibiting P-selectin–mediated interactions of platelets with carcinoma cell-surface mucin ligands (474). These studies suggest that the anti-inflammatory effects of heparin are due to a blockade of P- and L-selectins.

Table 7.1. Potential use of heparins beyond anticoagulation. Adapted from Mousa. Anticoagulants, Antiplatelets, and Thrombolytics, 2010 (429).

Heparin-sensitive disease state	Effects in experimental models	Clinical status
Adult respiratory distress syndrome	Reduces cell activation and accumulation in airways; neutralizes mediators and cytotoxic cell products; improves lung function in animal models	Controlled clinical trials
Allergic rhinitis arthritis	As for adult respiratory distress syndrome, it has not been tested in a specific nasal model	Controlled clinical trials; anecdotal reports
Asthma	Inhibits cell accumulation, collagen destruction, and angiogenesis	Controlled clinical trials
Cancer	Inhibits tumour growth, metastasis, and angiogenesis; increases survival time in animal models	Anecdotal reports plus recent clinical trials
Inflammatory bowel disease	Inhibits inflammatory cell transport in general; not tested in specific animal model	Controlled clinical trials

7.3.4 Heparins as possible Cancer Treatments

Initial studies have shown that administration of unfractionated heparin was able to attenuate metastasis of mammary carcinoma and melanoma cells in mouse models (482, 483). While a single dose of heparin effectively attenuated metastasis of human and mouse colon carcinoma and melanoma, mouse breast carcinoma and lung carcinoma, application of heparin either 24hrs before or after the tumour cell injection

had no effect (483, 484). In the majority of studies, the amount of heparin applied significantly exceeded the clinically used therapeutic dose. However, recent studies have provided evidence that heparin attenuated metastasis in two different mouse models at clinically relevant concentrations (485, 486). The observations from most animal studies indicate that heparin affects the initial phase of hematogenous metastasis. Furthermore, since the half-life of heparin in circulation ranges between 4 and 6hrs (487, 488), it has been suggested that heparin significantly modulates the metastatic capacity of tumour cells while they are still in the circulation. Possible mechanisms include inhibition of P- and L-selectin-mediated interactions, as previously disscussed (476, 489). Correlation between selectin ligand expression, typically on the cancer-associated mucins, and poor prognosis due to metastasis has been demonstrated in colon, gastric, lung, prostate, renal and breast cancer (490-494). Recent evidence has, however, shown that attenuation of metastasis was observed in the absence of P-selectin, and reduction of selectin ligands on tumor cells caused a decrease in platelet-tumour emboli formation and attenuation of metastasis (488, 495, 496). Furthermore, intravenous injection of a function blocking L-selectin antibody resulted in attenuation of metastasis (497). Varki *et al.*, have provided a working model to explain that heparin attenuates solid tumor metastasis through the inhibition of P-selectin and L-selectin, combined with an unknown degree of blockade of intravascular fibrin formation by the fluid-phase coagulation pathway (485).

As previously discussed, the relatively high doses of heparin administered in previous studies to attenuate metastasis are impractical to use clinically because of excessive anticoagulation. To circumvent this, many low molecular weight heparins have been created which possess better kinetics and bioavailability, and provide reduced incidence of side effects, such as heparin-induced thrombocytopenia (431,

498). Much variation is observed among low molecular weight heparins in their ability to inhibit both selectins, and unfractionated heparin was shown to the most effective at inhibiting selectins (485). It has been speculated that the reasons for this difference efficacy between unfractionated heparin and the LMWH is due to the extended dual-site nature of the P-selectin lectin domain. P-selectin (presented by either activated platelets or endothelial cells) is known to have two binding pockets: one for the sialyl Le^x moiety and another for the tyrosine sulfate–rich region of its native ligand PSGL-1, which is presented on leukocytes (499). Other P-selectin or L-selectin ligands can be sulfated, sialylated mucins presented on carcinoma cells, but all are notably molecules presenting high densities of negatively charged sulfates and carboxylates. It was suggested that heparins mimic these ligands due to their high density of sulfates and carboxylates (485). If the heparin chain is very short, it can only block one site at a time, making it a poor inhibitor. A longer heparin chain could interact with both binding sites on P-selectin and have some inhibitory activity. Conversely, the antithrombin-factor Xa complex only requires a single pentasaccharide to be sufficient to bind to antithrombin and catalyze the inactivation of Xa. Increasing the length of a heparin molecule would not change the outcome, unless there was more than one antithrombin-binding pentasaccharide in the sequence (485) (**Figure 7.1**).

Varki *et al.* demonstrated, using single boluses of heparin yielding clinically tolerable anti-Xa levels that are cleared from the system within a few hours, that the rank order (unfractionated heparin > Tinzaparin >> Fondaparinux, which are both LMWH) (**Figure 7.2**) of the ability of each heparin to inhibit P-selectin and L-selectin *in vitro* matched the effect on metastasis attenuation *in vivo* (485).

Figure 7.1. Proposed explanation for selectin inhibitory activity being concentrated in higher molecular weight heparin fractions. Fondaparinux and Tinzaparin are used as a comparative example (485).

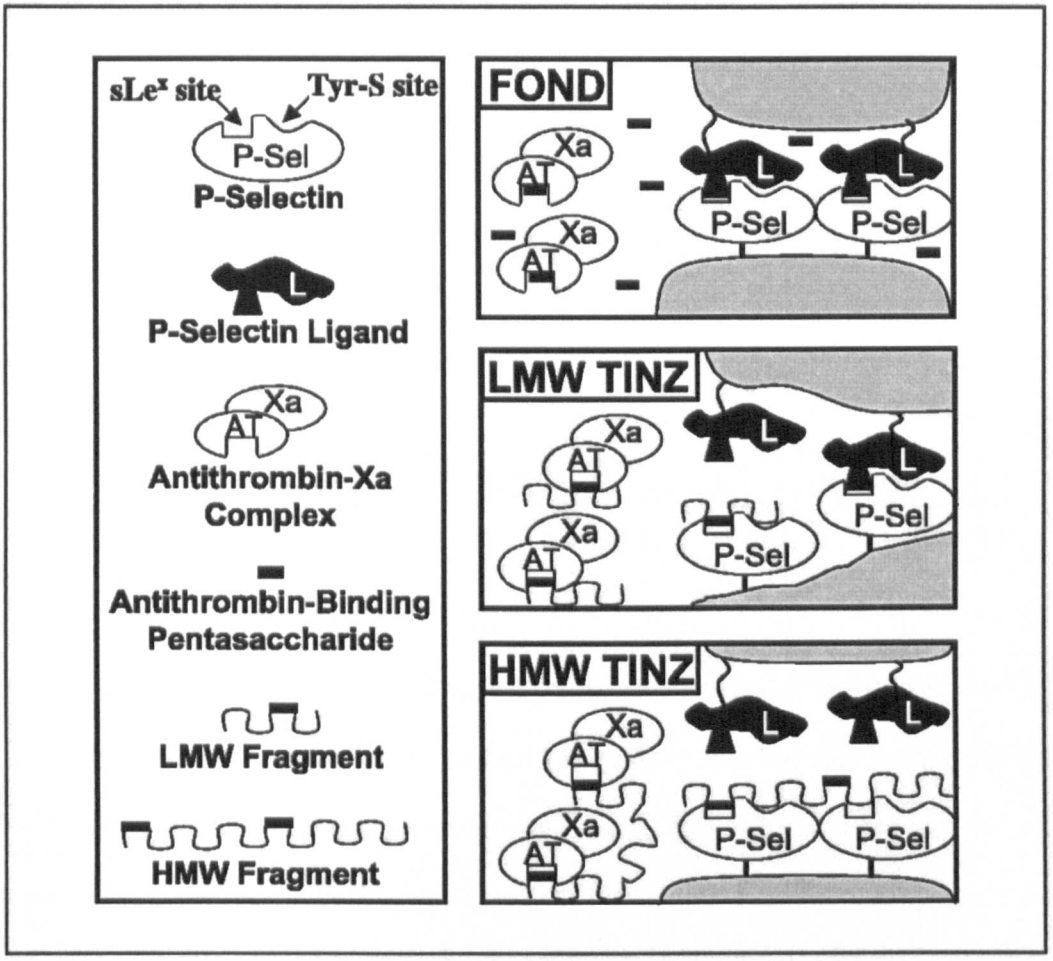
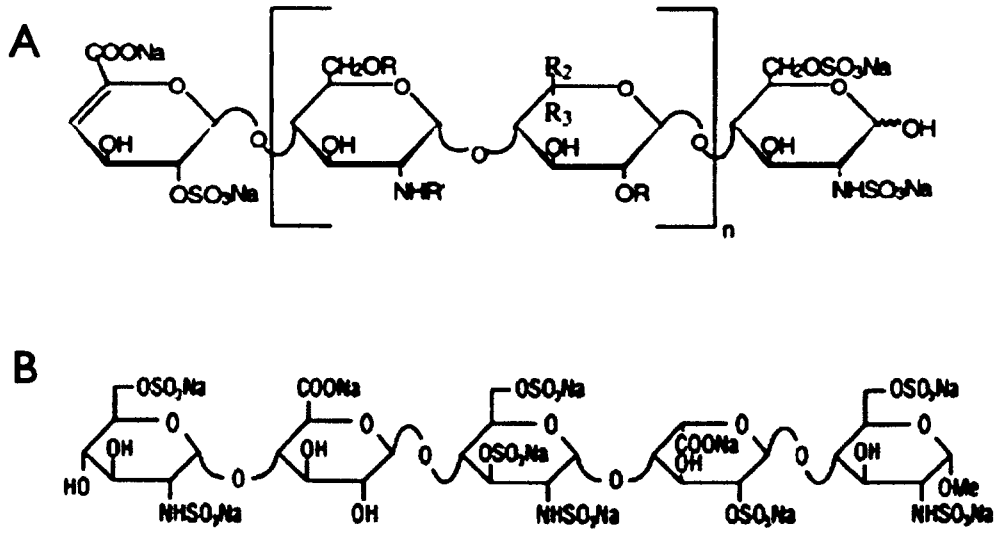


Figure 7.2. Molecular structures of LMW heparin derivatives. A.Tinzaparin. B. Fondaparinux.



Considering the effects of heparin upon the attenuation of metastasis, work is on-going to design new types of heparin to decrease anticoagulant activity whilst maintaining other activities (500-504). As heparin demonstrates inhibitory effects upon selectins, it could be hypothesized that heparin, and its chemically modified derivatives might have activity against other lectins, including galectins, and thus possess anti-cancer properties. The studies described in this chapter therefore investigate the effect of heparin and chemically modified heparins on galectin binding to TF-glycans and on galectin-mediated cancer cell adhesion and trans-endothelial migration.

7.4 METHODS

7.4.1 Materials

Biotinylated anti-galectin-3 antibody and recombinant galectin-3 were obtained from R&D Systems, Abingdon, UK. 96-well half-volume plates were obtained from Appleton Woods Ltd., UK. Asialofetuin was obtained from Sigma-Aldrich Ltd., UK. Matrigel was obtained from BD Biosciences, UK.

Chemically modified heparin derivatives were kindly provided by Prof. Jerry Turnbull, School of Biological Sciences, University of Liverpool, and were designated fractions as follows (505, 506):

A. Heparin

B. *N*-acetylated heparin

C. 2-de-*O*-sulfated heparin

D. 6-de-*O*-sulfated heparin

E. 2-de-*O*-sulfated heparin, *N*-acetylated heparin

F. 6-de-*O*-sulfated heparin, *N*-acetylated heparin

G. 2-, 6-de-*O*-sulfated heparin, *N*-sulfated heparin

H. 2-,6-de-*O*-sulfated heparin, *N*-acetylated heparin

I. Completely re-*O* and *N*-sulfated heparin (all hydroyl and amino-groups sulfated)

The anti-coagulation effects of these derivatives were analysed through the anti-Xa activity, and were as follows (**Figure 7.3**):

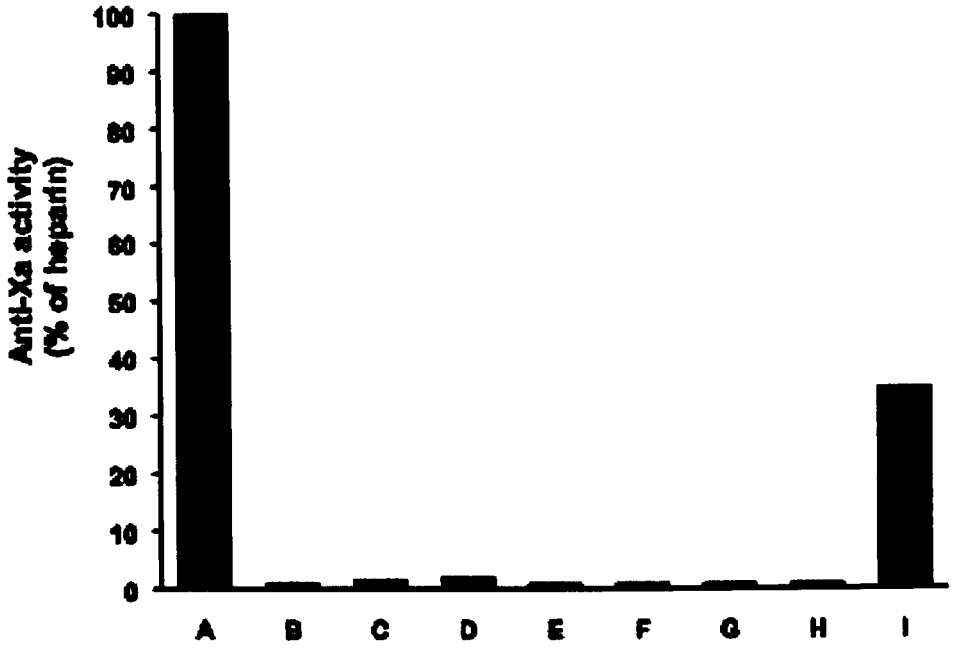


Figure 7.3. Anti-Xa activity, and therefore anticoagulation activity of the chemically modified heparin derivatives. (Data provided by Prof. Turnbull, University of Liverpool.) (505, 506)

7.4.2 Galectin-ligand binding ELISA

10μg/ml of asialo bovine mucin (ASM) in coating buffer (1.6g Na_2CO_3, 1.46g $NaHCO_3$ in 1L, pH9.6) was used to coat the wells of a 96-well half-volume plate overnight at 4°C. Before application to the ASM-coated wells, 1μg/ml galectin-3 was pre-incubated with 25μg/ml heparin derivative, or 100μg/ml lactose for 30mins. The protocol for the rest of this procedure was as described in **4.3.4.**

7.4.3 Cellular Adhesion

Endothelial Cell Plating:

HMVEC-L cells were released from a T25cm^2 flask with 2ml trypsin. HMVEC-Ls were diluted in complete EBM-2 to a plating density of 5.0x10^4 cells/ml. 100μl of cells were placed into the wells of a 96-well cell culture plate and incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air, for 2-3 days until a complete monolayer was formed.

Matrigel Coating:

Extracellular matrix components (Matrigel) (BD Biosciences, UK) were defrosted over night on ice, and pipette tips pre-cooled. 30μl undiluted Matrigel was directly pipetted into the wells of a 96-well plate to give a thin coating. Once the Matrigel had solidified, the wells were ready to be used.

A T25cm^2 flask of HT29-5F7 or SW620 human colon cancer cells were cultured as a monolayer to 70% confluence in complete DMEM at 37°C in a humidified atmosphere of 5% CO_2, 95% air, then washed twice with PBS, then

released from the flask with 2ml non-enzymatic cell dissociation solution to be used in cellular adhesion assays. 1μg/ml galectin-3 was pre-incubated with 25μg/ml heparin derivative for 30mins. The galectin-3/heparin mixture was then applied to $1x10^5$ cell/ml colon cancer cells for one hour at 37°C, before incubating the treated colon cancer cells with HMVEC-L monolayers or Matrigel coating in a 96-well cell culture plate at 37°C. The rest of this procedure was as described in **4.4**.

