# Imaging studies of glycan mediated breast cancer epithelial cell-endothelial cell interactions

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# ***ABSTRACT***

Relatively little is known about metastasis, the spread of cancer from the site of its origin to different organs in the body. Aberrant glycosylation of cell surface glycoproteins in cancer cells has been implicated in promoting cancer metastasis and consequent poor survival of cancer patients. In particular, aberrant glycans terminating in α-N-acetylgalctosamine (GalNAc), specifically recognized by the lectin *Helix pomatia* agglutinin (derived from the albumin gland of the Roman snail, *Helix pomatia*), have been described as a hallmark of aggressive cancers of the breast and several other sites. The research described in this thesis aims to study the role of HPA binding glycans in one of the most important, but poorly understood steps of the metastatic cascade namely cancer cell-endothelial cell adhesion and transendothelial migration, using a rocking adhesion assay system in a model of breast cancer metastasis.

Three breast cancer epithelial cell lines - MCF 7 and ZR 75 1, derived from breast cancer metastases cells, and BT 747, derived from primary breast cancer were studied during adhesion to, and migration across, monolayers of human umbilical vein endothelial cells (HUVECs) .

Initially, a confocal microscope compatible rocking adhesion assay system was developed and optimized in house. The rocking adhesion assay system was then used to study the role of HPA binding glycans in the adhesion of cancer cells to the endothelial cells. Results from the rocking adhesion assays showed a significant inhibition in the adhesion of MCF 7 and ZR 75 1 cells thereby

providing evidence that HPA binding glycans may play a functional role in the adhesion of breast cancer cell to endothelial cells.

Behavior of the cancer cells post adhesion was studied using 24 hour time series experiments with a smartslide system and confocal microscopy. Further high resolution imaging was also carried out using scanning electron microscopy. The results have provided evidence that the behavior of cancer cells during adhesion and transendothelial migration during metastasis is similar to that described for leucocytes during the inflammatory response. Like leucocytes, after adhesion, cancer cells were imaged moving along the endothelial cell layer. The imaging further showed the retraction of endothelial cells and the cancer cells settling and dividing in the gap produced by endothelial retraction. In addition to this, transcellular migration was also imaged using scanning electron microscopy.

# ***Acknowledgements***

As a doctor who had never stepped in to a laboratory ever in her life, I was dreading the two years I was going to spend away from patient contact and in a lab. Against all my expectations, I have had the best two years of my life and I have a lot of people to thank for it. My first and biggest thank you is to my supervisors - Dr. Susan Brooks, Professor.Munira Kadhim and Dr. John Runions. Susan, thank you for your guidance and friendship throughout my project. I cannot express in words how grateful I am for your endless patience and for your belief in me, particularly during the long writing up period, which was one of the busiest and hardest times of my life. Without you, I would never have finished. Munira, thank you for all the advice, support and training when I was learning the ropes and throughout my project. Thank you for not letting me take the easy way out, for insisting that I always do the right thing and for making sure scientific integrity came above everything else. You are truly a great scientist. John, you are one of the nicest people I have met and over the two years, you have become a really good friend. Thank you for teaching a surgeon how to look down a microscope and for always being ready to help, for always being nice and cheerful and for the countless hours you have spent making my imaging work.

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# Chapter 1:

# Introduction

## *1.1 Breast Cancer- UK statistics*

Breast cancer is the commonest cancer in the UK. The incidence rates of breast cancer have risen by 50% in the last 25 years. Breast cancer is now the second commonest cause of cancer deaths in the UK, killing approximately 12,000 women and 70 men each year (http://info.cancerresearchuk.org/cancerstats, 2010). The most common cause of cancer related deaths in patients with breast cancers and other cancers is metastasis, the spread of the cancer from the primary site to various organs in the body. For example, most patients with metastatic breast cancer at the time of initial diagnosis do not survive beyond 24 months (Gennari et al., 2005, Kiely et al., 2011). In comparison to this, 70-95% of patients presenting with cancers confined to the breast with no axillary or distant metastasis survive for 5 years and beyond (Rosen et al., 1993, Hanrahan et al., 2007).

## *1.2. Cancer Metastasis*

Metastasis is the spread of cancer from its primary site of origin to discontiguous nearby or distant sites where secondary, extracellular foci of tumours are formed (reviewed byWelch, 2006). Metastasis occurs most commonly via blood (haematogenous metastasis) or via lymphatics. In addition to these common routes of metastasis, some cancers also utilise, other, less common methods for their spread. For example, ovarian cancers metastasise by direct spread across the peritoneal cavity (Carmignani et al., 2003). Prostatic cancers and cancers of the head and neck spread by perineural invasion (Carter and Pittam, 1980, Villers et al., 1989). Malignant melanomas spread by

migrating outside the lumen of blood vessels into the space between endothelial cells and the basement membrane by a process called as extra luminal migratory metastasis (Lugassy et al., 2004). Common routes of cancer metastasis are illustrated in Figure 1.1.

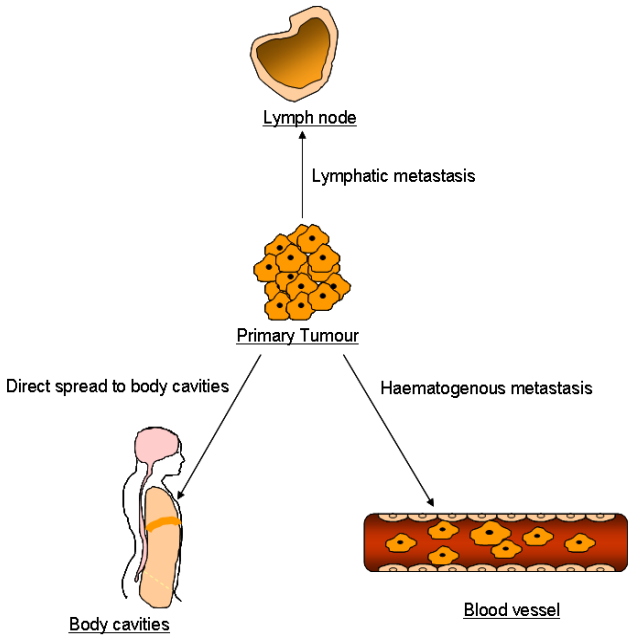



Figure 1.1: Routes of metastatic spread in the body: Lymphatic metastasis, haematogenous metastasis and direct spread across body cavities.

Metastasis is a complex, multi-step process. To successfully metastasize, cancer cells have to complete a series of interlinked steps in succession. Hence, the process of metastasis is often described as a cascade. The research project described in this thesis focuses on a specific step of the metastatic cascade utilised by breast cancers for haematogenous metastasis. Hence, a brief review of metastatic cascade with particular reference to haematogenous metastasis is given below. Steps 1-3 also occur in other modes of metastasis.

Metastatic cascade consists of the following key steps (Tarin and Matsumura, 1994):

1. Angiogenesis or formation of new blood vessels by the growing tumour.
2. Detachment of the tumour cells from the main tumour mass.
3. Migration of cancer cells through the basement membrane and extracellular matrix surrounding the tumour mass and intravasation in to the local blood vessels
4. Dissemination of the tumour cells to distant organs through blood.
5. Adhesion of the tumour cells to the endothelial cells and basement membrane of the blood vessels and subsequent extravasation in to the organs.
6. Seeding of the tumour cells in the organs and growth of metastatic tumour foci.

The steps of the metastatic cascade are shown in figure 1.2 below and described in detail in sections 1.2.1- 1.2.6

### *1.2.1. Angiogenesis*

Unregulated and autonomous growth is one of the hallmarks of cancers. However, despite possessing this potential for unrestrained growth, tumours cannot grow beyond 1-2mm in diameter by relying exclusively on the existing blood supply of the organ from which they originate (Gimbrone et al., 1972). For their sustenance and continued growth, it is essential for tumours to

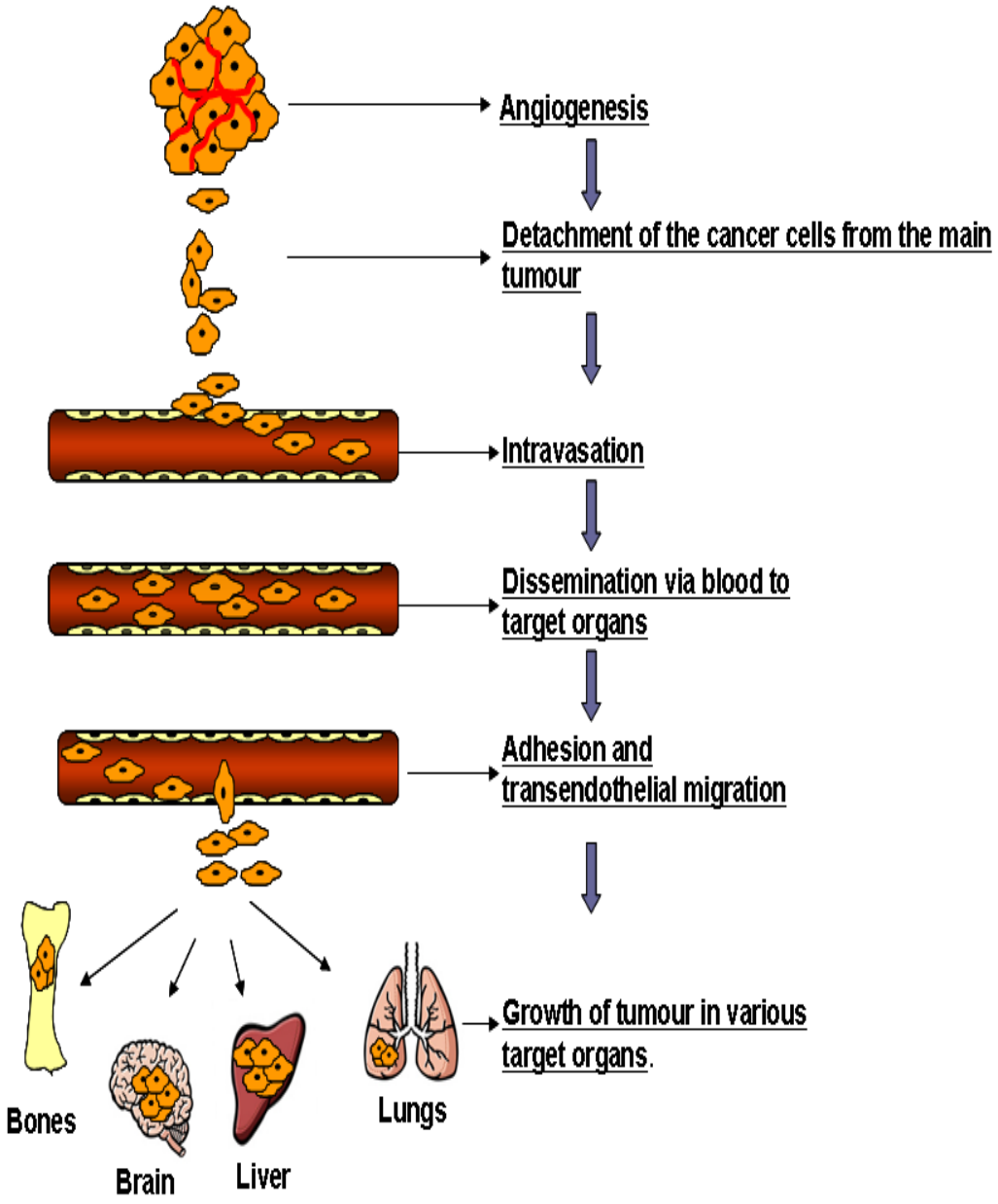



Figure 1.2.Steps of the metastatic cascade: Metastasis is a multistep process consisting of angiogenesis, detachment of tumour cells, intravasation, dissemination, adhesion and transendothelial migration and growth of metastatic tumour foci.