7.4.4 Cellular Migration

Endothelial Cell Plating:

HMVEC-L cells were released from a $T25cm^2$ flask with 2ml trypsin. HMVEC-Ls were diluted in complete EBM-2 to a plating density of $5.0x10^4$ cells/ml. 300μl of cells were placed into a cell culture insert, which was placed into a complete DMEM-containing well of a 24-well cell culture plate, and incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air, for 2-3 days until a complete monolayer was formed.

Matrigel Coating:

Extracellular matrix components (Matrigel) (BD Biosciences, UK) were defrosted over night on ice, and pipette tips pre-cooled. Matrigel was diluted 1:3 with serum-free, cold DMEM to ensure adequate gelling. 100μl of the diluted Matrigel was used to coat the chamber of a transwell insert, and incubated at 37°C for 4hrs.

Transwells were placed in complete DMEM containing wells of a 24-well cell culture plate.

As previously described, SW620 human colon cancer cells were used in trans-endothelial migration assays as they possess a higher metastatic potential than HT29-5F7 cells. A T25cm^2 flask of SW620 human colon cancer cells was cultured as a monolayer to 70% confluence in complete DMEM at 37°C in a humidified atmosphere of 5% CO_2, 95% air, then washed twice with PBS, then released from the flask with 2ml non-enzymatic cell dissociation solution to be used in cellular trans-endothelial migration assays. Galectin-3 was pre-incubated with 25μg/ml heparin derivative for 30mins and the galectin/heparin mixture was then applied to 1x10^5 cell/ml colon cancer cells for 1hr, before incubating the treated colon cancer cells with HMVEC-L monolayers in a cell culture insert in a 24-well cell culture plate. The rest of this procedure was as described in **4.5**.

7.5 RESULTS

7.5.1 De-O-sulfated fractions of chemically modified heparins inhibit galectin-3 binding to asialo bovine mucin.

Initial experiments to analyse the effects of chemically modified heparin fractions on the cellular adhesion and invasion-promoting properties of galectin-3 were performed. ELISA was used to determine the level of galectin-3 binding to asialo bovine mucin (ASM), a protein that was proven to be a strong ligand for galectin-3 in previous experiments (**6.5.1**). To assess any heparin derivatives as potential galectin-3 inhibitors, 25μg/ml of each heparin fraction, or 10mM lactose as a

control inhibitor, was pre-incubated with 1μg/ml galectin-3 for 1hr to ensure any galectin-3 CRDs were occupied by molecules of the potential inhibitor before applying the mixture to the ASM coated wells. Galectin-3 binding was observed through OD495, which was relative to the amount of biotinylated anti-galectin-3 antibody present within the well after washes, and therefore relative to galectin-3 bound to the ASM within the well.

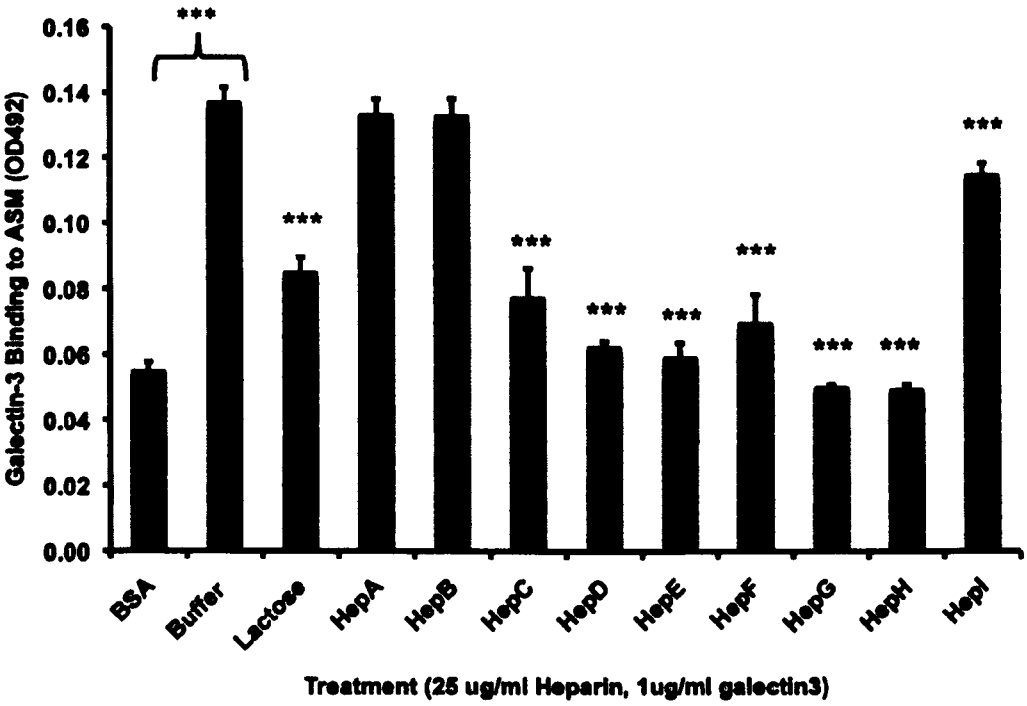


Figure 7.4. Level of binding of galectin-3 to asialo bovine mucin, with or without pre-incubation with chemically modified heparin derivatives. (* P<0.001, ** P<0.01, * P<0.05).**

ELISA was performed in a half-volume 96-well plate. Each well was coated with 50μl of 10μg/ml ASM. **BSA:** Bovine serum albumin, a control well with BSA as a coating in place of ASM. **HepA-I:** Heparin derivatives pre-incubated with galectin-

3. The experiment was performed twice, with each sample run in triplicate. Analysis with ANOVA, followed by Dunnett's multiple comparison with a control.

As previously demonstrated (**6.5.1**), galectin-3 showed stronger binding to ASM than to the negative control BSA, (0.137±0.006; P<0.0001, 0.054±0.003; (OD492) respectively). Pre-incubation of galectin-3 with lactose significantly abolished any galectin-3-ASM interaction (0.085±0.005, P<0.0001), corresponding with a 30.8±3.6% reduction in galectin-3 binding to ASM when compared to galectin-3 alone. At 25μg/ml, heparin derivative A and B (HepA and HepB) showed no significant effect on galectin-3 binding to ASM (0.133±0.006; P=0.229 and 0.136±0.005; P=0.379, (OD492) respectively) to that of galectin-3 alone. The presence of Heparin derivatives C-H significantly reduced the galectin-3 binding to ASM (0.074±0.012; P<0.0001, 0.062±0.003; P<0.0001, 0.058±0.005; P=<0.0001, 0.068±0.011; P<0.0001, 0.050±0.002; P<0.0001, 0.049±0.002; P<0.0001, (OD492) respectively), when compared to galectin-3 alone. This corresponded to a reduction in galectin-3 binding to ASM of 43.6±6.7%, 54.6±1.4%, 56.8±3.4%, 49.2±6.6%, 63.7±0.9% and 64.2±1.4%, for HepC-H, respectively. HepI also demonstrated a significant level of inhibition of galectin-3 binding (0.115±0.006; P<0.0001 (OD492)), which corresponded to a reduction of galectin-3 binding by 16.3±3.0% (**Figure 7.4**).

It is worth noting that the most significantly inhibitory heparin derivatives were the derivatives with the least anti-Xa activity, and therefore the least anticoagulation ability. When comparing the chemical modifications, it is apparent that the more inhibitory fractions had all been subjected to 2- and/or 6-de-*O*-sulfation, and some (HepE-H) possessed *N*-acetylation, or *N*-sulfation. The common factor

between these inhibitory heparins seems to be the de-*O*-sulfation modification, especially when we consider the less inhibitory HepB, which possesses de-*N*-acetylation alone, and the more inhibitory fractions HepE, F and H, which also possess de-*N*-acetylation.

7.5.2 All fractions of chemically modified heparins inhibit galectin-3 binding to asialo bovine mucin, but at different ranges of concentrations.

Considering the inhibitory effect heparin fractions C-H at 25μg/ml, further experiments were carried out to detect at which range these heparins were more effective. Three of the most significantly inhibitory modified heparin fractions, HepD, E and G were analysed through a range of concentrations, from 5μg/ml to 100μg/ml.

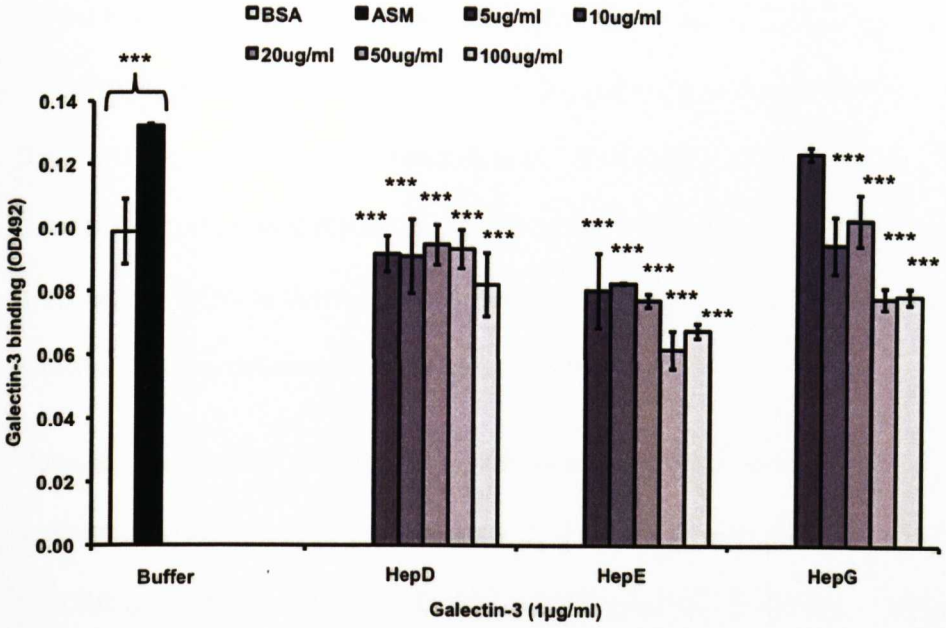


Figure 7.5. Level of binding of galectin-3 to asialo bovine mucin, with or without pre-incubation with chemically modified heparin derivatives D, E and G at a range of concentrations (µg/ml). (* P<0.001, ** P<0.01, * P<0.05).**

ELISA was performed in a half-volume 96-well plate, with each well coated with 50µl of 10µg/ml ASM. HepD, E and G at different concentrations diluted with PBS were pre-incubated with galectin-3 at 1µg/ml for 30mins, before applying to the wells for 1hr. The experiment was performed twice, with each sample run in triplicate. Analysis with ANOVA, followed by Dunnett's multiple comparison with a control.

All heparin derivatives showed a significant inhibitory effect on galectin-3 binding from 10µg/ml upwards to 100µg/ml when compared to the BSA control (0.099±0.010; P<0.0001 (OD492)) (**Figure 7.5**).

Heparin derivative D: HepD demonstrated a significant inhibitory effect on galectin-3 binding at all concentrations, 5, 10, 20, 50, and 100μg/ml (0.092±0.005; P=0.0002, 0.091±0.012; P=0.0002, 0.095±0.006; P=0.0001, 0.093±0.006; P=0.0002, 0.082±0.010; P<0.0001 (OD492)). However there seems to be limited variance in OD between the heparin derivative treatments. This may suggest that the effects of the heparin at these concentrations are already maximal.

Heparin derivative E: Hep E demonstrated a significant inhibitory effect on galectin-3 binding at all concentrations, 5, 10, 20, 50, and 100μg/ml (0.080±0.012; P<0.0001, 0.083±0.0002; P<0.0001, 0.077±0.002; P<0.0001, 0.0617±0.006; P<0.0001, 0.068±0.002; P<0.0001 (OD492)). There is a slight increase in inhibitory effect with increasing concentrations of the heparin derivative, particularly at 50μg/ml and 100μg/ml.

Heparin derivative G: Hep G demonstrated a significant inhibitory effect on galectin-3 binding at concentrations 10, 20, 50, and 100μg/ml (0.095±0.009; P<0.0001, 0.103±0.008; P<0.0001, 0.078±0.003; P<0.0001, 0.079±0.003; P<0.0001 (OD492)). There is a slight increase in inhibitory effect with increasing concentrations of the heparin derivative, particularly at 50μg/ml and 100μg/ml.

Further experiments were carried out on heparin derivatives D, E and G, as well as the other heparin derivatives that were shown to be inhibitory towards galectin-3 at 25μg/ml, to identify at what ranges they were functional.

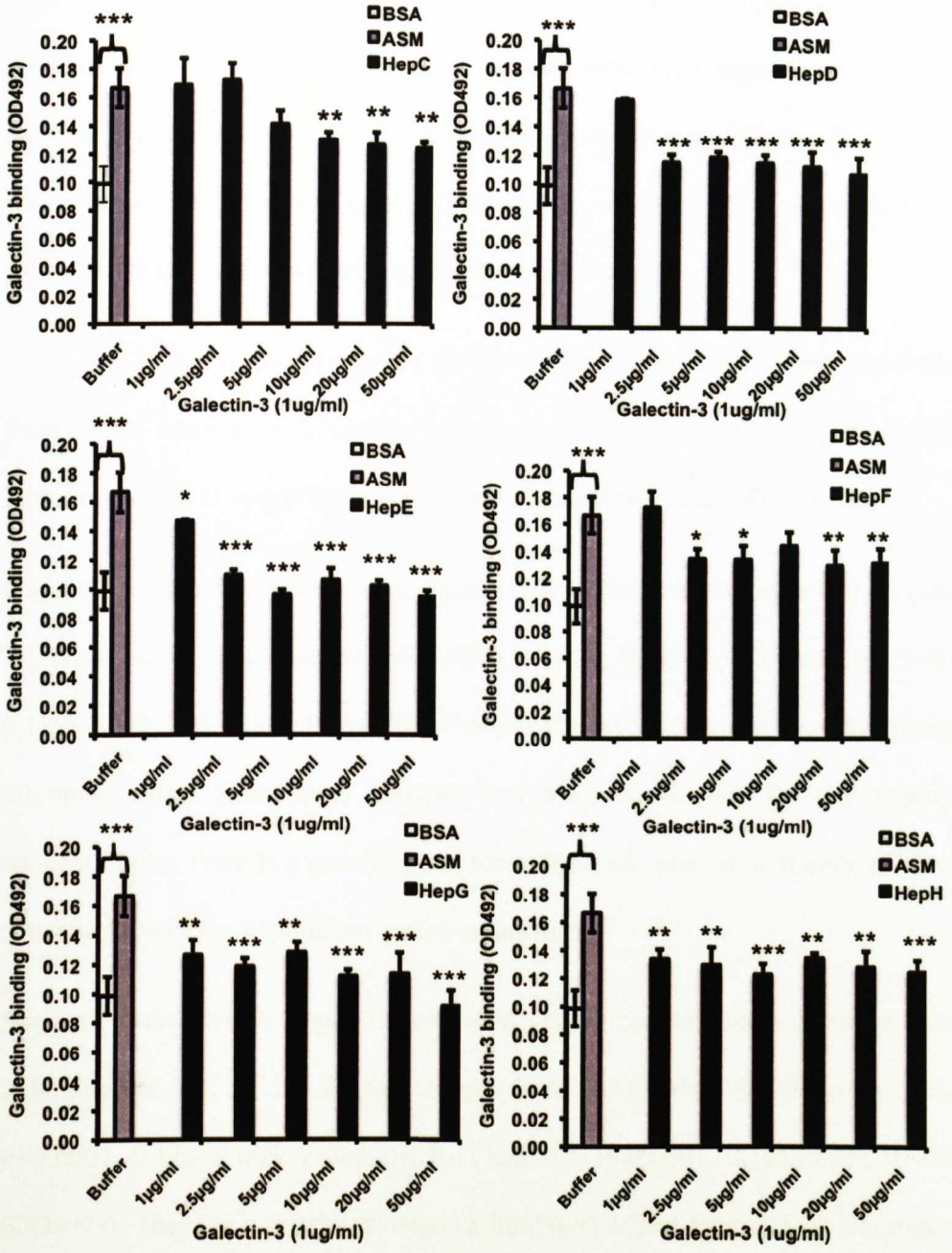


Figure 7.6. Binding of galectin-3 to asialo bovine mucin, with or without pre-incubation with chemically modified heparin derivatives at a lower range of concentrations (µg/ml). (* P<0.001, ** P<0.01, * P<0.05).**

ELISA performed in a half-volume 96-well plate, with each well coated with 50µl of 10µg/ml ASM. As before, BSA represents the negative control wells. ASM

represents a positive control with only galectin-3 added. Chemically modified heparin derivatives at different concentrations, diluted with PBS were pre-incubated with galectin-3 at 1μg/ml for 30mins, before applying to the wells for 1hr. The experiment was performed twice, with each sample run in triplicate. Analysis with ANOVA, followed by Dunnett's multiple comparison with a control.

All heparins demonstrated a significant inhibitory effect on galectin-3 binding from either 1μg/ml or 2.5μg/ml, with only one of the derivatives presenting an inhibitory effect at 5μg/ml when compared to galectin-3 alone (**Figure 7.6**).

Heparin derivative C: HepC demonstrated a significant inhibitory effect on galectin-3 binding at 5, 10, 20 and 50μg/ml (0.141±0.009; P=0.015, 0.130±0.005; P=0.003, 0.129±0.009; P=0.003, 0.124±0.004; P=0.003 (OD492)). There is a relative plateau in inhibitory effect from 10 to 50μg/ml, but when considering the whole range of concentrations, there is a general trend towards an increase in inhibitory effect when concentration of the heparin derivative is increased.

Heparin derivative D: HepD demonstrated a significant inhibitory effect on galectin-3 binding at 2.5, 5, 10, 20 and 50μg/ml (0.116±0.005; P<0.0001, 0.119±0.004; P<0.0001, 0.115±0.005; P<0.0001, 0.113±0.010; P<0.0001, 0.107±0.012; P<0.0001 (OD492)). There is a relative plateau in inhibitory effect from 2.5 to 50μg/ml, with little significant difference in inhibitory effect between the different concentrations.

Heparin derivative E: HepE demonstrated a significant inhibitory effect on galectin-3 binding at 1, 2.5, 5, 10, 20 and 50μg/ml (0.147±0.0007; P<0.0001, 0.110±0.005; P<0.0001, 0.096±0.004; P<0.0001, 0.106±0.005; P<0.0001, 0.102±0.010; P<0.0001, 0.095±0.012; P<0.0001 (OD492)). When considering the whole range of concentrations, there is a significant change in galectin-3 inhibition from 1μg/ml to

2.5μg/ml, but with increasing concentrations of HepE, a general plateau in the level of galectin-3 inhibition is observed.

Heparin derivative F: HepF demonstrated a significant inhibitory effect on galectin-3 binding at 2.5, 5, 10, 20 and 50μg/ml (0.134±0.012; P=0.007, 0.134±0.008; P=0.012, 0.144±0003; P=0.03, 0.129±0.011; P=0.009, 0.131±0.008; P=0.009 (OD492)). There is a general plateau in the level of galectin-3 inhibition is observed between the different concentrations.

Heparin derivative G: HepG demonstrated a significant inhibitory effect on galectin-3 binding at 1, 2.5, 5, 10, 20 and 50μg/ml (0.126±0.010; P=0.0006, 0.119±0.006; P=0.0002, 0.128±0.007; P=0.0003, 0.112±0.004; P<0.0001, 0.114±0.014; P<0.0001, 0.092±0.011; P<0.0001 (OD492)). When considering the whole range of concentrations, there is a general trend towards an increase in inhibitory effect when concentration of the heparin derivative is increased.

Heparin derivative H: HepH demonstrated a significant inhibitory effect on galectin-3 binding at 1, 2.5, 5, 10, 20 and 50μg/ml (0.134±0.007; P=0.0028, 0.130±0.012; P=0.0018, 0.123±0.008; P=0.001, 0.135±0.003; P=0.0012, 0.128±0.011; P=0.0018, 0.124±0.008; P=0.001 (OD492)). There is a general plateau in the level of galectin-3 inhibition is observed between the different concentrations.

As these heparin derivatives demonstrated an ability to inhibit galectin-3 binding at a variety of concentrations, the possibility that the heparin derivatives hepA, hepB which were not significantly inhibitory at 25μg/ml, could act as inhibitors also, but at a higher concentration. Attention was also paid to hepI, which demonstrated a significant inhibition of galectin-3 binding, but at a lower level than

the other effective fractions. Further experiments were carried out to investigate this hypothesis.

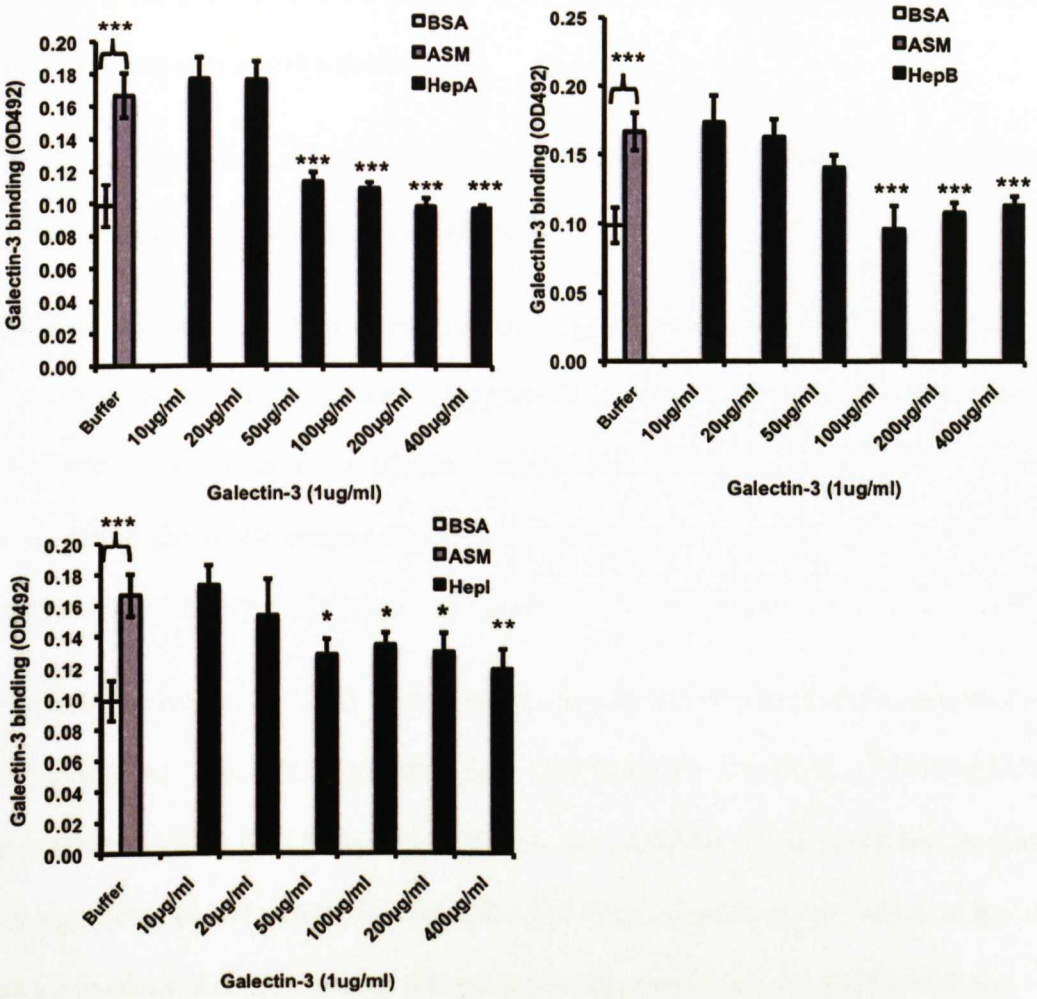


Figure 7.7. Binding of galectin-3 to asialo bovine mucin, with or without pre-incubation with chemically modified heparin derivatives A, B and I at a higher range of concentrations (µg/ml). (* P<0.001, ** P<0.01, * P<0.05).**

ELISA performed in a half-volume 96-well plate, with each well coated with 50µl of 10µg/ml ASM. As before, BSA represents the negative control wells. ASM represents a positive control with only galectin-3 added. HepA, B and I at different

concentrations diluted with PBS were pre-incubated with galectin-3 at 1μg/ml for 30mins, before applying to the wells for 1hr. The experiment was performed twice, with each sample run in triplicate. Analysis with ANOVA, followed by Dunnett's multiple comparison with a control.

At concentrations higher than 25μg/ml, HepA, B and I demonstrated an ability to inhibit galectin-3 binding to ASM (**Figure 7.7**).

Heparin derivative A: HepA demonstrated a significant inhibitory effect on galectin-3 binding at 50, 100, 200 and 400μg/ml (0.113±0.011; P<0.0001, 0.109±0.006; P<0.0001, 0.098±0.004; P<0.0001, 0.06±0.005; P<0.0001 (OD492)). When considering the whole range of concentrations, there is a general trend towards an increase in inhibitory effect when concentration of the heparin derivative is increased.

Heparin derivative B: HepB demonstrated a significant inhibitory effect on galectin-3 binding at 100, 200 and 400μg/ml (0.096±0.009; P<0.0001, 0.1085±0.017; P=0.0004, 0.114±0.007; P=0.0007 (OD492)), but inhibition at 50μg/ml HepB borders on significant (0.141±0.013; P=0.06 (OD492)) When considering the whole range of concentrations, there is a general plateau in the significance of galectin-3 inhibition.

Heparin derivative I: HepI demonstrated a significant inhibitory effect on galectin-3 binding at 50, 100, 200 and 400μg/ml (0.128±0.024; P=0.025, 0.134±0.010; P=0.0284, 0.130±0.008; P=0.0218, 0.119±0.012; P=0.0065 (OD492)). Previous experiments have already shown that HepI has a significant inhibitory effect upon galectin-3 at 25μg/ml. When considering the whole range of concentrations, there is a general plateau in the significance of galectin-3 inhibition.

These results suggest that galectin-3 does recognise native heparin, and its chemically modified derivatives, but may have weaker associations or binding constants to derivatives without certain modifications and binds preferentially to those with them.

Table 7.2. Binding of galectin-3 to asialo bovine mucin, with or without pre-incubation with chemically modified heparin derivatives at range of concentrations (μg/ml). Shaded boxes denote significant decrease of galectin-3 binding when compared to untreated control.

	Gal-3	Gal-3	1μg/ml	2.5μg/ml	5μg/ml	10μg/ml	20μg/ml	50μg/ml	100μg/ml	200μg/ml	400μg/ml
BSA	0.0986										
ASM		0.1666									
HepC			0.1688	0.1721	0.1411	0.1299	0.1264	0.1240			
HepD			0.1587	0.1155	0.1190	0.1152	0.1127	0.1069			
HepE			0.1469	0.1097	0.0963	0.1063	0.1023	0.0947			
HepF	z		0.1726	0.1343	0.1339	0.1440	0.1293	0.1313			
HepG			0.1263	0.1186	0.1280	0.1121	0.1136	0.0916			
HepH			0.1339	0.1297	0.1228	0.1345	0.1280	0.1242			
HepA						0.1768	0.1761	0.1134	0.1087	0.0977	0.0963
HepB						0.1735	0.1631	0.1406	0.0961	0.1085	0.1137
HepI						0.1731	0.1533	0.1281	0.1344	0.1297	0.1187

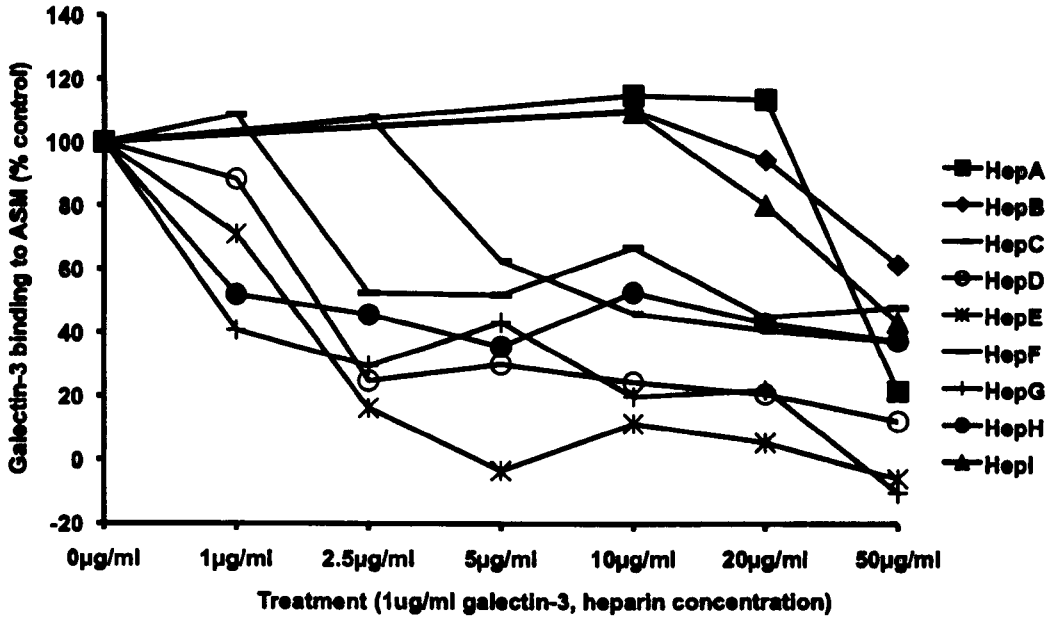


Figure 7.8. The inhibition of galectin-3 binding to ASM by chemically modified heparin derivatives.

7.5.3 Chemically modified heparins inhibit galectin-3-mediated colon cancer cell adhesion to HMVEC-L monolayers.

As chemically modified heparin derivatives showed inhibition to galectin-3 binding to ASM, further experiments were carried out to assess whether these heparin derivatives could also affect galectin-3-mediated cellular adhesion. 1µg/ml galectin-3 was pre-incubated with 25µg/ml heparin derivative for 30mins, before applying to the Calcein AM-labelled colon cancer HT29 or SW620 cells for 1hr. The cells were then incubated for 1hr with the HMVEC-L monolayers before the cell adhesion (fluorescence) was determined by fluorescence reading at (485nm).