establish a new network of blood vessels and this neovascularisation is achieved primarily by angiogenesis. Angiogenesis is a process in which new blood vessels are generated by utilising the dormant endothelial cells lining the existing vasculature of the organ.

Angiogenesis in tumours, as in normal tissues, is triggered by hypoxia in the tissue micro environment. Hypoxia triggers angiogenesis by stabilizing and activating the transcription factor, hypoxia inducing factor -1(HIF-1). HIF-1 in turn induces the action of the growth factor, vascular endothelial growth factor (VEGF), a potent stimulant of angiogenesis (Leung et al., 1989, Dery et al., 2005). In addition to VEGF, angiogenesis in tumours is also mediated by various cytokines and growth factors including epidermal growth factor (EGF), fibroblast derived growth factor (FGF) and transforming growth factor β (TGF-β), which stimulate angiogenesis directly, and other stimulants like tumour necrosis factor alpha (TNFα), interleukin (IL-1β), insulin derived growth factor (IGF) and epidermal growth factor (EGF), which stimulate angiogenesis indirectly by their action on HIF-1. The action of these stimulants on the dormant endothelial cells leads to an increase in vascular permeability and detachment of the endothelial cells. The detached endothelial cells then proliferate into the tumour stroma to sprout new capillaries and accomplish neovascularisation of the tumour mass (Hanahan and Folkman, 1996, Cook and Figg, 2010)

Angiogenesis in normal adults is a highly restricted process and occurs only in conditions like wound healing and during menstrual cycle where regeneration

of damaged blood vessels is essential. Under these physiological conditions, angiogenesis is regulated by a delicate, well regulated balance between pro and anti angiogenic factors. This balance is however, lost in tumour angiogenesis and results in the formation of poorly structured, leaky blood vessels which are not properly fortified by pericytes and basement membrane (Bockhorn et al., 2007). This defective structure of the blood vessels is utilised by cancer cells for intravasation and provides them access to blood stream and thereby entry in to systemic circulation.

### *1.2.2. Detachment of the cancer cells from the main tumour*

Tumour cells possess reduced cell-to-cell adhesion compared to normal cells and this has been attributed to the loss or down regulation of the adhesion molecule E-cadherin which is a trans membrane glycoprotein (Brooks et al., 2010). E-cadherin regulates cell to cell adhesion, interaction and polarity in normal cells and inactivation of E-cadherin is a predominant feature of cancers (Jeanes et al., 2008, Brooks et al., 2009, Berx and Roy, 2009) and results in reduced cell-to-cell adhesion. This facilitates the detachment of the cancer cells from the main tumour mass.

### *1.2.3. Migration of cancer cells through the basement membrane and extracellular matrix surrounding the tumour mass and intravasation in to blood vessels*

During invasion of the basement membrane by the cancer cells, several changes are brought about in the cancer cells and the surrounding stromal cells. The

cancer cells extend pseudopodia and several hydrolytic enzymes including matrix metalloproteinases (MMP's), urokinases and cathepsins are activated and released by these cancer cells. The basement membrane and extracellular matrix is digested by the action of these enzymes. The tumour cells then adhere to the degraded extracellular matrix and detach from the main tumour mass (François-Clément Bidard and Poupon., 2008, Brooks et al., 2009).

Several changes are also brought about in the basement membrane of the tumour and in the extracellular matrix. The basement membrane and the extracellular matrix are thinned out by the compression forces of the growing tumour, facilitating invasion by cancer cells. Compared to normal tissues, the extracellular matrix and stroma of tumours are stiffer. This increased stiffness is utilised by cancer cells to produce increased tractional forces resulting in remodelling of the extracellular matrix and linear rearrangement of collagen fibres. The tumour cells then form motility tracks in the extracellular matrix and migrate through these tracks to reach the basement membrane of blood vessels and lymphatics (Kumar and Weaver, 2009).

Invasion through basement membrane and into the blood vessels of the tumour is brought about by both active and passive mechanisms. The process is facilitated by the leaky and fenestrated structure of the newly formed blood vessels as described in section 1.2.1.

In the active mechanism, cancer cells manipulate the cytoskeleton to produce targeted migration towards the basement membrane. Then, they adhere to and pass through the basement membrane and endothelial cells to gain entry in to

the blood vessels. In the passive mechanism, the cancer cells gain entry in to blood vessels and lymphatics when these vessels collapse as a result of the mechanical stress produced by unregulated tumour growth in a confined space (Bockhorn et al., 2007).

### *1.2.4. Dissemination to distant organs*

Once cancer cells have gained entry in to the blood stream, they are subjected to shear forces from the flow of the blood and attack from the immune system. Though millions of tumour cells are shed in to the blood stream, it has been reported that less than 0.1% of the circulating cells are actually able to survive these attacks and successfully metastasize (Weiss, 2000). The surviving cancer cells reach various organs and arrest in the microvasculature of these organs.

### *1.2.5. Adhesion of the cancer cells to endothelial cells and transendothelial migration*

Once the cancer cells have reached the microvasculature of the organs that they metastasise to, they interact with the endothelial cells lining the blood vessels and migrate out of the blood vessels. This step, the interaction between the cancer cells and endothelial cells and the subsequent migration of cancer cells out of the blood vessels, is the focus of the research project described in this thesis. This step is explained in more detail in section 1.3.

Each type of cancer displays a distinctive pattern of dissemination. For example, breast cancers tend to metastasise to liver, bones, lungs and brain. The exact mechanisms underlying this organ-specific pattern of metastasis is

incompletely understood and two long-standing schools of thought still exist on this matter.

The first school of thought favours the 'seed and soil hypothesis', first proposed by Stephen Paget in 1889, in which tumour cells are compared to seeds, and the target organs to soil (as cited in Fokas et al., 2007). Tumour cells selectively metastasise to organs that have the right conditions for their growth.

The alternative, mechanical entrapment theory was first proposed by James Ewing in 1928 [as cited by (Chambers et al., 2002)] who suggested that tumour cells become mechanically entrapped in the capillary bed of the first organ they encounter and therefore preferentially metastasize to that organ.

It is now believed that mechanical entrapment alone, while it does occur, is not sufficient to explain the organ specific pattern of tumour metastasis (Glinskii et al., 2005) and that there are specific and selective molecular interactions between tumour cells and the cells of the organs to which they metastasise (Hart and Fidler, 1980, Fokas et al., 2007); however, this is an area that is not well researched and is incompletely understood.

### *1.2.6. Seeding of the tumour cells and growth of new tumour foci*

To be able to successfully metastasize, cancer cells have to manipulate the environment of the tissues that they metastasise to. For example, during bony metastasis, tumour cells destroy the balance between the activity of osteoblasts (cells that deposit bone) and osteoclasts (cells that remove bone) resulting in bone resorption. By thus manipulating the host environment, tumour cells

create conditions that are favourable for their growth and proliferation (Welch, 2006).

Once they have seeded in the target organs, the same factors that supported the growth of the primary tumours namely, limitless replicative potential, ability to mimic growth signals, insensitivity to anti-growth signals, resistance to apoptosis and sustained angiogenesis also help the metastatic tumours to flourish in the target organs (Hanahan and Weinberg, 2000).

## *1.3. Adhesion and transendothelial migration*

As described in the section 1.2.5, the tumour cells which have reached the microvasculature of the target organs have to adhere to, and interact with, the endothelial cells and the basement membrane of these vessels prior to extravasation and growth in the target organs.

During these interactions, tumour cells are believed to employ some of the molecular mechanisms that are utilized by leucocytes for adhesion and transendothelial migration during the normal process of inflammation. This process has been described as ‘leucocyte mimicry’ (Barthel et al., 2007, Albini, 1998). Though both cancer cells and leucocytes appear to, at least in part, utilize the same mechanisms for adhesion and transendothelial migration, the process has been extensively studied in leucocytes and relatively little studied in cancer cells. Hence, adhesion and transendothelial migration of leucocytes as a paradigm for metastasis is described below.

### *1.3.1. Leucocyte adhesion and transendothelial migration – a paradigm for metastasis*

Four key steps are involved in the adhesion and transendothelial migration of leucocytes. They are described by Barreiro and Sanchez-Madrid (2009) and are as follows:

1. Tethering and rolling of leucocytes by selectin-carbohydrate mediated interactions.
2. Activation of integrins.
3. Integrin mediated firm adhesions between leucocytes and endothelial cells and crawling of the leucocytes on the endothelial cell layer.
4. Transmigration of the leucocytes out of the blood vessel mediated by members of the immunoglobulin superfamily.

Selectins, integrins and members of the immunoglobulin superfamily (IgSF) play a vital role in the extravasation of leucocytes, and possibly of cancer cells. Hence a brief review of their structure, relevant types and function is given below.