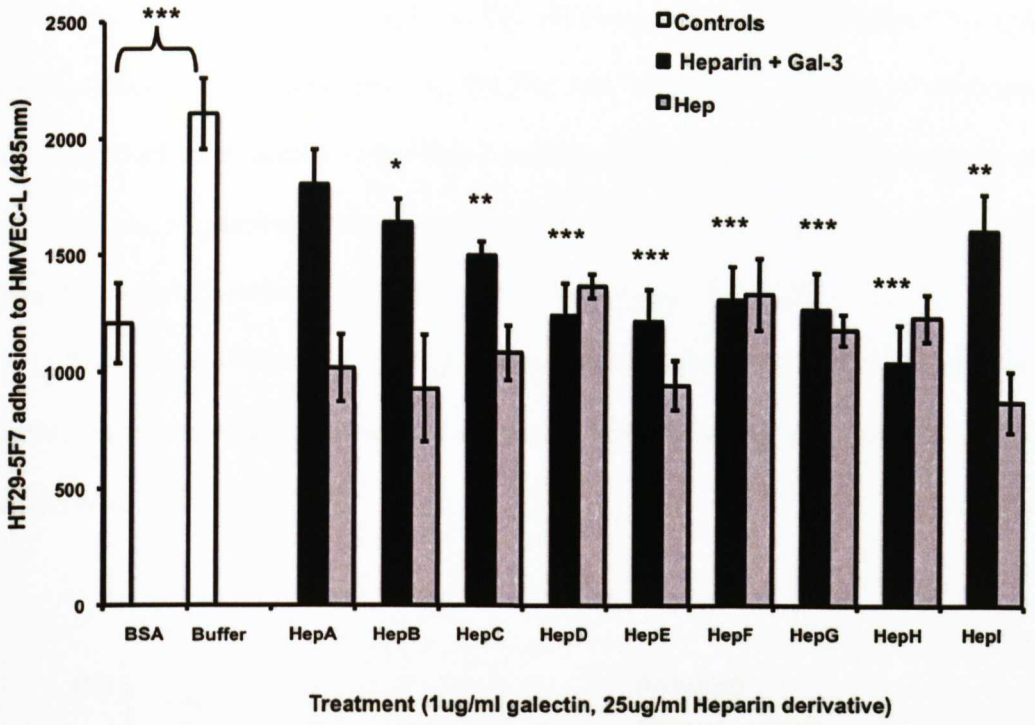


Figure 7.9. Inhibition of galectin-3-induced HT29-5F7 cell adhesion to HMVEC-L monolayers. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a continuous HMVEC-L monolayer. Heparin derivatives at 25μg/ml were pre-incubated with galectin-3 at 1μg/ml for 30mins, before applying to the wells for 1hr. The experiment was performed twice, with each sample run in triplicate. Analysis with ANOVA, followed by Dunnett's multiple comparison with a control.

As demonstrated in previous experiments, 1μg/ml galectin-3 significantly increased HT29-5F7 colon cancer cell adhesion to HMVEC-L monolayers when compared to untreated controls (1207±171; P<0.0001, 2108±152, (485nm) respectively). HepB-I induced a significant reduction in HT29-5F7 adhesion to HMVEC-L monolayers, (1641±98; P=0.0015, 1499±58; P=0.002, 1243±136; P<0.0001, 1216±138; P<0.0001, 1310±143; P<0.0001, 1270±155; P<0.0001,

1043±162; P<0.0001, 1612±156; P<0.0001 (485nm) respectively) (**Figure 7.9**). This corresponded to a 22±4%, 29±3%, 41±7%, 42±7%, 38±7%, 40±7%, 51±8% and 24±7% reduction in galectin-3-mediated cell adhesion to endothelial cell monolayers for HepB-I, respectively. HepD-H demonstrated the most significant inhibitory potential, which reflects the pattern identified within previous ELISA experiments.

To examine the generalisability of this galectin-3-mediated adhesion inhibition, further experiments were carried out in another MUC1-expressing colon cancer cell line, SW620.

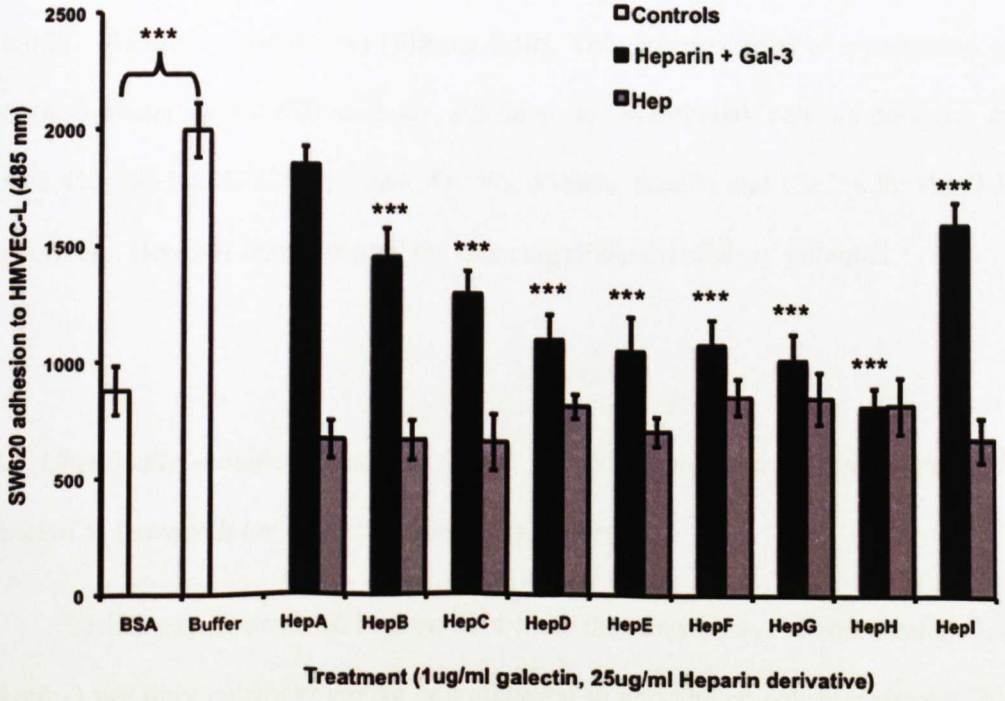


Figure 7.10. Inhibition of galectin-3-induced SW620 cell adhesion to HMVEC-L monolayers. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a continuous HMVEC-L monolayer. Heparin derivatives at 25μg/ml were

pre-incubated with galectin-3 at 1μg/ml for 30mins, before applying to the wells for 1hr. The experiment was performed twice, with each sample run in triplicate. Analysis with ANOVA, followed by Dunnett's multiple comparison with a control.

Again, treatment of SW620 colon cancer cells with 1μg/ml galectin-3 induced a significant increase of cancer cell adhesion to HMVEC-L when compared to the untreated control (1995±115; P<0.0001, 878±105, (485nm) respectively). As demonstrated in HT29-5F7 cell adhesion assays, HepB-I induced a significant inhibition of galectin-3-mediated SW620 cell adhesion to HMVEC-L monolayers (1450±124; P<0.0001, 1292±99; P<0.0001, 1097±110; P<0.0001, 1049±148; P<0.0001, 1075±107; P<0.0001, 1007±115; P<0.0001, 808±80; P<0.0001, 1585±95; P<0.0001 (485nm) respectively) (**Figure 7.10**). This corresponded to a reduction of galectin-3-mediated SW620 cellular adhesion to endothelial cell monolayers of 28.9±8.4%, 38±5%, 47±7%, 51±8%, 48±5%, 53±8%, 60±5% and 17±2% for HepB-I, respectively. HepD-H demonstrated the most significant inhibitory potential.

7.5.4 Chemically modified heparins inhibit galectin-3-mediated colon cancer cell adhesion to Extracellular Matrix Components (Matrigel).

Earlier experiments (**Chapter 6**) within this thesis have demonstrated that galectin-3 not only enhances cancer cell adhesion to endothelial cell monolayers, but also to extracellular matrix compenents (Matrigel) as well. Both cell-cell and cell-matrix interactions are involved in the process of cancer progression. Further experiments were carried out to assess whether this inhibitory effect of heparin derivatives on galectin-3 binding would affect galectin-3-mediated cellular adhesion to Matrigel. 30μl Matrigel (BD Biosciences, UK) was pipetted into the bottom of the

wells of a 96-well cell culture plate with a pre-cooled pipette tip, to form a thin layer which acted as a binding surface for the colon cancer cells, HT29-5F7 or SW620. Cancer cells and controls were treated as previously stated in the cell-cell adhesion assays. Cellular adhesion was observed through fluorescence (485nm), which was obtained by treating the cancer cells with Calcien AM, a cell-permeant green-fluorescent dye that labels live cells.

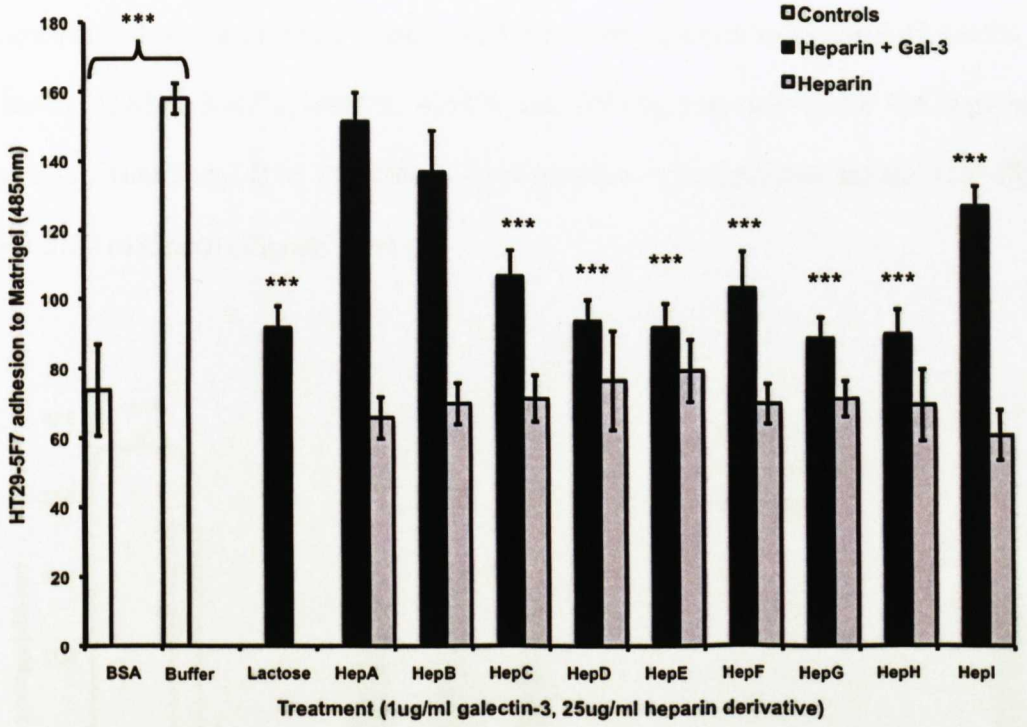


Figure 7.11. Inhibition of galectin-3-induced HT29-5F7 cell adhesion to Matrigel. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a thin layer of Matrigel. Heparin derivatives at 25μg/ml were pre-incubated with galectin-3 at 1μg/ml for 30mins, before application to the wells for 1hr. The experiment was performed twice, with each sample run in triplicate. Analysis with ANOVA, followed by Dunnett's multiple comparison with a control.

Treatment of HT29-5F7 colon cancer cells with 1μg/ml galectin-3 induced a significant increase of cancer cell adhesion to Matrigel when compared to the untreated control (158±4; P<0.0001, 74±13, (485nm) respectively). Heparin derivatives HepC-I decreased galectin-3-mediated cellular adhesion to Matrigel (107±7; P<0.0001, 94±6; P<0.0001, 92±7; P<0.0001, 103±10; P<0.0001, 89±6; P<0.0001, 90±7; P<0.0001, 127±6; P<0.0001 (485nm), respectively, which corresponded with a decrease in galectin-3 mediated adhesion to matrigel of 33±5%, 41±4%, 42±4%, 35±7%, 44±4%, 43±5% and 20±4%, respectively for the heparin fractions. HepA and B at 25μg/ml did not produce any significant change (151±8; P=0.2727 (485nm)) (**Figure 7.11**).

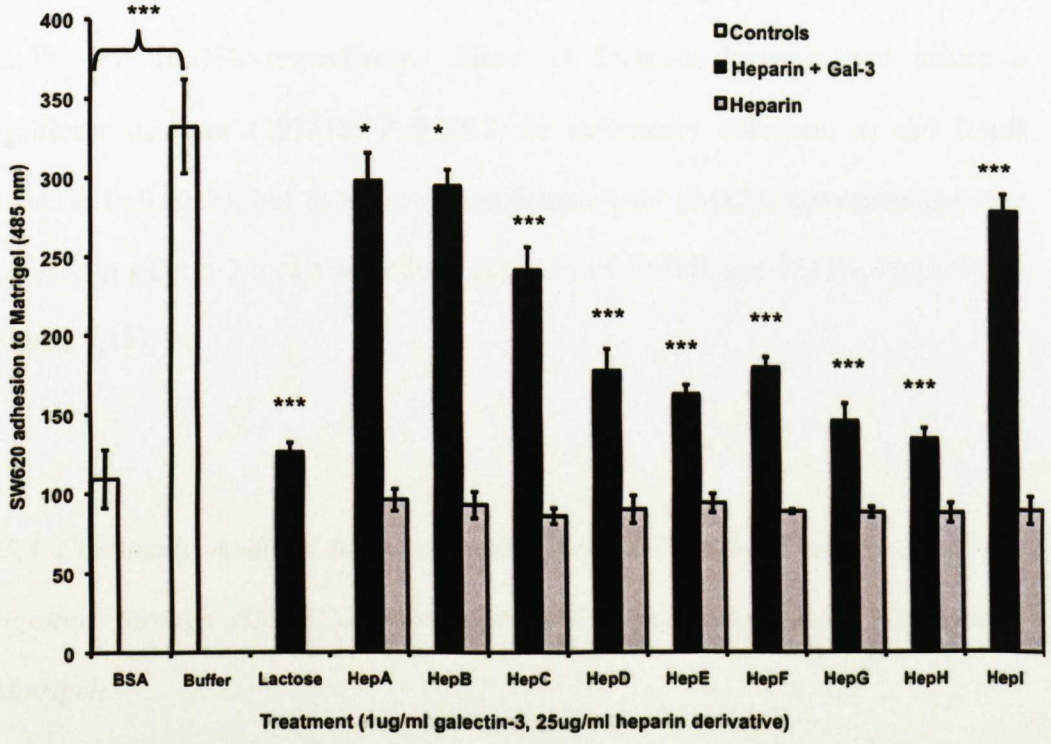


Figure 7.12. Inhibition of galectin-3-induced SW620 cell adhesion to Matrigel. (* P<0.001, ** P<0.01, * P<0.05).**

Adhesion assays performed in a 96-well cell culture plate, with each well containing a thin layer of Matrigel. Heparin derivatives at 25μg/ml were pre-incubated with galectin-3 at 1μg/ml for 30mins, before applying to the wells for 1hr. The experiment was performed twice, with each sample run in triplicate. Analysis with ANOVA, followed by Dunnett's multiple comparison with a control.

Treatment of SW620 colon cancer cells with 1μg/ml galectin-3 induced a significant increase of cancer cell adhesion to Matrigel when compared to the untreated control (332±30; P<0.0001, 109±18, (485nm) respectively). Heparin derivatives HepC-I induced the most significant decrease in galectin-3-mediated cellular adhesion to Matrigel (241±14; P<0.0001, 177±13; P<0.0001, 162±6; P<0.0001, 179±6; P<0.0001, 145±11; P<0.0001, 134±7; P<0.0001, 278±10; P<0.0001 (485nm) respectively), which corresponded to a decrease in galectin-3-mediated cellular adhesion to matrigel of 28±4%, 47±4%, 51±2%, 46±2%, 56±3%, 60±2% and 16±3%, respectively. HepA at 25μg/ml, however, did induce a significant decrease (297±18; P=0.0012) in cell-matrix adhesion, as did HepB (293±10; P=0.0013), but at a lower significance than HepC-I, corresponding to a decrease in galecin-3-mediated cellular adhesion of 11±5% and 12±3%, respectively **(Figure 7.12)**.

7.5.5 Chemically modified heparins inhibit galectin-3-mediated colon cancer cell migration through HMVEC-L monolayers and Extracellular Matrix Components (Matrigel).

Further experiments were carried out to assess whether the inhibitory effect of heparins on galectin-3 binding would affect galectin-3-mediated trans-endothelial

migration through HMVEC-L monolayers, and Matrigel coated well inserts, in the same way that they inhibit galectin-3-mediated adhesion to both HMVEC-L monolayers and Matrigel coatings. SW620 colon cancer cells were used for these trans-endothelial migration experiments as previous finding in this thesis have demonstrated that HT29-5F7 have a lower metastatic potential than SW620 cells. HMVEC-L endothelial cells were grown at the bottom of 24-well transwell insert as a monolayer. 1μg/ml galectin-3 was pre-incubated with 1 x 10^5 cell/ml of colon cancer cells, and this was used as a control to measure any other experimental treatments against. Alternatively, 100μl diluted Matrigel (BD Biosciences, UK) was pipetted into the bottom of a 24-well transwell insert with a pre-cooled pipette tip, to form a gelled layer which presents a matrix component target to which colon cancer cells, SW620 could bind. Cancer cells and controls were treated in the same way as previously stated in the cell-cell adhesion assays. Cellular trans-endothelial migration through both endothelial cells and Matrigel were observed through fluorescence (485nm), which was obtained by pre-labelling with Calcein AM.

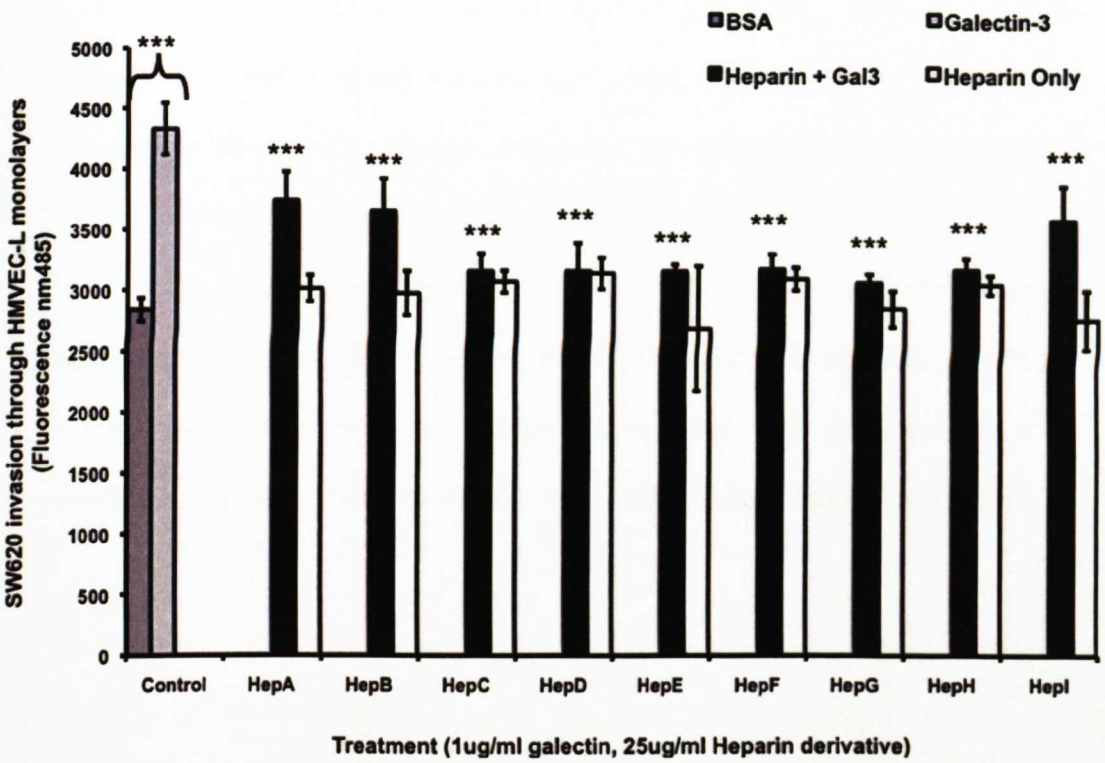


Figure 7.13. Inhibition of galectin-3-mediated SW620 cell trans-endothelial migration through HMVEC-L monolayers. (* P<0.001, ** P<0.01, * P<0.05).**

Invasion assays were performed in a 24-well cell culture insert, with each transwell containing a gelled layer of Matrigel. Heparin derivatives at 25μg/ml were pre-incubated with galectin-3 at 1μg/ml for 30mins, before applying to the wells for 1hr. The experiment was performed twice, with each sample run in triplicate. Analysis with ANOVA, followed by Dunnett's multiple comparison with a control.

Treatment of SW620 colon cancer cells with 1μg/ml galectin-3 induced a significant increase of cancer cell trans-endothelial migration through HMVEC-L monolayers when compared to the untreated control (4329±214; P<0.0001, 2838±96, (485nm) respectively). Heparin derivatives HepC-H induced the most significant decrease in galectin-3-mediated cellular adhesion to Matrigel (3153±147; P<0.0001,

3155±232; P<0.0001, 3157±55; P<0.0001, 3176±125; P<0.0001, 3065±70; P<0.0001, 3170±93; P<0.0001 (485nm) respectively), which corresponded to a decrease in trans-endothelial migration through endothelial cell monolayers of 27±3%, 27±5%, 27±1%, 27±3%, 29±2% and 27±2%, respectively for the heparin fractions. HepA, HepB and HepI at 25μg/ml, also induced a significant decrease in galectin-3-mediated trans-endothelial migration (3735±239; P<0.0001, 3650±267; P<0.0001, 2758±243; P<0.0001 (485nm) respectively), which corresponded with a decrease in trans-endothelial migration of 14±6%, 16±6% and 17±7%, respectively (**Figure 7.13**).

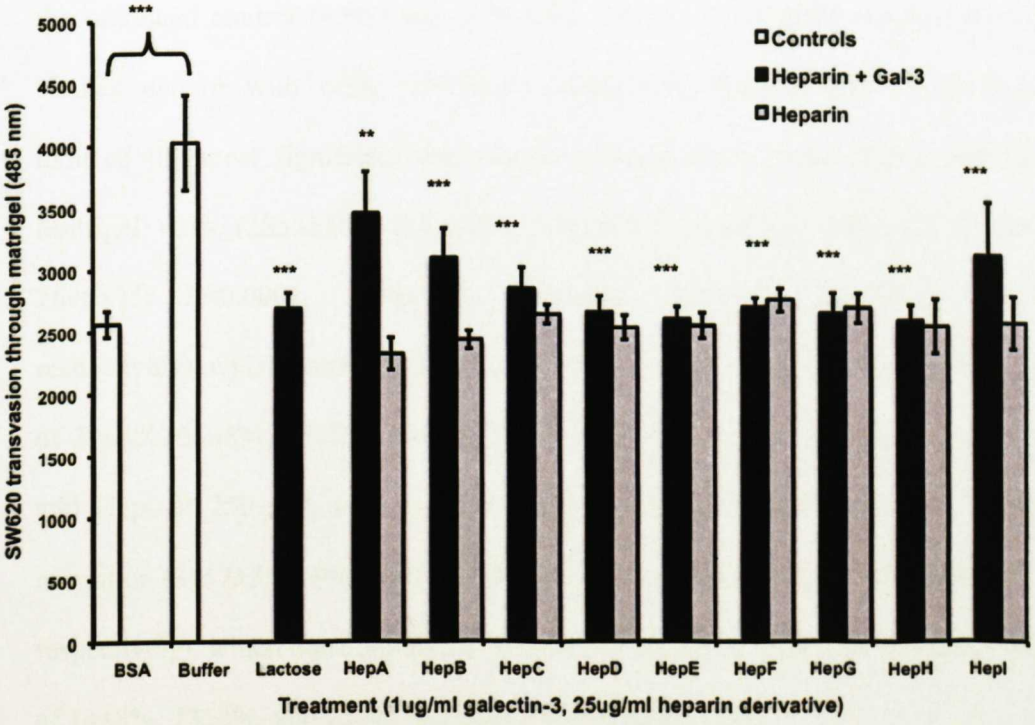


Figure 7.14. Inhibition of galectin-3-induced SW620 cell invasion through Matrigel. (* P<0.001, ** P<0.01, * P<0.05).**

Trans-matrigel migration assays performed in a 24-well cell culture insert, with each well containing a gelled layer of Matrigel. As previously described, BSA represents the negative control wells, with no galectin-3 pre-incubation, to give a basal level of SW620 cell migration through Matrigel, and Galectin-3 represents the positive control wells with untreated galectin-3 added. Heparin derivatives at 25μg/ml were pre-incubated with galectin-3 at 1μg/ml for 30mins, before applying to the wells for 1hr. The experiment was performed twice, with each sample run in triplicate. Analysis with ANOVA, followed by Dunnett's multiple comparison with a control.

Treatment of SW620 colon cancer cells with 1μg/ml galectin-3 induced a significant increase of cancer cell migration through Matrigel layer when compared to the untreated control (4025±382; $P<0.0001$, 2565±106, (485nm) respectively). In a similar pattern with other inhibitory experiments, heparin derivatives HepC-H induced the most significant decrease in galectin-3-mediated cellular adhesion to Matrigel units (2855±164; $P<0.0001$, 2655±135; $P<0.0001$, 2601±97; $P<0.0001$, 2696±75; $P<0.0001$, 2645±169; $P<0.0001$, 2591±122; $P<0.0001$ (485nm) respectively), which corresponded to a decrease in cellular migration through matrigel of 29±4%, 34±3%, 35±2%, 33±2%, 34±4% and 36±3%, respectively. HepA, HepB and HepI at 25μg/ml, also induced a significant decrease in galectin-3-mediated migration (3457±332; $P<0.0001$, 3093±238; $P<0.0001$, 3109±431; $P<0.0001$ (485nm) respectively), which corresponded to a decrease in cellular migration through matrigel of 14±8%, 23±6%, and 23±11%, respectively (**Figure 7.14**).

7.6 DISCUSSION

Previous work described in this thesis has established that circulating galectin-3 (as well as other members of the mammalian galectin family) may exert pro-cancer properties when they are in circulation. Specifically, galectin-3 has been shown to bind to cancer-associated MUC1 via galectin-3-TF antigen interactions. Following galectin binding, MUC1 is polarised, breaking the continuous 'shield' of MUC1 seen on cancer cells, and cell-surface adhesion molecules are consequently exposed (315, 316). Previous experiments have shown that, through this mechanism, galectins can induce a significant increase in cell-cell, and cell-matrix adhesion and trans-endothelial migration, and previous work within our group has also demonstrated that circulating galectins can also induce a significant increase in cell-cell homotypic aggregation (382). Considering the role of galectin-3, with its pro-cancer inducing properties, and the potential role of heparins in the human circulation, with its anti-cancer potential, heparins represent an attractive target for the inhibition of galectin-3, and a subsequent negation of cancer-associated cellular adhesion and trans-endothelial migration.

Native heparin is a complex 'molecular soup', with a variety of repeating disaccharide units. Although it is well acknowledged that galectins generally recognise the terminal galactose on a carbohydrate structure, previous experiments within this thesis using simply glycosylated glycoproteins versus complex glycosylated glycoproteins, have demonstrated that members of the galectin family bind more successfully to targets with a more complex array of carbohydrates, quite possibly including *N*-glycans. With this in mind, this chapter aimed to investigate if the heparin complex sugar mixture and derivatives could inhibit galectin-3 binding thereby inhibit galectin-3-mediated cellular adhesion and trans-endothelial migration.

Specific focus on chemically modified heparin derivatives was undertaken for a number of reasons. Due to the diverse range of effects heparin has on a wide variety of disease states, chemically modified heparins are under development and investigation to possess the parts of the polymeric heparin chain, or modified structures, necessary to enhance the certain effects applicable for each disease therapy. To block the dissemination of metastatic tumour cells, heparin can be administered only at a relatively low concentration *in vivo* due to its anticoagulant potency and potential for inducing hemorrhagic complications (507). The chemically modified heparins which are used in this chapter are selectively de-*O*-sulphated, and have been shown to lack their anti-coagulation anti-Xa activity (508). They have also been shown to be anti-inflammatory (508), and anti-allergic (509).

Chemically modified heparin fractions, notably fractions that have undergone de-*O*-sulfation presented attractive binding targets to galectin-3 at a concentration of 25μg/ml. Native heparin, *N*-acetylated fractions, and re-*O*- and *N*-sulfation also represented galectin-3 binding targets, but only at a higher range of concentrations. Heparin fractions with *N*-acetylation as well as de-*O*-sulfation did not present any increase in inhibitory effect. Therefore, additional modifications did not augment the effect of de-*O*-sulfation, as galectin-3 did not seem to have a significantly increased recognition of these fractions. It would be beneficial to investigate the relevant recognition of the TF structure by galectin-3 in order to assess whether the galactose residue is truly essential. Findings within this chapter suggest that this may not fully be the case, as heparin chains very rarely contain any galactose residues within their structure, yet act as binding partners for galectin-3. With a similar trend to the ELISA experiments, the de-*O*-sulfated fractions significantly inhibited HT29-5F7 and SW620 colorectal cancer cell adhesion to both HMVEC-L monolayers and extracellular

matrix components (Matrigel). Furthermore, de-*O*-sulfated fractions significantly inhibited SW620 colorectal cancer cell invasion through HMVEC-L monolayers and a gelled Matrigel layer. The removal of the sulfate group presumably has allowed the exposure of the carbohydrate antigen underneath, enhancing the level of galectin-3 binding. Thus chemically modified heparins present an attractive possibility for the inhibition of circulating galectin-3-mediated cancer cell metastatic spread. Further experiments must be undertaken to support these findings. Future experiments should go on to include other members of the galectin families, such as galectin-4, as it too is elevated significantly in the sera of colorectal cancer patients. An essential evolution of the experiments within this chapter would be to move on to *in vivo* experiments in mouse model. This would provide more information of the efficacy of chemically modified heparins on inhibition of galectin-3-mediated metastasis.

CHAPTER 8

THE EFFECT OF CORE 1 TRANSFERASE KNOCKDOWN ON EXPRESSION OF CELLULAR *O*-GLYCOSYLATION

8.1 HYPOTHESES

Suppression of the core 1 galtransferase that controls the biosynthesis of TF antigen regulates galectin-mediated cancer cell adhesion.

8.2 AIMS

- To assess the effect of core 1 galtransferase suppression on expression of other *O*-linked glycans in human colon cancer HT29 and SW620 cells.

- To assess the influence of core 1 galtransferase suppression on galectin-mediated cell adhesion

8.3 INTRODUCTION

8.3.1 Core 1 Galtransferase

Mucin-type *O*-glycosylation is a widespread post-translational modification of proteins and is found throughout the entire animal kingdom. The structures of *O*-glycans are diverse and can be classified according to their core structure. The core 1 and core 3 structure are synthesised by the addition of Gal and GlcNAc, respectively, to a GalNAc residue with a β1-3 linkage. It is generally recognised that the core 1 and core 3 structures are in competitive relationship to each other because these structures are formed by the addition of Gal or GlcNAc to the same 3-position of a GalNAc residue (510). The GalNAc of GalNAcα-Ser/Thr (Tn) can also be modified with a sialic acid residue by a sialyl-transferase (ST6GalNAc-T) to form sialic acid-β1,6GalNAcα- (sialyl-Tn) antigen, which is also in competition with the core 1 and core 3 structures (**Figure 8.1**). Particular emphasis has been directed to research on *O*-glycans based on sequences deriving from the Tn antigen, as this antigen is expressed abnormally in several diseases and disorders including cancer (511-516). The Tn antigen is a normal endogenous substrate for several enzymes, most notably Core 1 Galtransferase (C1GalT), also known as T-synthase, a β3-galactosyltransferase that transfers Gal from uridine diphosphate galactose (UDP-Gal) to the Tn antigen to form the core 1 *O*-glycan (262, 517).