#### *1.3.1.1 Selectins*

Selectins are a family of type-1, transmembrane glycoproteins consisting of three members, P-selectin, E-selectin and L-selectin. P-selectin is expressed by platelets and endothelial cells, E-selectin by endothelial cells and L-selectin by leucocytes. The three members of the selectin family share a similar structure consisting of an extracellular domain, a transmembrane sequence and a

cytoplasmic domain. A schematic representation of selectin structure is shown in the Figure 1.3.

The extracellular domain has a N-terminal carbohydrate recognition, lectin-type domain (which is similar to that of C-type or $Ca^{2+}$-dependant type lectin), an epidermal growth factor like or EGF- like domain and a multiple short consensus repeat or SCR domain. The SCR domain is followed by the trans membrane sequence and a short cytoplasmic, C-terminal domain (Bevilacqua and Nelson, 1993, Symon and Wardlaw, 1996).

L-selectin is present on the projections of microvilli on unstimulated neutrophils. Unlike L-selectin, P- and E-selectins are up-regulated during inflammation by the cytokines, thrombin or histamine (Tedder et al., 1995).

The binding partners for selectins include fucosylated and sialylated carbohydrates including ligands with heavily O-glycosylated, mucin like domains, sialyl $Lewis^a$ and sialyl $Lewis^x$ antigens (Varki and Varki, 2001, Symon and Wardlaw, 1996).

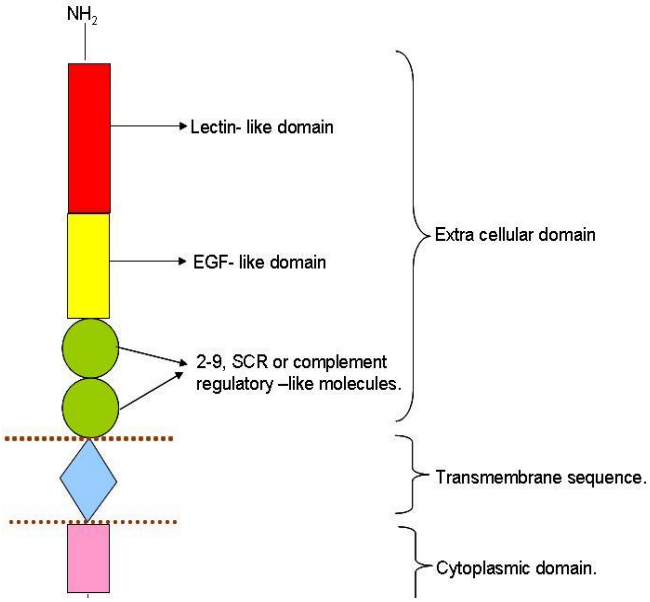



Figure 1.3.Schematic representation of the structure of selectins: Selectins are made up of an extra cellular domain, a transmembrane sequence and a cytoplasmic domain. Adapted from (Bevilacqua and Nelson, 1993).

*1.3.1.2. Integrins*

Integrins are a family of more than 20 cell surface receptors that are vital in cell-cell and cell-extracellular matrix interactions (Barreiro and Sanchez-Madrid, 2009). Structurally, integrins are heterodimeric, composed of two noncovalently linked sub units- α chains and β chains. The two chains contain an extracellular domain, a transmembrane sequence and a cytoplasmic domain (Brooks et al., 2009)

α chain has a head region made up of a β propeller domain and a thigh domain. The thigh domain, in turn, is linked to two calf-domains, calf -1 and calf- 2. In

some integrins, a domain with 200 amino acids called the α1 domain is inserted into the β propeller domain.

The extracellular domain of the β chains contains a hybrid domain, a plexin-semaphorin integrin (PSI) domain, a β1 domain and four epidermal growth factor (EGF) repeats (Barczyk M, 2010). A schematic representation of integrin structure is shown in the figure 1.4.

Integrins perform their functions by dynamically changing their conformation. The α chain is responsible for ligand binding. The β chain is closely linked with the cytoskeleton through its cytoplasmic tail and helps mediate the interactions with the cytoskeleton.

In their resting or inactive state, integrins are present in a compact, bent form. The action of integrins is initiated when talin, a protein present in the cytoskeleton, binds to the cytoplasmic tail of the β chain. This binding sends a signal to the α chain of the integrin, causing it to extend to a small degree. This partly extended state is called the primed position and helps to expose the ligand binding site on the α chain. The ligand then binds to the α chain and extends the α chain further, making it achieve its active form. The tractional force produced by this extension is transmitted to the cytoplasmic tail of the β chain and through it to the cytoskeleton. This switches on various intra cellular signalling cascades mediating cell to cell interactions and various other functions (Askari et al., 2009 ).

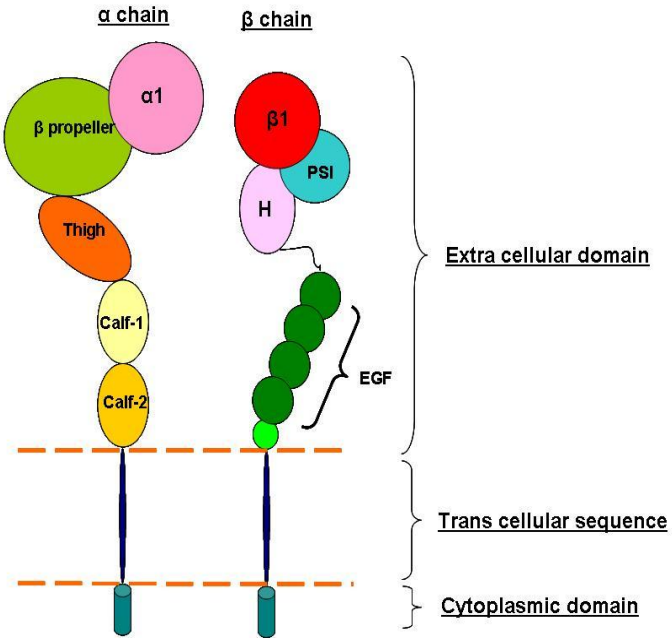



Figure 1.4: Schematic representation of the structure of integrins. The α and β chains have an extra cellular domain, a transmembrane sequence and a cytoplasmic domain. The components of the individual chains are explained in the section 1.3.12. Adapted from (Barczyk et al., 2010).

*1.3.1.3. Immunoglobulin super family (IgSF) adhesion molecules*

Binding partners of integrins are the members of the immunoglobulin superfamily. These include intercellular adhesion molecules (ICAMs) 1-5, vascular adhesion molecule- 1 (VCAM-1), junctional adhesion molecules (JAMs) A, B and C and platelet-endothelial adhesion molecules (PECAMs).

Functionally, as the binding partners of integrins, these adhesion molecules play a central role in the adhesion and transendothelial migration of leucocytes during inflammation. ICAM-1 and VCAM-1 are involved in integrin mediated leucocyte adhesion. ICAM-1, and VCAM -1 along with ICAM-2, JAM-B,

JAM-C and PECAM are involved in transendothelial migration (Barreiro and Sanchez-Madrid, 2009).

## *1.4. The leucocyte adhesion cascade*

### *1.4.1. Tethering and rolling of leucocytes by selectin-carbohydrate mediated interactions*

Tethering and rolling of leucocytes, the first step in the events leading to leucocyte extravasation is largely mediated by selectins and their ligands. Leucocyte rolling on endothelial cells begins minutes after tissue injury. In response to injury, several mediators of inflammation – for example, histamine, thrombin and cytokines - are generated at the site of injury. These mediators activate P-selectin from intracellular stores which mediates the early phase of rolling (Tedder et al., 1995).

There are two phases to rolling, both requiring a close interaction of selectins and their ligands. Initially, the leucocytes form transient association-dissociation interactions with the blood vessel wall. This process is called tethering and is mediated by P- selectin and its ligand, P-selectin glycoprotein ligand 1 (PSGL1). Tethering captures the circulating leucocytes and slows them down.

Tethering is converted to more stable rolling by the interaction between E-selectin and its ligand E-selectin ligand1 (ESL1). Slow rolling correctly orientates the L- selectin on the leucocytes and promotes their binding to PSGL1 (Barreiro and Sanchez-Madrid, 2009). These processes help the next

step in the leucocyte adhesion cascade, namely activation of integrins on the surface of the leucocytes.

### *1.4.2. Activation of integrins*

As explained in the previous section, selectin mediated interactions recruit leucocytes and cause them to roll along the endothelial cells. This allows them to come in to contact with various chemokines, for example, TNFα and interleukin-8 (IL-8) expressed on the surface of the endothelial cells. These, in turn, activate integrins on the surface of the leucocytes. In addition to activation by chemokines, integrins are also activated by intracellular signalling produced by the binding of L-selectins (Crockett-Torabi, 1998). As explained in the section 1.3.12, these signals bring about conformational changes in the integrins and initiate the next step in the adhesion cascade, namely integrin mediated firm adhesion.

### *1.4.3. Integrin mediated firm adhesion*

Integrin mediated leucocyte adhesion takes place in two stages. In the first stage, the leucocytes crawl along the endothelial surface. This is essential for the leucocytes to reach the site appropriate for transendothelial migration. Once the leukocytes have reached the appropriate place on the endothelial cell layer, the integrins react with the activated adhesion molecules ICAM-1 and VCAM-1 (described in the section 1.3.13) and adhere firmly to the endothelial cell layer (Gahmberg et al., 1997, Penberthy et al., 1997). This adhesion is believed to be reinforced by ICAM-1 and VCAM-1 by the production of 'leukocyte docking structures' which prevent the adherent leukocytes from

detaching under the shear forces produced by blood flow and aid their transmigration through the endothelial cell layer (Barreiro and Sanchez-Madrid, 2009).

### *1.4.4. Transmigration of leucocytes*

Following integrin mediated adhesion, the next stage is the transmigration of the leucocyte across the endothelial layer. During this process, microvilli on the endothelial cells actively surround the adherent leucocytes in a cup like structure called the transmigratory cup. The formation of transmigratory cup is mediated by ICAMs.

Leucocytes then employ two routes of transmigration. In the first route, leucocytes use diapedesis to squeeze out between adjacent endothelial cells aided by the action of JAMs and PECAMs (described in section 1.3.13). This mechanism is called Para cellular migration. In the second route, leucocytes pass through the endothelial cells themselves to migrate out. This is called transcellular migration (Rahman and Fazal, 2009). Both mechanisms are believed to play an equally important role in transendothelial migration of leucocytes. Adhesion and transendothelial migration of leucocytes is illustrated in the Figure 1.5

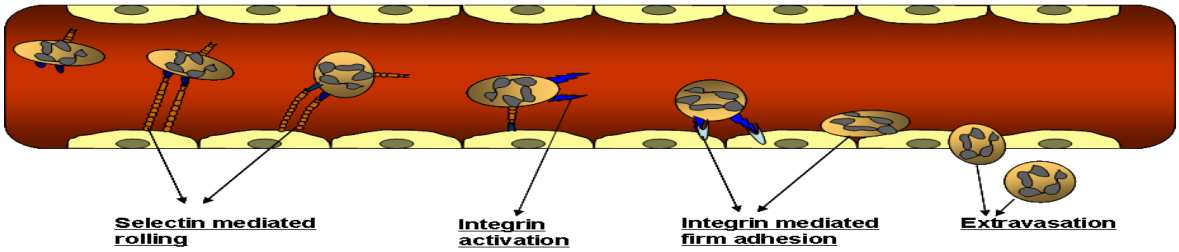



Figure.1.5. Leucocyte adhesion cascade. Leucocytes captured from blood stream roll along endothelial cells by selectin-mediated interactions. Rolling activates integrins and they cause firm adhesion of the leucocytes to the endothelial cells. The adherent leucocytes extravasate in to the site of their action. Adapted from (Vestweber and Blanks, 1999).

## *1.5. Adhesion and transendothelial migration of tumour cells*

As explained in the section 1.3, metastasising cancer cells are believed to employ mechanisms similar to those used by leucocytes during inflammation (Strell and Entschladen, 2008). Selectins appear to be the main mediators of rolling in cancer cells and expression of selectin ligands has been shown to result in enhanced metastatic capability (Kannagi et al., 2004, Strell and Entschladen, 2008). Expression of E- selectin ligands which mediate E-selectin mediated binding to endothelial cells has been shown in metastatic prostate carcinoma cells (Dimitroff et al., 2005). In addition to this, cancer cells are believed to induce endothelial E-selectin expression (Kannagi et al., 2004) and facilitate selectin mediated rolling. In colon cancer cells E, P and L-selectins

have been shown to mediate slow, intermediate and fast rolling respectively (Hanley et al., 2006). Similar to the events during leucocyte adhesion and transendothelial migration, P-selectin binding has been shown to produce integrin activation in colon carcinoma cells (Reyes-Reyes et al., 2006). In addition to this, N-cadherins have been shown to mediate the rolling of cells of the breast cancer cell line MDA-MB-468, thereby indicating that in some cancers mediators other than selectins might be involved in cancer cell rolling on endothelial cell surface (Strell et al., 2007). In addition to their role in mediating cancer cell rolling on endothelial cell layer, selectins have also been shown to mediate diapedesis of cancer cells (Tremblay et al., 2008) .

In leucocytes, integrins mediate firm adhesion of leucocytes to the endothelial monolayer and this process has been described in sections 1.4.2 and 1.4.3. Similarly, integrin mediated firm adhesion has also been shown to occur in cancer cells. In nude mice, very late antigen integrin 4 (VLA-4) mediated binding of melanoma cells to its ligand VCAM-1 was demonstrated, and resulted in an increase in the formation of metastatic colonies (Garofalo et al., 1995). In addition to integrins, galectins have been implicated in mediating firm adhesion of breast and prostate cancer cells to the endothelium (Glinsky et al., 2001).

Following firm adhesion of cancer cells, endothelial retraction is brought about to facilitate cancer cell transmigration across the endothelial layer. The tumour cells then degrade the basement membrane by the action of several enzymes, predominantly collagenase IV, and migrate into the stroma of the target organ

(Albini, 1998). The steps of tumour adhesion and extravasation are shown in the Figure 1.6.

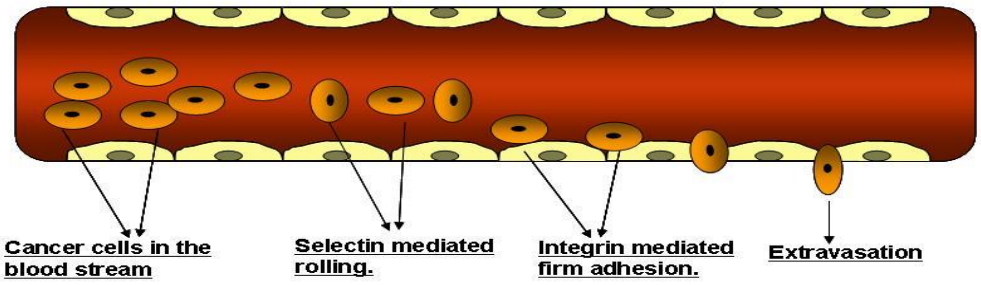



Figure.1.6.Adhesion and transendothelial migration of cancer cells: Cancer cells initially roll along the endothelial layer by predominantly selectin mediated interaction. This results in integrin mediated firm adhesion following which the cancer cell migrate out across the endothelial cell layer.

## *1.6. Glycosylation in human cells*

It is clear from the description of metastatic cascade in the previous sections that metastasis is an extremely complex process which relies on the interplay of a diverse family of cell surface receptors and their ligands. Of particular importance to this project is the fact that several of these molecules are either glycoproteins or substances which interact with them. Abnormal glycosylation of cell surface glycoproteins is a salient feature of cancers. This project aims to investigate the role played by abnormal cell surface glycans present on cancer cells during adhesion and transendothelial migration step of the metastatic cascade, focussing on breast cancer as a model system. It is of paramount

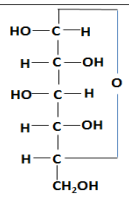
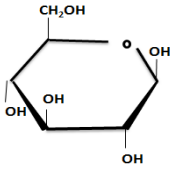
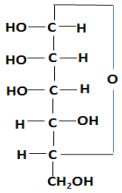
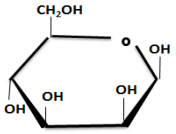
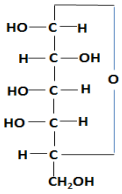
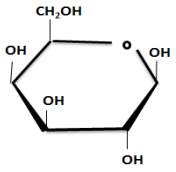
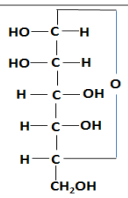
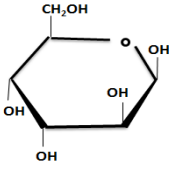
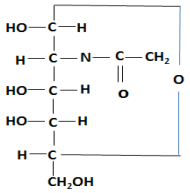
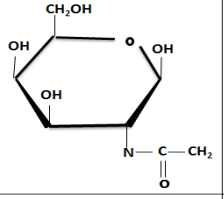
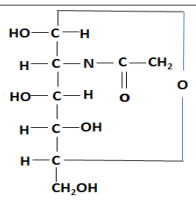
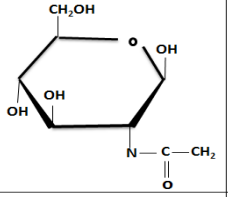
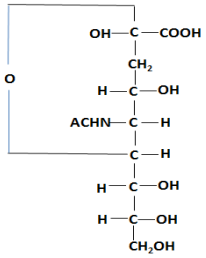
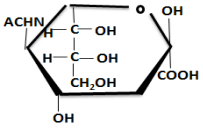
importance, therefore, to have a clear understanding of the fundamental concepts of glycobiology, including basic carbohydrate chemistry. It is beyond the scope of this thesis to describe carbohydrate chemistry in detail. For an in depth, explanation of carbohydrate chemistry and glycosylation, please refer to a standard text book of glycobiology [for example (Brooks, 2002, Varki, 1999)]. However, some fundamental concepts essential to understand the process of cell surface glycosylation and its abnormalities is outlined in the following sections.

### *1.6.1. Structure of Glycoproteins*

Glycoproteins are proteins in which glycans, either in linear or branching chains, are linked covalently to the polypeptide backbone usually via N- or O-linkages. Glycan is a generic term utilised in glycobiology to describe a carbohydrate in mono, oligo or poly saccharide form (Varki, 2009). N- and O-linkages are described in detail in sections 1.6.2 - 1.6.4

In humans, the carbohydrate moiety of glycoproteins is formed by a combination of seven monosaccharides, glucose (Glc), galactose (Gal), mannose (Man), fucose (Fuc), N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc) and sialic acids (SA). The 7 monosaccharides, their Fischer and Haworth projection formulae and the symbols and abbreviations used to represent them in this thesis are given in the Table 1.1.

Table.1.1.The seven monosaccharides utilised in cell surface glycosylation in humans. Adapted from (Brooks et al., 2002).

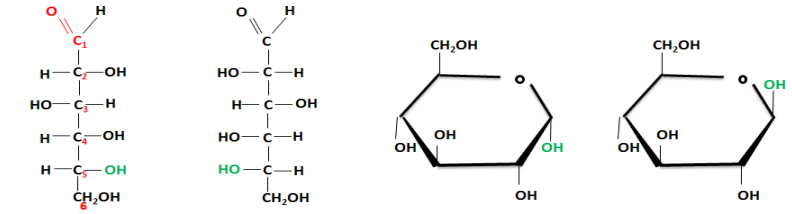
| Name | Haworth formula | Fischer formula | Symbol |
|---|---|---|---|
| Glucose (Glc) |  |  |  |
| Mannose (Man) |  |  |  |
| Galactose (Gal) |  |  |  |
| Fucose (Fuc) |  |  |  |
| N-acetylgalactosamine (GalNAc) |  |  |  |
| N-acetylglucosamine (GlcNAc) |  |  |  |
| Sialic acids (SA) |  |  |  |

### *1.6.2. Carbohydrate chemistry*

The complexities of carbohydrate chemistry are well reviewed by Varki et al 1999 and Brooks et al, 2002. Carbohydrates are defined as polyhydroxy aldehydes or poly hydroxyketones or their derivatives, or substances which can be hydrolysed to produce these compounds. Monosaccharides are the simplest form of carbohydrates and are the building blocks of more complex carbohydrates. Monosaccharides occur in open chain or ring forms, the structurally stable ring forms being more prevalent. The structural backbone of a monosaccharide is formed by an unbranched chain of carbon atoms covalently linked to each other and in turn linked to groups including–CHO, -H, -OH and -$CH_2OH$.

The carbon atoms of a monosaccharide are conventionally numbered 1,2,3 and so on with the carbon atom linked to the carbonyl or C=O group being numbered 1(Figure.1.7 a).