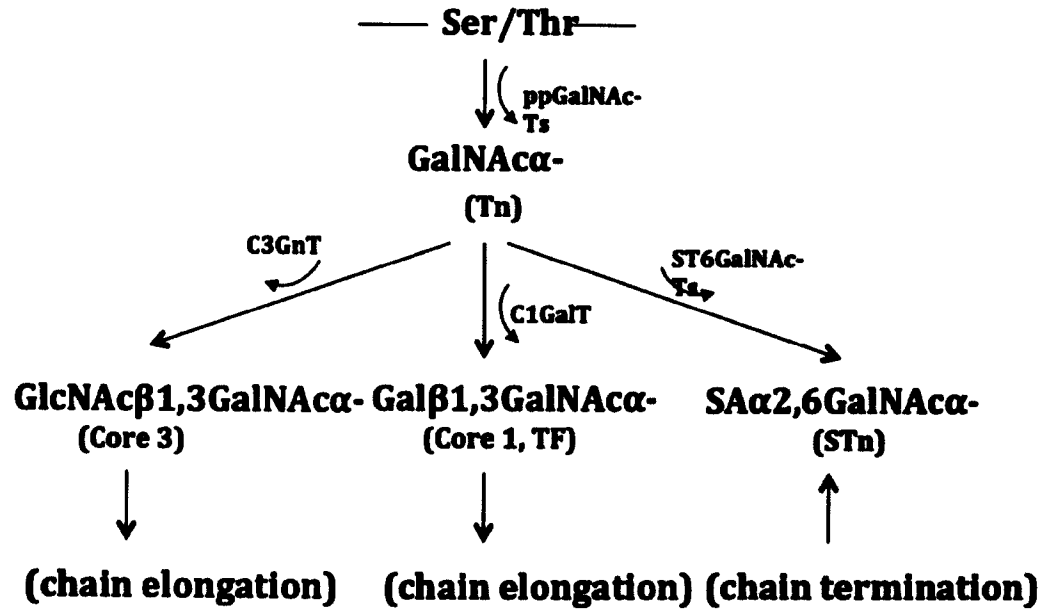


Figure 8.1. Inititation and elongation of the mucin type O-linked glycans.

Studies have shown that a unique and specific molecular chaperone, Cosmc, is required for the formation of the active C1GalT (260, 262). Cosmc interacts with partly unfolded and inactive C1GalT, and has demonstrated an ability to restore C1GalT activity after <5mins incubation (518), with Cosmc binding directly to C1GalT, and to ATP (262). Cosmc has the ability to restore the activity of denatured C1GalT activity independent of other co-chaperones *in vitro*, it is also suggested that co-chaperones may play an important role *in vivo* by regulating the kinetics of the refolding of C1GalT in the crowded environment of the ER by eliminating or removing the inactive and oligomeric C1GalT from the ER lumen (518). In cells lacking Cosmc, it has been shown that inactive C1GalT forms oligomeric aggregates in the ER and is eventually degraded by ubiquitin-dependent pathways in the cytosolic proteasome (260, 262).

Acquired mutations in Cosmc have been shown to lead to loss of C1GalT activity and the expression of abnormal Tn and sialyl Tn antigens, which are known as tumour associated carbohydrate antigens (260-262). Preliminary studies have shown that cervical tumour cells that demonstrate heavy Tn and Sialyl Tn expression typically possess Cosmc mutations (262). Furthermore, disruption of C1GalT in mice results in embryonic lethality, typically caused by defective angiogenesis and lymphangiogenesis (263, 264). Currently, RNA silencing is used to suppress C1GalT and prevent the formation of core 1, and any further elongation of the TF antigen. Therefore, by using this siRNA technology to suppress the formation of core 1, the balance and competition between core structures can by analysed.

8.3.2 RNA Interference and RNA Silencing

RNA interference (RNAi) pathway, a phenomenon originally named cosuppression, was first detected in petunias where the introduction of a pigment producing gene under the influence of a powerful promoter caused the suppression of both the introduced gene and the homologous endogenous gene (519). This process was also identified in other species of plants and fungi where it was named quelling (520). Further discoveries were made following the injection of dsRNA into the gonads of *Caenorhabditis elegans*, which resulted in potent gene silencing and established dsRNA as the inducer of RNAi (521). Short interfering RNAs (siRNAs) were first implicated in plants as part of the post-translational gene silencing in plants (PTGS)(522) which led to subsequent work on the *Drosophila* system, which indicated that dsRNA was processed into siRNAs of 21-25bp in length which could

cause mRNA cleavage corresponding to the introduced dsRNA, forming the basis of the RNAi pathway (523).

Higher eukaryotes have many antimicrobial defence mechanisms that are ultimately based upon the recognition of conserved molecular patterns, including double stranded RNA (dsRNA). The RNAi pathway is an antiviral immune response to dsRNA, which is a key component in the genomic replication of viral DNA and not typically seen in eukaryotic cells as a normal activity (524). This innate cellular response can be induced artificially by the introduction of exogenous dsRNA or short interfering RNA (siRNA), which stimulates the RNAi pathway. When early attempts to utilise the RNAi pathway for gene expression were unsuccessful, it was discovered that dsRNA molecules larger than 30bp caused an interferon-mediated response, which led to apoptosis of the cells. The RNAi mechanism is extremely sequence specific, and responds to dsRNAs by selectively degrading mRNAs that are homologous in sequence to the dsRNA inducer. RNAi, therefore, is capable of blocking the expression of not only viral, but host cell genes, upon the introduction of homologous dsRNA. This allows the specific knockdown of proteins by the introduction of sequence specific dsRNAs.

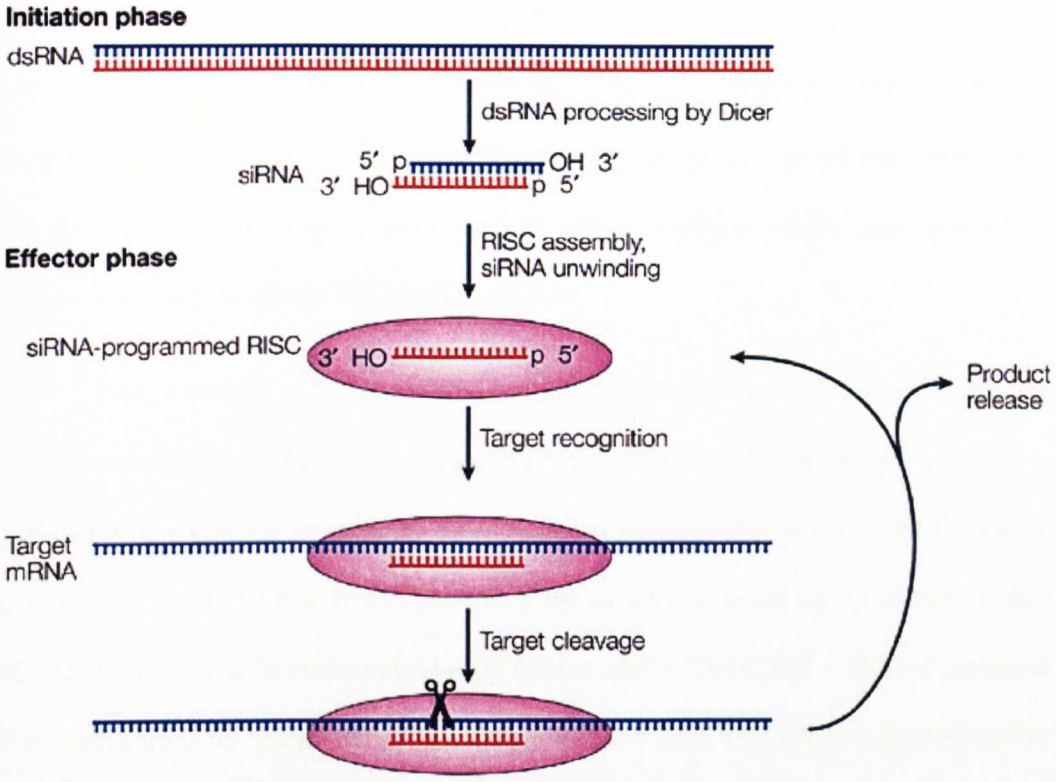


Figure 8.2. The general pathway of RNAi *in vitro* (525).

In vitro, the mechanism for RNA silencing by inducing the RNAi pathway in mammalian cells begins with the introduction of dsRNA, which is cleaved into 21-23bp siRNA duplexes by the ribonuclease-III enzyme Dicer (526). siRNAs bare 5'-phosphate groups and 2-bp 3' overhangs, both of which are important for subsequent siRNA-induced silencing complex (siRISC) assembly (525). The siRNAs can be chemically synthesised and can be directly introduced into the cytoplasm with the use of transfection reagents. During the effector phase of the RNAi pathway, the siRNA is unwound (527) by an unwindase enzyme, which leads to the assembly of RISC. This is the activated effector complex, and it recognises the target by siRNA to mRNA base pairing, and cleavage of the mRNA strand is achieved through its

endoribonuclease activity (528) (**Figure 8.2**). This process is accelerated by the presence of ATP, which increases the enzyme turnover by promoting siRNA-product unwinding and product release (529). The remaining 'guide' strand associates with the PAZ domain of an Argonaute (Argo2) protein (530) at which point the RISC complex is ready to cleave the specific mRNA.

How the RISC complex works is still being elucidated, but the main points are understood with work being carried out within the *Drosophila melanogaster* system. Following the dsRNA processing the siRNA will either stay attached to the Dicer (Dcr2)-R2D2 heterodimer it was generated by, or will be taken up by another Dcr2-R2D2 complex in a formation previously known as R1. The R2D2 will bind the more thermodynamically stable end of the siRNA and the Dcr2 will bind to the less stable end. This asymmetric binding facilitates the determination of the guide strand of siRNA from the strand which will be loaded into the RISC complex later on. The siRNA then enters the RISC-loading complex (RLC) which was known before as R2, and associated with other as yet unknown RISC factors and one of the siRNA strands (the 'passenger' strand) is discarded (**Figure 8.3**).

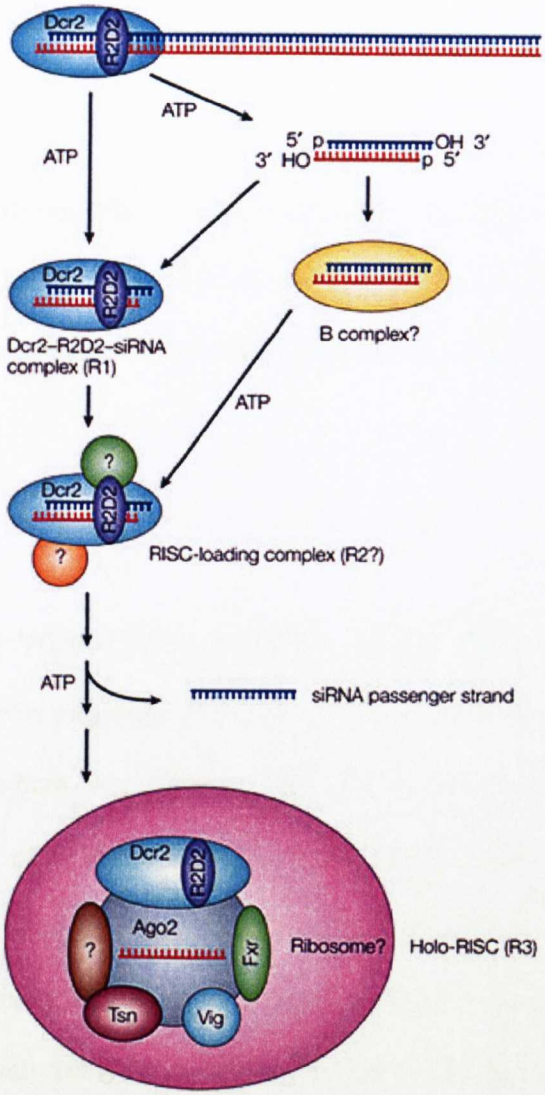


Figure 8.3. The assembly of RISC in the *Drosophila melanogaster* (525).

8.4 METHODS

8.4.1 SiRNA Galtransferase

Cell Plating:

Both HT29 cells and SW620 cells were diluted in antibiotic-free DMEM with 5% FCS to a plating density of $5.0x10^4$ cells/ml. 100µl of cells were placed into each well of a 96 well plate (Sigma-Aldrich Ltd., UK) and incubated for 24hrs at 37°C.

Transfection:

A 2µM either targeted or the scrambled control siRNA solution in siRNA buffer (Dharmacon) was prepared, from which 17.5µl was added to 17.5µl serum-free/antibiotic free medium. In a separate tube, 1.4µl of DharmaFECT4 (Dharmacon) was added to 33.6µl of FCS-free/antibiotic-free DMEM. These tubes were gently pipetted up and down and left for 5mins before mixing them together and leaving them to stand for a further 20mins. To this solution 280µl antibiotic free DMEM was added and to each well 100µl was added. The plate was incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air for 48hrs.

8.4.2 Slot Blotting

Slot blotting was carried out as described in chapter **4.8.**

8.5 RESULTS

8.5.1 Suppression of Core 1 GalTransferase in human colon cancer cells by siRNA treatment.

siRNA suppression of C1GalT was conducted in human colon cancer HT29 and SW620 cells. The efficiency of C1GalT suppression was monitored by assessing the expression of TF expression with anti-TF antibody, TF5 (531).

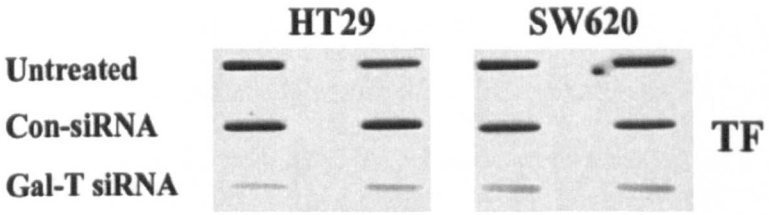


Figure 8.4. Slot blot assessments of cellular TF expression with anti-TF TF5 antibody.

Colon cancer cells were treated with the appropriate siRNA for 48hrs at 37°C before lysing with slot blot buffer (4X stacking buffer 2.5ml, glycerol 1.0ml (Sigma-Aldrich Ltd., UK), mercaptoethanol 0.5ml (Sigma-Aldrich Ltd., UK), 20% SDS on H_2O 1.0ml). Cell lysate was then diluted 1:1 with Tris buffer. 50µl of the diluted sample was loaded on to the respective well within the slot blot rig after further dilution with 100µl of Tris buffer to ensure even distribution of proteins. The blot was probed with anti-TF TF5 antibody at a concentration of 0.2µg/ml, diluted in blocking buffer (5% BSA, 0.5% tween-20 in PBS).

C1GalT siRNA treatment of HT29 cells for 48hr caused effective suppression of C1Gal1T expression as manifested by a 86% (86±3%, mean±SD) reduction of cellular TF expression. A similar reduction of the TF expression (84±2%) was also observed in SW620 cells after C1GalT siRNA treatment for 48hrs (**Figure 8.4**).

8.5.2 Suppression of C1GalT is associated with an increase in Tn, Sialyl-Tn, and Core 3 structures.

Considering that treatment of colon cancer cells with siRNA targeted for C1GalT expression successfully suppressed the C1GalT expression, further experiments were carried out to assess the cellular expressions of sialyl-Tn, Core 3 and Tn glycans. The Tn and sialyl-Tn antigens were assessed with Anti-Tn (clone HB-Tn1) and Anti-Sialyl Tn (clone HB-STn1) antibodies (Dako Pathology Products, Ely, UK). Core 3 was assessed with GlcNAc-binding *Griffonia simplicifolia* lectin II (GSL-II). GSL-II is a lectin isolated from *Griffonia (Bandeiraea) simplicifolia* and recognizes exclusively α- and β-linked GlcNAc residues on the non-reducing terminal of oligosaccharides (532). Working concentrations of antibodies and lectins are as listed in **Table 4.7 and 4.8**.