Linear forms of carbohydrates are present in L- or D-configurations depending upon whether the -OH group linked to the highest numbered asymmetric carbon atom is to the left or right of the chain respectively. The ring forms are present in α and β anomeric forms depending on whether the -OH group linked to the anomeric carbon atom, C1, lies below the plane of the ring or above it. The linear and ring forms of monosaccharides in their various conformations are illustrated in figures 1.7a-d with glucose as an example.




Figures.1.7: Structure of D- and L-glucose: The carbon atom linked to the carbonyl group (shown in red) is numbered 1. The -OH group attached to the highest numbered asymmetric carbon atom (in green) is to the right in the D-form (1.7.a) and to the left in the L-form (1.7.b). The OH group linked to the anomeric carbon atom (in green) is below the plane of the ring in the α form (Figure 1.7.c) and above the plane of the ring, in the β form (Figure1.7.d). Adapted from (Brooks et al., 2002)

Oligosaccharides are made up of linear or branching chains consisting of a variable number of monosaccharides. The chains are formed by a reaction between the -OH groups of two monosaccharides with elimination of a molecule of water. The resulting bond containing acetal oxygen is called a glycosidic bond. If the glycosidic bond is axial, i.e. below the plane of the ring of the left hand monosaccharide involved in the reaction, it is termed α-glycosidic linkage. If the bond is equatorial i.e. above the plane of the ring of the left hand monosaccharide involved in the reaction, it is termed a β-glycosidic linkage (Varki et al., 2009., Brooks et al., 2002).

As described above, monosaccharides in a chain can be linked with α and β glycosidic bonds, the linkages can be formed between different carbon atoms of a monosaccharide chain (for example, to form a 1→3, 1→6, 2→3 linkage etc.) and glycan chains of variable length and complexity that are linear or branched can be formed. This can potentially give rise to a vast array of glycans. In

addition to this, glycan synthesis is not under direct genetic control. A protein may have several potential glycosylation sites but the choice of glycosylation site that is actually utilised for glycosylation is governed by several factors including the availability of appropriate carbohydrate substitutes, cellular environment, like pH, and availability and activity of the specific enzymes required for glycosylation. All these factors result in immense and extremely complex structural diversity of glycoproteins.

### *1.6.3. Glycoproteins Synthesis*

Glycoproteins are synthesized in the endoplasmic reticulum (ER) and the Golgi apparatus (GA). The glycans may be N-linked or O-linked to the polypeptide. An insight in to the biosynthesis of N-linked and O-linked glycosylation is of paramount importance in understanding the abnormal glycosylation changes in cancer described in sections 1.7-1.8 of this thesis. Hence, a brief review of the biosynthesis of N and O-linked glycosylation is given below.

### *1.6.4. Synthesis of N-linked glycans*

N-linked glycosylation is a co-translational event being initiated during the synthesis of the polypeptide chain. It is initiated in the endoplasmic reticulum (ER) with the GlcNAc molecule from an oligosaccharide forming a β-N glycosidic linkage with the amide group of the amino acid asparagine (Asn). It is a prerequisite in N-linked glycosylation that this asparagine is within a consensus amino acid sequence asparagine-X-serine/threonine (where X may

be any amino acid other than proline). The steps in the N-linked glycosylation are shown in the Figure 1.8. and are described below:

1. The first step in N-linked glycosylation is the synthesis of dolichol linked oligosaccharide intermediate, $Man_5GlcNAc_2$-phosphate-dol (consisting of 5 mannose residues and two GlcNAc residues), in the cytoplasm by the action of corresponding glycosyltransferases. At the same time, the growing polypeptide chain, still under active construction on the ribosome, is fed into the rough endoplasmic reticulum (RER) lumen.
2. The oligosaccharide intermediate is then flipped across the RER membrane into the lumen.
3. In the RER, the oligosaccharide intermediate is further extended by addition of 3 glucose and 5 mannose residues to form $Glc_3Man_9GlcNAc_2$-phosphate-dol.
4. The oligosaccharide intermediate is then cleaved from the dolichol molecule and linked instead to the asparagine residue of the nascent polypeptide chain (with the previously described consensus amino acid sequence). This reaction is catalysed by the action of the enzyme complex, oligosaccharyltransferase (OST).
5. Following the attachment to the polypeptide chain, the oligosaccharide intermediate is trimmed by glucosidase and mannosidase enzymes which remove 3 glucose residues and one mannose residue, respectively, resulting in the formation of $Man_8GlcNAc_2$-Asn. This

trimming is essential for the correct folding of the polypeptide chain and is a vital step in the biosynthesis of N-linked glycoproteins.

6. The glycoprotein is then transferred to the GA where further mannose residues are cleaved giving rise to a range of structures, most commonly $Man_5GlcNAc_2$-Asn. Central to the structure of all N-linked glycoproteins is the presence of a trimannosyl core consisting of $Man_3GlcNAc_2$-Asn.
7. These trimmed structures are then further extended to give three main types of N-linked glycoproteins. They are high mannose type, complex type and hybrid type. The high mannose type glycans have five to nine mannose residues attached to the trimannosyl core. The complex type glycans are characterized by the presence of lactosamine units GlcNAc(β1→4)GlcNAc. These lactosamine units make up the polylactosamine units (GlcNAc(β1→4)GlcNAc)n which in turn form the antennae of the branched N-linked chains. The hybrid glycans contain features of both the high mannosyl and complex type glycans.

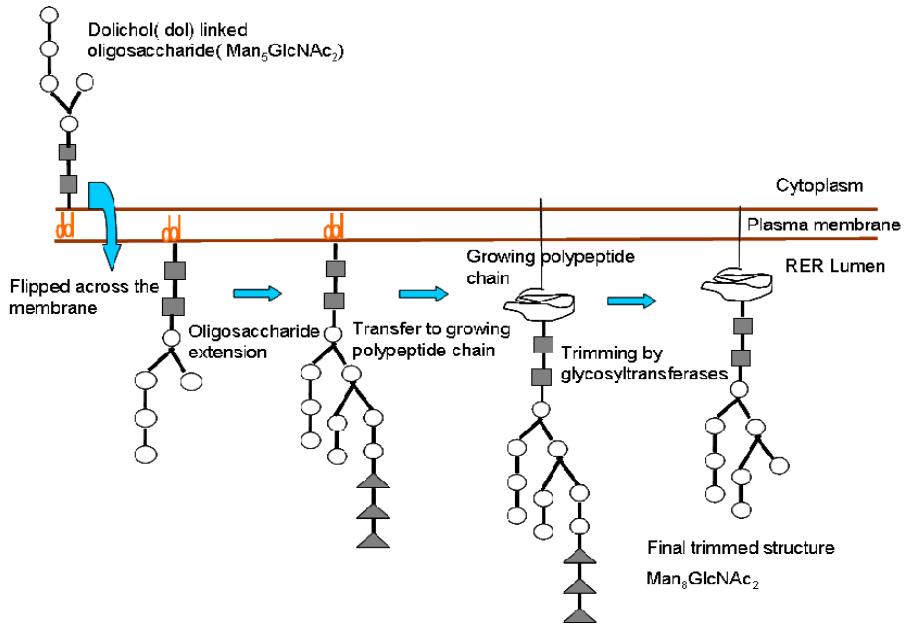



Figure.1.8.Schematic representation of N-linked glycosylation: Dolichol linked $Man_5GlcNAc_2$-phosphate is formed in cytoplasm and flipped into the lumen. The oligosaccharide chain is extended to form $Glc_3Man_9GlcNAc_2$-P-dol, cleaved from dolichol and attached to nascent polypeptide chain. The oligosaccharide is trimmed to $Man_8GlcNAc_2$. Monosaccharide symbols are given in Table.1.1. Adapted from (Brooks et al., 2002).

### *1.6.5. Synthesis of O-linked glycans*

O-linked glycosylation is simpler than N-linked glycosylation. It takes place in the Golgi apparatus. Unlike N-linked glycosylation, O-linked glycosylation is an entirely post transational event, i.e. it takes place after the synthesis and folding of the protein chain.

Individual monosaccharides are added in a step wise manner to the protein chain and addition of each monosaccharide is catalysed by a specific glycosyltransferase. O-linked glycans usually contain the monosaccharides

GalNAc, GlcNAc, Gal, Fuc and sialic acids, although other monosaccharides can also be present. The steps in O-linked glycan synthesis are described below.

1. O-linked glycosylation begins in the cis-compartment of the Golgi apparatus, most usually by the addition of a GalNAc to serine or threonine residue of the polypeptide chain to give rise to GalNAc-O-Ser/Thr. This structure is termed Tn antigen. The structure of Tn antigen is shown in Figure1.9. The reaction is catalysed by a family of GalNAc polypeptide transferases (ppGalNAc-Ts). Unlike N-linked glycosylation, a consensus amino acid sequence is not required for O-linked glycan attachment, although features characterising the sites of attachment have been described (Brooks et al., 2008b).

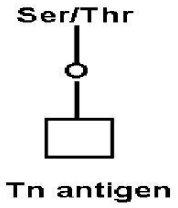



Figure.1.9.Schematic representation of the structure of Tn antigen: Tn antigen is the first glycan synthesized in O-linked glycosylation. For monosaccharide symbols, see Table 1.1. Adapted from (Brooks et al., 2002)

2. Tn antigen is normally not exposed in healthy cells, although it is often left unelaborated and exposed in cancer cells (Lisowska, 1995). It is usually either terminated by sialylation or extended by the stepwise addition of more monosaccharides. For example, addition of Gal by a

(β1→3) linkage to the Tn antigen gives rise to Gal(β1→3) GalNAc-Ser/Thr as shown in Figure 1.10. This structure is called core 1 or Thomsen-Friedenreich (TN or T) antigen.

3. In total, 8 O-linked core structures have been described, each resulting from the addition of Gal and/or GlcNAc to Tn antigen. Core 1-core 4 structures are more common compared to the other core structures. The 8 core structures are shown in Figure1.10
4. The core structures can be either sialylated / fucosylated, by the addition of SA or Fuc respectively, terminating further extension or they can be extended to form linear or branching O-linked chains. These linear and branched chains in turn can also be terminated by fucosylation or sialylation.
5. O-linked glycans can be very simple, for example, consisting of a single monosaccharide or they can be more complex. In general, O-linked glycoproteins tend to be less branched compared to N-linked glycoproteins. They are usually composed of simple linear or bi antennary chains.

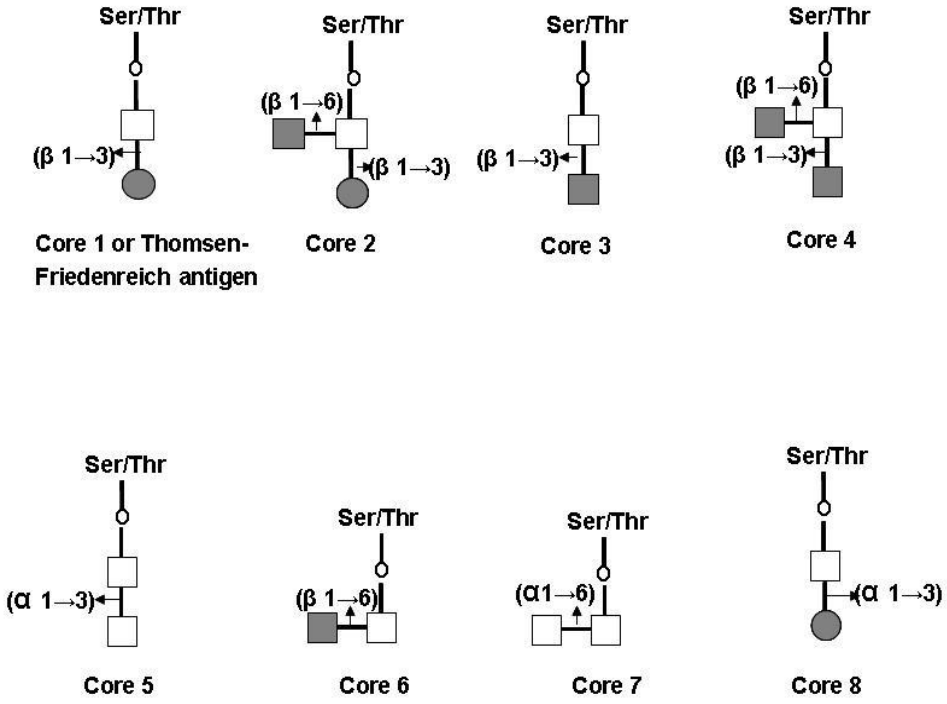



Figure.1.10: The eight core structures formed during O-linked glycosylation:

The core structures are formed by the addition of Gal and GlcNAc to Tn antigen. For monosaccharide symbols please see Table 1.1. Adapted from (Brooks et al., 2002).

### *1.6.6. N- and O-linked chain extension*

The N and O-linked structures described above can be extended by the addition of monosaccharides to give two types of glycoprotein chains. Type 1 chain is formed by the addition of Gal to a terminal GlcNAc residue by (β1→3) linkage. Type 2 chain is formed by the addition of Gal to a terminal GlcNAc by (β1→4) linkage and is called a lactosamine unit. Both these chains can be terminated by sialylation /fucosylation or can be extended further. Type 1 and type 2 chains are shown in the figure 1.11.


Figure.1.11: Schematic representation of type 1 and type 2 chains: The chains are formed during the extension of N and O-linked glycan chains. For monosaccharide symbols please see table 1.1

## *1.7. Aberrant glycosylation in cancer*

Glycoproteins play a key role in cell-cell and cell-extracellular matrix adhesion and interactions. Glycoproteins function as cell adhesion molecules *per se* and they also modulate the function of receptors of adhesion molecules like selectins and integrins which recognize them. Selectins and integrins have been described in the section 1.3.11 – 1.3.12. Aberrant glycosylation alters these functions and can confer cancer cells with novel properties, for example altered invasiveness and metastatic capability (Hakomori, 1996). While the changes in glycosylation reported in malignancy are widespread and complex, they may be categorised according to defined themes, described below:

1. Incomplete synthesis of glycans or synthesis of truncated glycans.
2. Exposure of Lewis antigens
3. Increased β1→6 branching of N-linked glycans
4. Changes in sialylation and fucosylation

5. Emergence of HPA-binding glycans.

### *1.7.1. Incomplete synthesis of glycans*

As described in section 1.6.5, the first step in the biosynthesis of O-linked glycans is the formation of GalNAcα Ser/Thr or Tn antigen. Following its synthesis, Tn antigen is either sialylated to form sialyl Tn (which terminates further chain extension) or more commonly, it is extended by the sequential addition of monosaccharides to form various core structures and further extension. Highly specialised enzyme families catalyse each step of O-glycosylation and these include, among others, members of the ppGalNAc –Ts, sialyl transferases and several core transferases.

Enzymes of O-linked glycosylation show altered level of activity in cancers. For example, among the members of the ppGalNAc –T enzyme family (which catalyse the first step of O-linked glycosylation) ppGal NAc- T1 and T2, believed to be 'housekeeping' enzymes, have been detected in normal breast epithelial cell lines while, in addition, other members of the ppGalNAc-T family, notably T3 and T6, whose expression is normally tightly regulated, are detectable in breast cancer cell lines (Brooks et al., 2007). Similar changes have also been found in colorectal cancers (Kohsaki et al., 2000) and gastric carcinomas (Gomes et al., 2009). Increased activity of α2,3 sialyl transferase (ST3Gal I), which catalyses the addition of SA to core 1, is seen in breast cancers (Burchell et al., 1999). Such alteration in the expression as well as the activity of these normally highly regulated enzymes results in modification of

the usual pathway of glycosylation resulting in the formation of incomplete or truncated glycans.

One such incomplete glycan that has been extensively researched in cancer glycobiology is Tn antigen. In normal tissues, Tn antigen is always extended and hence, masked. Exposure of Tn antigen is a well-known feature of cancers and was described for the first time in breast cancer (Springer et al., 1975). Tn antigen exposure is associated with poorly differentiated and metastatic carcinomas and hence is considered an established marker of poor prognosis. Tn antigen and another truncated glycan, core 1 or Thomsen-Freidenreich antigen (T antigen, described in section 1.6.5), have now been shown to be pan carcinoma markers present in more than 90% of all carcinomas (Springer, 1995).

Sialyl Tn (N-acetylneuraminic acid α(2→6)-N-acetylgalactosamine) which is formed by the sialylation of Tn antigen is also a feature of several cancers. It has been described in breast, gastric, oesophageal and colorectal cancers (Itzkowitz et al., 1989, Werther et al., 1994, Cho, 1994, Freire et al., 2005).

### *1.7.2. Exposure of Lewis antigens*

Lewis antigens are a family of small, fucosylated, histo blood group antigens consisting of four members, namely Lewis a ($Le^a$), Lewis b ($Le^b$), Lewis x ($Le^X$) and Lewis y ($Le^y$).

Lewis antigens are normally found on erythrocytes. In addition to this, $Lewis^a$ and $Lewis^b$ are also found on secretory epithelia like those in the gastro intestinal tract. During foetal life, Lewis antigens play a crucial role in

embryogenesis and organogenesis. In adults, they are involved in the protection of mucosal surfaces and binding of bacteria. In addition to this, Lewis antigens are strong binding partners of selectins. Therefore Lewis antigens present on neutrophils aid the recruitment of neutrophils and their selectin mediated rolling during inflammation as explained in sections 1.3 -1.4 (Sanders and Kerr, 1999, Ohyama et al., 1999). As explained in section 1.3, during metastasis, tumour cells may employ mechanisms that are similar to those used by leukocytes in inflammation. This process is thought to be mediated by Lewis antigens. Lewis antigens, when present on cancer cells, may be implicated in cancer cell adhesion and transendothelial migration, and thus be functional in metastatic mechanisms (Ohyama et al., 1999). Lewis antigens a and x and their sialylated counterparts are shown in Figure.1.12.

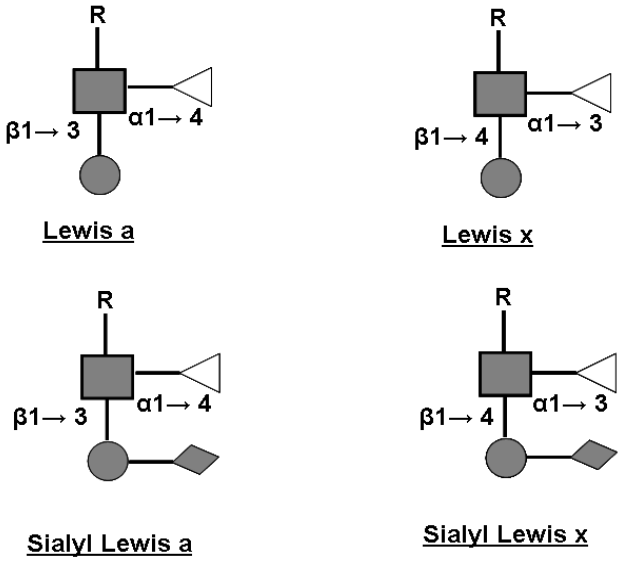



Figure 1.12.Schematic representation of Lewis antigens, a and x and their sialylated forms. R represents linkage to the polypeptide chain. Monosaccharide symbols are shown in Table 1.1

Lewis antigens and their sialylated forms are synthesised by several cancers. $Le^y$ and $Le^b$ antigen are elevated in breast carcinoma and have been shown to be a bad prognostic sign when present in lymph node negative tumours (Madjd et al., 2005). Sialyl $Le^a$ has been shown to be a prognostic indicator for outcome and survival in colorectal cancers (Shimono et al., 1994). Loss of $Le^b$ antigen has been described in breast cancer and $Le^y$ antigen has been associated with poorly differentiated cancers of colon, rectum, liver and uterus (reviewed in Brooks et al., 2008b).

### *1.7.3. Increased branching*

As explained in section 1.6.6., glycoprotein extension often takes place through the formation of type 1 and type 2 chains. Type 1 chains are frequently extended by the addition of Fuc, GalNAc, SA and Gal residues to give several commonly occurring glycoproteins. The Lewis group of antigens, described in the previous section, is one such derivative of N-linked glycosylation. Malignant transformation usually leads to synthesis of larger N-linked glycans which frequently demonstrate increased branching at the trimannosyl core i.e. -GlcNAcβ1-6Manαl-6Manβ- (Easton et al., 1991). The β(1→6) branches of tri and tetra antennary N-linked glycans are typically attached to polylactosamine chains. These polylactosamine chains in turn carry Lewis antigens (Brooks et al., 2008b). Increased β(1→6) branching therefore increases Lewis antigen content. As described in the previous section 1.7.2, the presence of increased Lewis antigens are associated with enhanced metastatic capability of cancers and poorer prognosis.