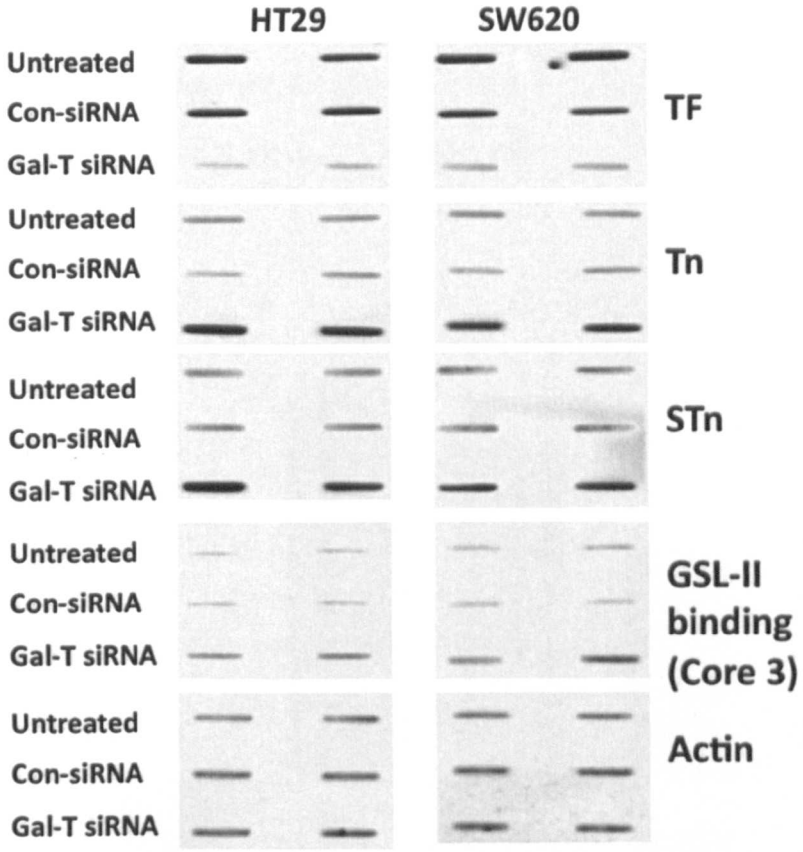


Figure 8.5. Slot blot detecting the presence of carbohydrate structures following C1GalT knockdown.

Cellular glycan expression in response of the cells to siRNA C1CalT. After treatment of the cells with siRNA C1GalT or control non-targeting siRNA (con-siRNA). HT29 and SW620 colon cancer cells were treated with C1GalT for 48hrs and assessed for the relative expression of carbohydrate structures. **Tn:** GalNAc-α-Ser/Thr. **STn:** Sialyl-Tn, sialic acid-β1,6GalNAc-Ser/Thr. **Core 3:** GlcNAcβ1,3GalNAcα-Ser/Thr. **Actin:** Control blot to ensure equal loading. Blots were run in tandem and slots were loaded in duplicate.

Confirmation of relative expressions of the carbohydrate structures was analysed by subsequent densitometry quantification of the density of the blots using Image Lab software (Bio-Rad, Hemel Hempstead, UK).

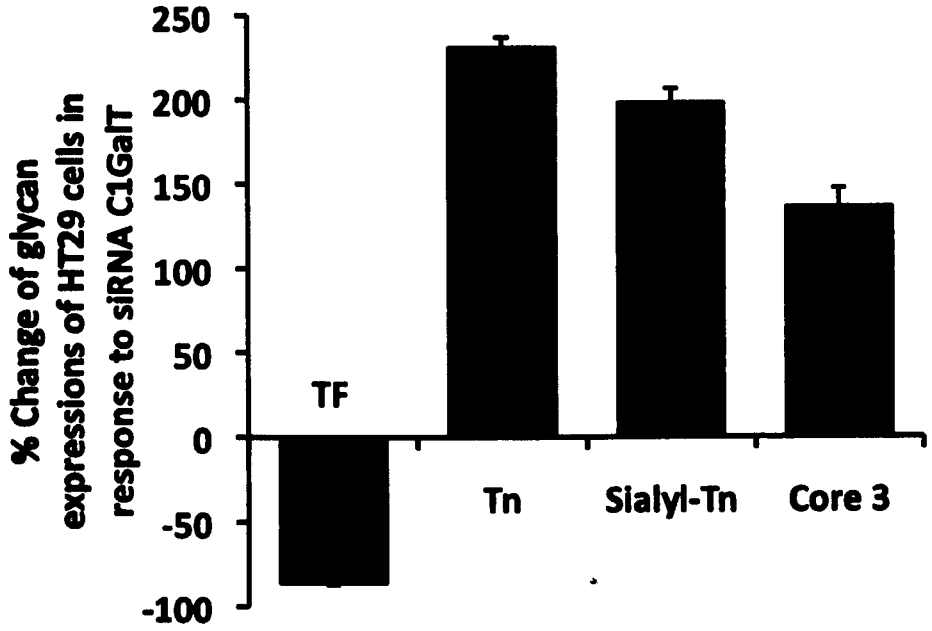


Figure 8.6a. Quantification of the expression of cellular carbohydrate structures following the treatment of HT29 colorectal cancer cells with siRNA targeted to C1GalT.

Quantification of the expression of cellular TF, Tn, sialyl-Tn, sialic acid and Core 3 glycans in response of the cells to siRNA C1GalT in HT29 colon cancer cells. Densities of the slots blots were quantified and the glycan expressions are expressed as percentage change to the non-siRNA control after normalization with protein loading.

Following C1GalT knockdown in HT29 colon cancer cells, which was confirmed by a reduction in TF antigen expression, there was a noted increase in Tn

antigen (231±6%), sialyl Tn (198±8%), and an increase of the core 3 structure (136±24%) (**Figure 8.6a**).

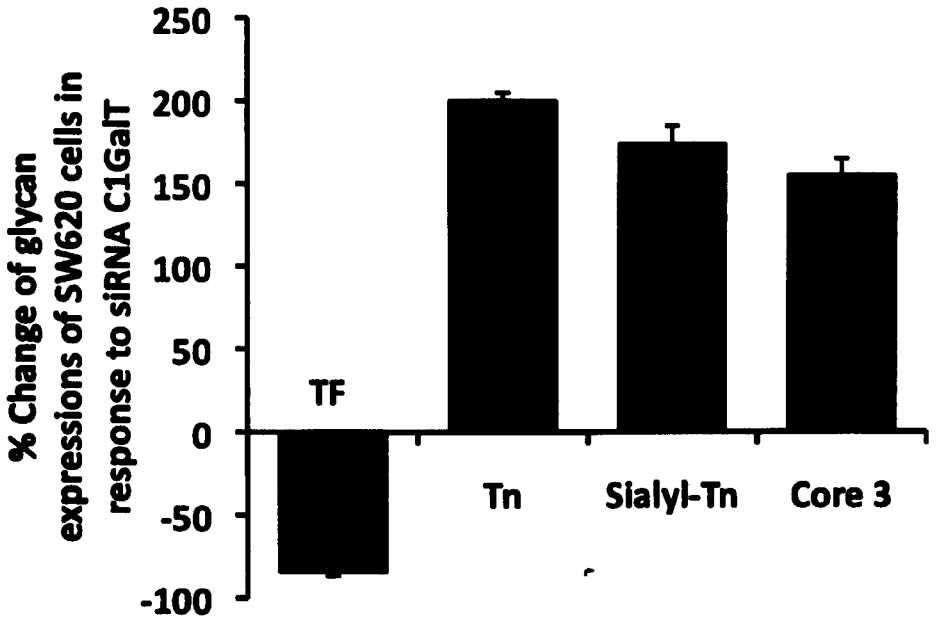


Figure 8.6b. Quantification of the expression of cellular carbohydrate structures following the treatment of SW620 colorectal cancer cells with siRNA targeted to C1GalT.

Quantification of the expression of cellular TF, Tn, sialyl-Tn, sialic acid and Core 3 glycans in response of the cells to siRNA C1GalT in SW620 colon cancer cells. Densities of the slots blots were quantified and the glycan expressions are expressed as percentage change to the non-siRNA control after normalization with protein loading.

Following C1GalT knockdown in SW620 colon cancer cells, which was confirmed by a reduction in TF antigen expression, there was a similar pattern of

carbohydrate structure increase as seen in siRNA treatment of HT29 cells. There was a noted increase in Tn antigen (200±5%), sialyl Tn (174±11%), and an increase of the core 3 structure (155±34%) (**Figure 8.6b**).

8.6 DISCUSSION

The biosynthesis of *O*-linked mucin type glycans is a multi-stepped, sequential, post-translational process catalysed by the expressions and activities of an array of glyco-transferases. It has been speculated that the C1GalT, C3GnT and ST6GalNAc-T compete to modify the GalNAc residue of the newly-synthesised GalNAcα-Ser/Thr for the formation of TF, Core 3 or sialyl-Tn structures in living cells(54, 96). Mutation or inactivation of Cosmc, an ER-localized molecular chaperone that is required for the enzyme activity of C1GalT(533), has been shown to be associated with the Tn syndrome, a rare autoimmune disease in which subpopulations of the blood cells carry the incompletely glycosylated Tn antigen(534). Furthermore, treatment of human cancer cells with the O-glycosylation inhibitor Benzyl-GalNAc, a competitive inhibitor for C1GalT transferase and alpha-2,3-sialyltransferase, decreases the expression of cellular sialic acids and increases the expression of TF (535).

The results within this chapter demonstrate that suppression of the C1GalT that controls the biosynthesis of the Core 1 structure of mucin type O-linked glycans is associated with increased expressions of sialyl-Tn and Core 3 glycans in human colon cancer cells. This provides direct evidence of the long-suspected competition between C1GalT, C3GnT and ST6GalNAc-T transferases for modification of the

GalNAc residue of GalNAcα-Ser/Thr in the biosynthesis of complex O-linked mucin type glycans.

The ultimate formation of cellular TF, Tn, sialyl-Tn and Core 3 glycans are controlled not solely by the activity of these 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(536). The relative positioning of the glyco-transferases within the Golgi is also reported to be an important determinant. Work by Kellokompu and colleagues (537, 538) and by ourselves (539) 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. Furthermore, the expression and action of ER-localized molecular chaperones can also play a role in the expression of the oncofetal glycans by controlling the folding and hence the activity of the relevant glyco-transferases(533). Thus, the overall cellular expressions of Tn, sialy-Tn, TF and Core 3 structures are the consequence of a range of complex factors that include competition between the relevant glyco-transferases, the spatial arrangement of the glyco-transferases within the Golgi, the availability of nucleotide sugar-donors in the Golgi apparatus and actions of relevant molecular chaperones.

The Tn, TF and sialyl-Tn antigens are all known as oncofetal carbohydrate structures. They are expressed in fetal epithelia then become concealed by other sugar residues in healthy adult tissue but reoccur in cancerous and pre-cancerous dysplastic cells. It is estimated that up to 90% of all human cancers carry these oncofetal carbohydrate antigens (540-543). Increased occurrence of these oncofetal carbohydrate structures is associated with the development and progression of various

human cancers including breast (544), colon (540, 545) and pancreatic (546) cancers. Increasing evidence suggests that alteration of these oncofetal glycans may play an active role in metastasis. Over expression of sialyl-Tn antigen by cancer cells has been shown to cause more aggressive cell behaviour such as increased adhesion to extra-cellular matrix and increased migration and invasion *in vitro* (547). An increased interaction between TF expressed on cancer-associated mucin protein MUC1 and circulating galectin-3, as a result of the increased expression of TF-expressing MUC1 by cancer cells and also the increased release of galectin-3 by cancer/stromal/immune tissue/cells into the circulation, both of which are common features in cancer, has been shown to promote cancer cell metastatic spread in an nude mice metastasis model (548, 549). This effect of the TF/MUC1-galectin-3 interaction occurs as a result of the increased cancer cell heterotypic adhesion to vascular endothelium(549) and also as a result of cancer cell homotypic aggregation to form micro-tumour emboli that prolong tumour cell survival in the circulation and allow lodging within capillaries at the metastatic site(550, 551). It has also been reported that breast cancer patients with higher levels of anti–TF antibody show better prognosis than the patients with lower anti-TF levels(552). Targeting these oncofetal glycans by immunotherapy with TF-mimicking peptides for potential cancer treatment has shown promising results in mice (553).

As galectin-mediated cancer cell adhesion to endothelial cells act via binding to the TF structure on MUC1, further experiments are required to analyse the effect of C1GalT knock down on galectin-mediated cell-cell interactions. Due to time restrains, this was not carried further in this study.

CHAPTER 9

SUMMARY OF THE MAIN FINDINGS IN THIS THESIS

- The expression of circulating galectin-2, -3, -4, and -8 increases in the sera of patients with colorectal cancer, and the expression of circulating galectin-1, -2, -3, -4, -8 and -9 increases still further in the sera of patients with colorectal cancer and liver metastasis.

- The expression of circulating galectin-2, -3, and -8 increases in the sera of patients with breast cancer, while the expression of circulating galectin-1 decreases in the sera of patients with breast cancer.

- As a serum cancer marker, CEA-based ELISA demonstrates more specificity and sensitivity than galectin-3 and -4 combined ELISA when comparing sera from patients with colorectal cancer and sera from healthy individuals.

- Galectin-3 and -4 combined ELISA demonstrates more specificity and sensitivity than CEA-based ELISA when comparing sera from patients with colorectal cancer and liver metastasis and sera from healthy individuals, and when comparing sera from patients with colorectal cancer only and those with liver metastasis.

- All the galectin members tested in this study show binding to TF expressing glycoproteins (antifreeze glycoprotein, asialofetuin, and asialo bovine mucin) although with different affinities. Removal of the TF antigen by *O*-glycanase significantly reduced the level of galectin-binding to asialo bovine mucin.

- Galectins induce increase of MUC1-expressing cancer cell (HT29-5F7, SW620 and HCA1.7+) adhesion to endothelial cell monolayers (HUVEC and HMVEC) and also to extracellular matrix components (matrigel), but not of MUC1-negative cell lines (HCA1.7-) or cells weakly expressing MUC1 (parental HT29 cell line).

- Galectins induce an increase of MUC1-expressing cancer cell trans-endothelial migration and migration through extracellular matrix components (Matrigel).

- Galectin-mediated cellular adhesion to endothelial cell monolayers is inhibited by pre-incubation of galectins with lactose or asialofetuin.

- The removal of TF antigen by *O*-glycanase, or the presence of anti-TF-antibody negates galectin-mediated cancer cell adhesion to endothelial cell monolayers.

- Pre-incubation of MUC1-expressing cancer cells with each of the galectins induces MUC1 cell surface polarisation.

- Pre-fixation of MUC1-expressing cancer cells negated galectin-mediated adhesion to endothelial cell monolayers.

- The knockdown of MUC1 expression by siRNA treatment negates galectin-mediated cancer cell adhesion to endothelial cell monolayers.

- Several chemically modified heparin derivatives inhibit galectin-3 binding to asialo bovine mucin, but at different concentration ranges.

- Several chemically modified heparin derivatives inhibit galectin-3-mediated colon cancer cell adhesion to HMVEC-L monolayers and Extracellular Matrix Components (Matrigel).

- Several chemically modified heparin derivatives s inhibit galectin-3-mediated colon cancer cell migration through HMVEC-L monolayers and Extracellular Matrix Components (Matrigel).