Increased GlcNAc β(1→6)Man branching has been demonstrated on colon and breast cancers. The glycans with increased β(1→6) branching are specifically recognised by a lectin (lectins, which are naturally occurring glycan-binding molecules, are described in the section 1.8.) isolated from the common bean *Phaseolus vulgaris* (PHA-L). PHA-L binding glycans were reported to be significantly increased in clinical specimens of breast and colon cancers and the levels correlated with the grading of the tumors (Fernandes et al., 1991). Increased β(1→6) branching has also been demonstrated in breast cancers with lymph node metastasis and is associated poorer prognosis (Handerson et al., 2005).

### *1.7.4. Altered sialylation*

It has been explained in the section 1.6.5., that sialic acids are often present at the ends of glycan chains and the addition of sialic acids terminates further chain extension. Sialic acids are negatively charged and play an important role in cell to cell communication, cell-extracellular matrix communication, adhesion and interaction with cell membrane proteins.

Altered sialylation is brought about by the alteration of expression or activity of sialyl transferases, the enzymes that catalyse addition of sialic acids in specific linkages to the glycans chain. Altered activity of sialyl transferases has been demonstrated in cancers including breast cancer (Whitehouse et al., 1997, Burchell J, 1999 ) and gynaecological cancers (Wang, 2004).

### *1.7.5. HPA binding glycans*

Abnormal glycosylation changes recognised specifically by the lectin *Helix pomatia* agglutinin (HPA) are a well-documented feature of several cancers and are described in detail in 1.8.2-6. This type of glycosylation change, and in particular, the putative functional role of the glycans in the metastatic cascade, are of particular interest to this thesis. Hence, a brief description of lectin chemistry, HPA and its binding partners, HPA binding glycans in cancers in general and breast cancer in particular is given below.

## ***1.8. Lectin chemistry***

Lectins are proteins or glycoproteins that bind to carbohydrates in a selective and specific fashion. They are ubiquitous in nature and present in micro-organisms, invertebrates, vertebrates and plants. Lectins are multivalent (i.e. they possess multiple carbohydrate binding sites) and are of non-immune origin. Hundreds of purified lectins are available commercially, many from plants sources, and with differing and often incompletely defined carbohydrate-binding properties. Like HPA, they have been utilised widely in glycobiology research. They are of particular interest in the study of abnormal glycosylation in cancers because they possesses highly selective binding pattern to specific glycans for e.g. abnormal glycans present on cancer cell surface.

Human lectins are classified in to 5 lectin families which include C-Type lectins, I-Type lectins, S-Type lectins or Galectins and P-Type and L-Type lectins. Among these 5 lectin families, the C-Type lectins which include selectins and I-Type lectins which include members of the immunoglobulin

superfamily like ICAM-1 and PECAM are of particular relevance to this thesis (see also sections 1.3.13 and (Brooks et al., 2002) .

### *1.8.1. HPA and its binding partners*

*Helix pomatia* agglutinin (HPA) is a glycoprotein that is derived from albumin gland of the edible Roman snail, *Helix pomatia*. HPA is structurally a hexamer and displays a binding specificity to the monosaccharide, α-D-GalNAc (Hammarstrom and Kabat, 1969, Sanchez et al., 2006). In addition to GalNAc, it also binds to the monosaccharides α-D-GlcNAc and α-Gal, but with lower affinity. In experimental systems, HPA identifies and specifically binds terminal GalNAc residues in normal substances including blood group A antigen. It also recognises cancer-associated glycans including Tn antigen (discussed in Sections1.6.4 and 1.7.1) and Forssman antigen (αGalNAc1→3αGalNAc), a cancer-associated epitope present in glycolipids (Lescar et al., 2007). The recognition by HPA of (as yet incompletely defined) glycans synthesised by cancers has been described as being associated with metastasis and poor patient prognosis, as described in section 1.8.3 and is a particular focus of this thesis.

### *1.8.2. HPA binding glycans and cancer prognosis*

HPA binding glycans on cancer cell surface as a marker of poor prognosis was described for the first time by Leathem and Brooks in 1987. They conducted a retrospective study of 176 patients who had in undergone mastectomy for primary breast cancer and labelled the tumour samples of these patients for

HPA binding glycans. Positive or negative HPA binding in the tumour samples was compared to patient prognosis defined in terms of disease free interval post- surgery, 5 year and 10 year survival rates. It was shown that patients with positive HPA-binding had shorter survival periods, with an average 5 year survival rates for HPA-positive patients being 40% and 10 year survival rates being 35%. This was in comparison to 5 year survival rate of 90%, and 10 year survival rates of 75% in non-HPA-binding cases. This study thereby provided evidence that presence of HPA binding glycans correlated with shorter disease free interval and poorer prognosis.

The same researchers continued with an investigation of the association between HPA binding glycans and positive lymph node metastasis in breast cancer in an extended retrospective study of 373 patients (Brooks and Leathem ,1991). Subsequently, a multivariate analysis was also carried out in the same patient group to analyse the relationship between HPA binding glycans and other, well established prognostic indicators including lymph node status, patient age at diagnosis, tumour size, histological grade and S-phase fraction (Brooks et al, 1993). Both studies established a strong correlation between HPA binding glycans and lymph node metastasis. In addition to this, as a prognostic factor, the presence of HPA binding glycans was demonstrated to be independent of all the other prognostic factors tested. Hence, HPA binding glycans emerged as vital indicators of metastatic potential and poor patient prognosis in breast cancer (Brooks and Leathem, 1991, Brooks et al., 1993).

Following on from these pioneering studies, abnormal HPA binding glycans as a hallmark of cancers and a feature of poor prognosis has been subsequently confirmed by several other independent studies both for breast cancer (Fenlon et al., 1987, Fukutomi et al., 1989, Alam et al., 1990, Thomas et al., 1993, Noguchi et al., 1993, Chen et al., 2007) and for cancers of the oesophagus (Yoshida et al., 1993), stomach (Kakeji et al., 1991, Maehara et al., 1995), colon (Schumacher and Adam, 1997), lungs (Laack et al., 2002), prostate (Shiraishi et al., 1992) and malignant melanomas (Kjonniksen et al., 1994, Thies et al., 2001) .

### *1.8.3. Characterisation of abnormal HPA binding glycans*

Despite a strong body of evidence linking HPA binding glycans to cancers in general and metastatic cancers in particular as described in the previous section, the exact nature and identity of HPA binding glycans remains undefined. Some early reports linked the metastatic potential of abnormal HPA binding glycans first described by Leathem and Brooks in 1987 to the presence of Tn antigen (Springer, 1989) and to blood group A substance (Grundbacher, 1987), both established binding partners of HPA. However, immunohistochemical studies utilising HPA and monoclonal antibodies reactive with Tn antigen and blood group A substance in breast cancer specimens has established that while these two epitopes are present in a minority of the cancer specimens and are promptly recognised by HPA, in majority of the specimens, HPA also identified an additional diverse group of abnormal glycans with a markedly different labelling and distribution pattern compared to blood group A substance and Tn

antigen (Brooks and Leathem, 1995). Furthermore, western blot analyses comparing HPA binding glycoproteins with blood group A- and Tn antigen-bearing glycoproteins in breast cancer specimens and colon cancer cell lines indicated that the unidentified epitope bound by HPA represents a global glycosylation abnormality that is different in distribution to both Tn antigen and blood group A substance (Brooks and Leathem, 1995, Saint-Guirons et al., 2007).

Extraction and purification by affinity chromatography of cell membrane glycoproteins from breast cancer cell lines and clinical breast cancers specimens has shown that the HPA binding glycans in these glycoconjugates contain GalNAc and sialic acid. Similar experiments in colorectal cancer cell lines followed by competitive inhibition studies with GalNAc, GlcNAc and sialic acids has shown that the glycan- HPA interaction in these cells occurs through binding of HPA to glycoconjugates consisting of GalNAc, GlcNAc and sialic acid and their location on the glycan chain is either terminal or sub terminal (Dwek et al., 2001, Saint-Guirons et al., 2007).

Protein elution from HPA affinity chromatography and subsequent characterisation of these proteins by proteomic analysis has revealed that HPA binding proteins include molecules like integrin α6, integrin α5 and annexins, which are involved in cell adhesion and migration, filament proteins like α-tubulin, β-tubulin, cytokeratins and actin which are involved in remodelling, and HSP-70, HSP-90, HSP-96 and TNFR-1 which are involved in antiapoptotic pathways. While several of these molecules have been associated with cancer

and might be subject to abnormal glycosylation and hence recognised by HPA, the exact mechanism of binding of these complexes to HPA is still unclear. Some of these molecules might bind HPA directly through the lectin-glycan interactions described earlier, while others might bind HPA indirectly by forming complexes with HPA binding glycoproteins, for example, by binding O-GlcNAc glycoproteins in the cytoplasm (Saint-Guirons et al., 2007).

### *1.8.4. Functional role of HPA binding glycans in the metastatic cascade*

It is clear from the discussions in sections 1.8.2.that exposure of HPA binding glycans on cancer cell surface both in breast cancers and several other cancers is a prominent feature of aggressive and metastatic cancers and a well-recognised marker of poor prognosis. As a consequence of this association, studies to explore if HPA binding glycans played a part in any, or all, of the steps of metastatic cascade were initiated.

Brooks and Hall (2002) carried out a matrigel invasion assay to investigate whether HPA binding glycans were involved in the adhesion of cancer cells to the vascular basement membrane and subsequent invasion through it (Brooks and Hall, 2002). This process of adhesion to, and invasion through, basement membrane is a vital component of both intravasation in to blood vessels and extravasation out of blood vessels during blood borne metastasis and is described in detail in sections 1.2.1-1.2.5.

In this study, seven breast epithelial cell lines were selected to include normal breast epithelial cells along with a range of malignant cells to represent a disease spectrum varying from primary, minimally invasive breast cancers to

aggressive, metastatic breast cancers. The HPA binding profiles of these cell lines had been well characterized previously and was found to correlate with HPA binding glycan profile present in clinical breast cancer specimens (Brooks et al., 2001). The cells were assessed for their ability to adhere to, and invade through basement membrane matrix in the presence of HPA binding glycans and this was compared to adhesion and invasion of cancer cells after the inhibition of HPA binding glycans. There was no difference in the ability of the cancer cells to adhere or to invade through basement membrane in the presence or absence of HPA inhibition in this study for all the seven cell lines studied thereby indicating that HPA binding glycans were unlikely to be involved in this aspect of the metastatic cascade.