- Treatment of human colon cancer cells with Core 1 GalTransferase siRNA is associated with an increase in Tn, Sialyl-Tn, and Core 3 structures.

CHAPTER 10

GENERAL DISCUSSION AND IMPLICATIONS FOR FUTURE STUDIES

The relationship between cancer and the glycome is intriguing. The distinct changes provide a potential binding target for carbohydrate-binding lectins, such as the β-galactoside-binding galectins. The human digestive tract is rich in galectins, and in the colon and rectum, four galectins, galectin-1, -3, -4 and -8, are known to be present(281-283). The wide body of earlier research that constitutes the knowledge of galectin presence and the subsequent cellular effects they produce is largely limited to galectins found intracellularly, and those that are extracellular but still associated with the cell membrane. Recent research on galectins by our group (315, 316, 382) has been focused upon those galectins in the circulation. Iurisci *et al.*(287) demonstrated earlier that the concentrations of circulating galectin-3 are increased up to 5-fold in the serum of colorectal cancer patients (287)and the concentrations of circulating galectin-3 are shown to be higher in patients with metastasis than those with localized tumours (287). Investigations within this thesis have shown that circulating galectin-2, -3, -4, and -8 increase in the sera of patients with colorectal cancer, and the expression of circulating galectin-1, -2, -3, -4, -8 and -9 increase in the sera of patients with colorectal cancer with liver metastasis. Furthermore, when comparing colorectal cancer patients with or without liver metastasis, there was a general increase in the group with metastasis, seen with all galectins investigated. It was also demonstrated that the expression of circulating galectin-2, -3, and -8 increases in the sera of patients with breast cancer, while the expression of circulating galectin-1 decreases in the sera

of patients with breast cancer. The source of the increased serum galectins is not known. However, at least for galectin-3, it has been speculated that the galectin is generated and secreted by the tumour cells themselves, as well as the peri-tumoral inflammatory and stromal cells (287). Whichever the case, this marked cancer-associated increase of circulating galectins presented two research pathways, as investigated within this thesis: the potential of galectins as biomarkers, and the significance of the increased circulating galectins as important metastasis promoters.

Among those galectins, galectin-3 and -4 both presented the most significant increase in the serum of colorectal cancer groups when compared to healthy samples. Neither of them are, however, as sensitive or specific as the CEA measurements for cancer biomarkers. However, combined galectin-3 and -4 measurements seems to provide a better measurement than CEA for separate colorectal cancer patients with and without liver metastasis, although CEA measurements demonstrated a higher level of specificity and sensitivity when comparing colorectal cancer patients and healthy individuals. This suggests that a combined galectin-3 and -4 measurements may hold potential as a useful test for monitoring cancer progression. A most recent study has suggested that galectin-4 could be useful as a complementary marker when combined with CEA/CA19-9 to improve CRC follow-up, considering that combined use of galectin-4 with CEA and/or CA19-9 markedly increased the proportion of CRC patients who were positive for tumor markers when compared to CEA or CA19-9 (381).

It has been shown earlier by studies in our laboratory that the increased circulation of galectin-3 in cancer patients is an important promoter in metastasis by inducing heterotypic cancer cell-endothelium adhesion and homotypic aggregation (315, 330), an effect that is associated with galectin-3 interaction with the TF antigen

on cancer associated-MUC1 and induction of MUC1 cell surface polarization and exposure of the smaller cell surface adhesion molecules (316). This study shows that those galectins showing increase in the blood circulation of cancer patients binds to MUC1 and all produced similar increases of cancer cell adhesion to endothelial monolayers, as galectin-3. This is associated with the ability of the galectins to induce the formation of multimeric 2D and 3D lattice structures of MUC1. It is known that lattice formations induced by galectins provides stability and subsequent biologic functionality (279). All members of the galectin family, whether prototype, chimera or tandem repeat, possess this feature of multivalency, and are shown to dimerise, or form higher-order oligmers in solution (554), making them well suited for mediating cellular adhesion and eliciting signaling (555, 556). This could be corroborated by the fact that PNA, a tetrameric protein containing two carbohydrate recognition domains (271), induces the same MUC1 polarisation response that the galectin family have demonstrated.

These galectin-mediated effects on cancer cell adhesion are associated with their binding to TF on MUC1. All those galectins showing increase in the blood circulation of cancer patients binds to TF disaccharides. Lectin–monosaccharide interactions are known to be relatively weak (dissociation constants 10^4 M^{-1}) (557-559), and that galectins demonstrate a preference for β-galactoside-containing glycans comprised of repeating units of N-acetyllactosamine (Galβ1,4GlcNAc; LacNAc), as disaccharide units at the termini of complex N- glycans or as repeating backbone units in a poly-N-acetyllactosamine chain on N-glycans or O-glycans (558-560). Glycoproteins, (and in the case of this functional model, MUC1) bear multiple copies of the carbohydrate ligands that are recognized by galectins. Again in this case the target is TF antigen, a weak binding partner. Whereas galectin binding to a single

ligand is typically low-affinity, the multivalent nature of galectin–carbohydrate interactions produce high overall avidity (association constants 10^6 M^{-1}) (558, 561). Furthermore, despite the TF antigen being a weaker binding target of galectins this study shows that the galectin-TF/MUC1 interactions increase cell adhesion.

Galectin-3 has been the most clearly defined circulating galectin in terms of the MUC1-based pro-cancer properties, and has previously been shown by our laboratory to increase the likelihood of metastasis *in vivo* (315, 316, 382). Due to this, galectin-3 represents an attractive target for therapy through its inhibition. This thesis demonstrates that chemically modified heparin derivatives can block galectin-3 binding and galectin-3-mediated cell adhesion thus represents a therapeutic candidate. Heparin has already attracted attention as a possible treatment for cancer, and it has been shown that heparin treatment negates cancer promoting steps most efficiently when it is in circulation (474, 484, 496). Native heparin is a complex 'molecular soup', with a variety of repeating disaccharide units, which by its very nature could provide galectin-3 with a wide selection of possible binding targets, in turn increasing the likelihood of galectin-3 inhibition. This thesis has demonstrated that selectively de-*O*-sulphated heparin derivatives successfully negate galectin-3 mediated adhesion to and migration through endothelial cells as well as matrix components. Less modified derivatives were shown to produce inhibition, but only at higher ranges of concentration. Furthermore, the most effective heparin derivatives that showed inhibition of galectin-3 binding also demonstrated significantly reduced anti-coagulation effects., This suggests that chemically modified heparins present an attractive target for the inhibition of circulating galectin-3-mediated cancer cell metastatic spread.

A feature of colon carcinogenesis is shown to be accompanied by an increase in carbohydrate structures of less complexity, such as the core antigens Tn, TF and their siaylated counterparts sialyl-Tn and sialyl-TF, as well as the blood group antigens Lewis^a, Lewis^b, Lewis^x and Lewis^y (199). Recent estimates suggest that up to 90% of all human cancers carry oncofetal carbohydrate antigens (540-543), and their occurrence is associated with the development and progression of various human cancers including breast (544), colon (540, 545) and pancreatic (546) cancers. Furthermore, the TF-galectin-3 interaction has been shown to be the pivotal mechanism by which galectin-3 can induce cancer cell adhesion and aggregation. It presents an interesting concept, that carbohydrate changes during disease states act synergistically with the cancer-associated increase of galectins to promote the spread of cancer by providing more binding targets. The results within this thesis have demonstrated that suppression of the C1GalT, and therefore the Core 1 structure of O-linked glycans, results in increased expressions of sialyl-Tn and Core 3 glycans in human colon cancer cells. Not only does this provide direct evidence of competition between C1GalT, C3GnT and ST6GalNAc-T transferases for modification of the GalNAc residue of GalNAcα-Ser/Thr within proteins, but a potential therapy. It been reported that breast cancer patients with higher levels of anti–TF antibody show better prognosis than the patients with lower anti-TF levels (552), and that targeting the oncofetal glycans by immunotherapy with TF-mimicking peptides for potential cancer treatment has shown promising results in mice (553). Furthermore, these results are an early indication that the suppression of C1GalT is a potential alternative method to reduce cancer-associated antigens, and to potentially restore the normal glycan structures in the gut.

IMPLICATIONS FOR FUTURE STUDIES

This thesis has raised many interesting questions that require further investigation. Further work is required to expand the serum data set and determine any correlations between circulating galectin expression and cancer stages. Furthermore, further investigations should be carried out that combines galectins and other colorectal cancer serum markers in ELISA-based tests. These experiments could potentially uncover novel serological tests that could provide enhanced sensitivity and specificity for diagnosis and predicting prognoses.

Galectins have been shown to bind to cell-surface receptors, and become involved in the regulation of receptor segregation and turnover, which leads to modulation of cell growth, differentiation and survival (422). In a similar manner, galectin-3 binding and subsequent repolarisation of MUC1, may induce changes in cell signaling and downstream cellular effects. It has been demonstrated that MUC1 can bind intercellular adhesion molecule-1 (ICAM-1) on surrounding accessory cells and facilitates trans-endothelial migration of MUC1-bearing cells (155, 157, 159). Furthermore, it was acknowledged that MUC1 triggers Rac1- and Cdc42-dependent actin cytoskeletal protrusive activity (562). It would be an interesting concept to see whether the influence of galectin-3-MUC1 interaction has an effect on the activation of members of the small GTPase Rho family, as these family members are crucial regulators in directing actin cytoskeletal reorganization and formation of motile lamellipoldial or filopodial protrusions (563), which would influence cell surface MUC1 localization in cell reponse to galectin binding.

Galectins have emerged as promising molecular targets for cancer therapy, and galectin inhibitors have the potential to be used as anti-tumour and anti-metastatic agents. Within this thesis, chemically modified heparin derivatives have demonstrated themselves to be effective galectin-3 inhibitors. Future experiments should go on to assess the effectivity of heparins on inhibiting other members of the galectin families, such as galectin-4, as it also is elevated significantly in the sera of colorectal cancer patients and increase cancer cell adhesion to vascular endothelium in vitro. An essential evolution of the experiments within this chapter would be to move on to *in vivo* experiments in a mouse model. This would provide more information of the efficacy of chemically modified heparins on inhibition of galectin-mediated metastasis.

Suppression of C1GalT has provided interesting confirmation of the competition of enzymes involved in O-glycan chain elongation. It would be a useful confirmation to determine the level of expression of C1GalT transcripts by QPCR, as well as the level of expression of the other enzymes (ST6GalNAc I, Core3 synthase) to confirm that no change occurred in treated cells. Further structural characterization of O-linked glycans should be carried out by mass spectrometry. A further facet to investigate would be to detect any potential changes in the level of galectin-binding to C1GalT siRNA-treated cells and begin assessment of the hypothesis that changes in expression of oncofetal carbohydrate structures change the ability and affinity of galectin binding. This would provide interesting information on the likelihood of transferase-based therapy in the diseased gut.

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APPENDIX 1

Components of Cell Culture Medium

Dulbecco's Modified Eagles Medium (DMEM)

Components	[1x] g/L
Inorganic salts:	
Calcium Chloride	0.2
Ferric Nitrate • $9H_2O$	0.0001
Magnesium Sulfate (anhydrous)	0.09767
Potassium Chloride	0.4
Sodium Bicarbonate	3.7
Sodium Chloride	6.4
Sodium Phosphate Monobasic (anhydrous)	0.109
Amino acids:	
L-Arginine • HCl	0.084
Glycine	0.03
L-Histidine • HCl • H_2O	0.042
L-Isoleucine	0.105
L-Leucine	0.105
L-Lysine • HCl	1.46
L-Phenylalanine	0.066
L-Serine	0.042
L-Threonine	0.095
L-Tryptophan	0.016
L-Tyrosine • 2Na •$2H_2O$	0.10379
L-Valine	0.094
Vitamins	
Choline Chloride	0.004
Folic Acid	0.004
myo-Inositol	0.0072
Niacinamide	0.004
D-Pantothenic Acid (hemicalcium)	0.004
Pyridoxine • HCl	0.004
Riboflavin	0.0004
Thiamine • HCl	0.004
Other components	
D-Glucose	4.5
Phenol Red • Na	0.0159
Pyruvic Acid • Na	0.11

Lonza Clonetics™ Endothelial Cell Medium Products

EGM™ Endothelial Growth Medium & EGM™ BulletKit™

Basal media developed for Normal Human Endothelial Cells in a low-serum environment.

EGM™ and EGM™-BulletKit™

BBE (Bovine Brain Extract), with heparin
hEGF
Hydrocortisone
GA-1000 (Gentamicinm Amphotericin B)
FBS (Fetal Bovine Serum) 10ml

Final serum concentration is 2%

EGM™-2 Endothelial Growth Medium

Refinements to basal medium and growth factors.
Further defined, does not contain BBE

Final serum concentration is 2%

EGM™-2 BulletKit™

hEGF
Hydrocortisone
GA-1000 (Gentamicinm Amphotericin B)
FBS (Fetal Bovine Serum) 10ml
VEGF
hFGF-B
R3-IGF-1
Ascorbic Acid

APPENDIX 2

LIST OF FIGURES

LIST OF TABLES

6.1 Quantification of MUC1 repolarisation in MUC1 transfected HCA1.7+ with or without pre-incubation with galectins.

6.2 MUC1 repolarisation in MUC1 transfected HCA1.7+ with or without pre-incubation with PNA.

7.1 Potential use of heparins beyond anticoagulation

7.2 Binding of galectin-3 to asialo bovine mucin, with or without pre-incubation with chemically modified heparin derivatives at range of concentrations (μg/ml).

PUBLISHED WORK AND PRESENTATIONS

Circulating galactoside-binding galectins are increased in colorectal cancer patients, promote cancer cell adhesion to HUVEC and may have an important role in cancer metastasis.
Hannah Barrow, Jonathan Rhodes and Lu-Gang Yu
Oral presentation, BSG, Glasgow, 2009

Circulating galactoside-binding galectins are highly elevated in colorectal cancer patients with liver metastasis and promote cancer cell adhesion to HUVECs.
Hannah Barrow, Jonathan Rhodes and Lu-Gang Yu
Poster presentation, DDW, Chigago, 2009

Circulating galectins and metastasis
Hannah Barrow, Jonathan Rhodes and Lu-Gang Yu
Cancer Research Seminar, Liverpool, 2011

Circulating galectins and metastasis
Hannah Barrow, Jonathan Rhodes and Lu-Gang Yu
NWCRF Symposium, Liverpool, 2009-2011

The role of galectins in colorectal cancer progression (Review).

Barrow H, Rhodes JM, Yu LG.
Int J Cancer. 2011 Jan 20 [Epub ahead of print]

Interaction between circulating galectin-3 and cancer-associated MUC1 enhances tumour cell homotypic aggregation and prevents anoikis.

Zhao Q, Barclay M, Hilkens J, Guo X, Barrow H, Rhodes JM, Yu LG.
Mol Cancer. 2010 Jun 18;9:154.

PAPERS IN PROGRESS

Circulating galectin-2, -3, -4 and -8 are highly increased in the blood circulation of colon and breast cancer patients and promote cancer-endothelial cell adhesion and metastasis.
Hannah Barrow, Xuli Guo, Hans H. Wandall, Bo Fu, Qicheng Zhao, Jonathan M. Rhodes and Lu-Gang Yu

Suppression of Core 1 Gal-T transferase is associated with reduction of Thomsen Friedenreich (TF) antigen and reciprocal increase of Tn, sialyl-Tn and Core 3 glycans in human colon cancer cells.
Hannah Barrow, Benjamin Tam, Jonathan M Rhodes and Lu-Gang Yu

Midway upon the journey of our life
I found myself within a forest dark,
For the straightforward pathway had been lost.
Ah me! How hard a thing it is to say
What was this forest savage, rough, and stern,
Which in the very thought renews the fear.

- Dante Alighieri, *Inferno*