A further pilot study then investigated the role of HPA binding glycans in another crucial step of the metastatic cascade namely, adhesion of cancer cells to vascular endothelial cells lining the microvasculature and their subsequent transmigration across the vessels (adhesion and transendothelial migration of cancer cells has been described in detail in sections 1.3-1.5). The adhesive interactions of six breast cancer epithelial cell lines in the presence and absence of HPA binding glycans to cultured endothelial cells lines HMMEC7 and HUVEC were studied using a rocking adhesion assay system. Inhibition of HPA binding glycans was found to significantly reduce the adhesion of breast cancer cells to the endothelial cell layer in this study thereby suggesting that HPA binding glycans present on cancer cell surface might contribute to

adhesion and transendothelial migration of these cells during metastasis (Valentiner et al., 2005).

### *1.8.5. Role of HPA binding glycans in adhesion and transendothelial migration step of the metastatic cascade*

The very limited pilot study by Valentiner et al described above in section 1.8.4. was further expanded by Dr. Hannah Lomax-Browne, one of the PhD students in our group to investigate the adhesion and transmigration step of the metastatic cascade by utilising a model of breast cancer metastasis consisting of breast cancer cell lines and human microvascular endothelial cell lines (Lomax-Browne, 2009). The 7 breast epithelial cell lines employed in this model included HMT3522 derived from normal breast and 6 breast cancer cell lines namely BT474, MDA MB 435, MDA MB 468, ZR751, MCF7 T47D and DU4475. The microvascular endothelial cell lines were human brain microvascular endothelial cells (HBRMEC), human lung microvascular endothelial cell line (HLMEC), human peripheral lymph node endothelial cell line (HPLNEC) and human mesenteric lymph node endothelial cells (HMLNEC) derived from human brain, lung and lymph nodes respectively. However, due to technical faults with the microvascular endothelial cell lines, the breast cancer metastasis model used by Dr. Lomax-Browne was altered to incorporate the primary endothelial cells, human umbilical vein endothelial cells (HUVECs). Furthermore, the adhesion assays were further developed and optimised to incorporate live cell imaging.

Three breast cancer cell lines were utilized in the project described in this thesis and these were BT 474, ZR 75 1 and MCF 7. The process of adhesion and transendothelial cell migration of these 3 cell lines was studied by performing adhesion assays to a monolayer of commercially available, primary endothelial cells, human umbilical vein endothelial cells (HUVECs).

The three breast cancer cell lines BT 474, ZR 75 1 and MCF 7 were a kind gift from the genomic instability research group at Oxford Brookes University and HUVECs were procured from Lonza, UK. The three breast cancer cell lines utilized in this project were selected to represent a spectrum of behavioural patterns seen in breast cancers ranging from primary, non-metastatic tumours to aggressive, metastatic tumours. BT 474 is a breast cancer epithelial cell line derived from a non-metastatic primary ductal carcinoma of the breast (Lasfargues et al., 1978). ZR 75 1 is derived from malignant ascites secondary to ductal carcinoma of the breast (Engel et al., 1978). MCF 7 was originally derived from a malignant pleural effusion secondary to infiltrating ductal carcinoma of the breast (Soule et al., 1973). The HPA binding glycan profiles of the cell lines have been carefully documented and are consistent with their derivation. Thus, the metastatic MCF 7 cells synthesize high levels of HPA binding glycans, ZR 75 1 cells synthesize moderate levels of HPA binding glycans and BT 474 cells synthesize negligible levels of HPA binding glycans (Brooks et al., 2001). HPA has been clearly shown to recognize a similar profile of glycosylated proteins in these cell lines and clinical breast cancer samples and hence, these cells form appropriate tools for the study of breast

cancer cell behaviour during metastasis (Dwek et al., 2001, Brooks et al., 2001).

HUVECs are primary, macro vascular endothelial cells commonly obtained by collagenase digestion of the umbilical vein from freshly delivered placenta. The cells possess morphological and immunological properties similar to other macro vascular endothelial cells in the body (Gimbrone et al., 1974, Jaffe et al., 1973). They are commonly utilised in the study of adhesion and transendothelial migration in both cancer cells and leucocytes. One of the main limitations of using HUVECs in the study adhesion and transendothelial migration is that HUVECs are not derived from a site which is physiologically relevant to cancer cell metastasis. The molecular interactions during adhesion and transendothelial migration in cancer cell metastasis and the analogous process involving leucocytes during inflammation occur predominantly in the terminal capillaries of the body where the cancer cells or leucocytes interact with the micro vascular endothelial cells lining the capillaries. However, HUVECs are commonly employed as a substitute to micro vascular endothelial cells in the study of adhesion and transendothelial migration in both cancer cells and leucocytes because they are relatively inexpensive and easy to grow in comparison to their micro vascular counterparts. In addition to this, HUVECs, when grown as primary cultures, also express a range of relevant adhesion molecules e.g. selectins and integrins involved in the process of adhesion and transendothelial migration, and expression of these key molecules is usually lost in immortalised cell lines.

The optimisation of model of breast cancer metastasis to incorporate HUVECs and to facilitate live cell imaging and the study of adhesion and transendothelial migration step of the metastatic cascade by utilising this are the focus of the research project described in this thesis.

## ***1.9. Aims of the project***

The aims of the project are as follows.

- To optimise a confocal microscope compatible rocking adhesion assay system to facilitate live cell imaging
- To determine the optimum duration of rocking to be utilised for the rocking adhesion assays.
- To employ the rocking adhesion assay system to evaluate the role of HPA binding glycans in the adhesion of breast cancer epithelial cells to endothelial cells
- To study the post adhesive behavior of the cancer cells using confocal microscopy and scanning electron microscopy

# Chapter 2: Materials and Methods

## *2.1. Introduction*

The experiments described in this thesis utilise three breast cancer cell lines BT 474, ZR75 1 and MCF 7 and the endothelial cells HUVECs.

In the following sections, a brief description of the techniques employed for routine culture and maintenance of the cancer and endothelial cells used in this project is given along with a brief outline of the important quality control steps. Detailed description of individual experiments is then given in the chapters pertaining to those experiments.

## *2.2. Routine cell culture techniques:*

### *2.2.1: Retrieving cells from liquid nitrogen storage*

Cryovials containing frozen cell suspension were removed from liquid nitrogen storage and sterilised with 70% ethyl alcohol. The cell suspension was thawed by rolling the cryovials in the palm of the hand. The thawed cell suspension from each vial was pipetted out in to a 15ml Falcon tube containing 5ml of fresh, warmed culture media. The different cell culture media utilised for the culture of the various cells types is detailed in Table 2.1. The Falcon tubes were centrifuged in a Jouan B4 centrifuge at 260 ×g for 10 min if retrieving breast cancer epithelial cells and 7 min if retrieving endothelial cells. The supernatant was discarded and the tubes were flicked several times to loosen the pellet of cells. The cells from each tube were resuspended in 7ml of cell culture media and transferred to a 25cc tissue culture flask. The cells were incubated in a 37oC, 5%, CO2 incubator. 24 h after retrieval, spent culture media was pipetted

out from the flasks to remove any dead cells and replaced with fresh cell culture media to facilitate the recovery of the remaining cells. The cells were grown to confluence and were either transferred to 75cc flasks or sub cultured as described in section 2.2.3.

### *2.2.2: Routine maintenance of cell cultures:*

The breast cancer epithelial cells and endothelial cells were cultured in 25cc or 75cc tissue culture flasks. Cell culture media was routinely changed every 48-72 h by pipetting out old media and replacing it with fresh, warmed media (7ml for 25cc flasks and 15ml for 75cc flasks) until the cells were ready to be sub cultured. The cell culture media used for each cell type and the relevant supplements is listed in Table 2.1.

### *2.2.3: Routine sub culturing of the cells:*

Cells were sub cultured when the confluence of the cells was between 85-100%. Confluence was estimated by visual assessment of the percentage of the culture surface covered by cell growth. For example if half of the culture surface was covered by cell growth, then confluence was taken to be 50%, if the whole surface was covered by cells, then 100% and so on.

To subculture cells, spent media was pipetted out and discarded from culture flasks and the cells were washed thoroughly with phosphate buffered saline (PBS, pH: 7.2-7.4, Gibco, UK). PBS was discarded and the cells were rinsed thoroughly with a solution of porcine trypsin in EDTA (Gibco, UK), (1ml for 25cc flasks and 2ml for 75cc flasks). Breast cancer cells were trypsinised using

0.05% w/v of trypsin/EDTA solution and after removing the trypsin, cells were incubated at $37^0$C for 7-10 minutes. A similar method of trypsinisation was utilised for HUVECs but using 0.025% w/v solution of trypsin/EDTA solution and an incubation time of 2-5 min. The trypsin was inactivated and the cells dissociated from the culture flasks by rinsing the culture surface with cell culture media. The cell suspension was pipetted up and down several times to disaggregate clusters of cells and the cells were seeded into tissue culture flasks at a ratio of 1:5-1:10.

Fresh vials of HUVECs were retrieved from liquid nitrogen prior to each set of experiments. The cancer cells were grown for a maximum of 5 passages and HUVECs were grown for a maximum of 3 passages to minimise phenotypic variation.

### *2.2.4: Freezing down cells in liquid nitrogen storage:*

Cell suspension was obtained from confluent 75cc flasks as described in section 2.2.2-3. The cell suspension was centrifuged at 1200rpm in a Jouan B4 centrifuge at 260 ×g for 10 min (7 min for endothelial cells). Supernatant was discarded and the cell pellet was loosened by flicking the tube. The cells were suspended in 2ml of 1:9 solution of dimethyl sulphoxide in culture media and transferred to 2x 1.5 ml cryovials. The cryovials were placed in $-20^0$C freezer for 2 hours, $-80^0$C freezer overnight and were then transferred to liquid nitrogen store the next morning.

Table 2.1: Media and supplements used for cell culture

| Cells | Media | Supplements |
|---|---|---|
| BT 474 | IMDM<br>(PAA, UK) | Heat inactivated FCS-13% v/v (Sigma, UK)<br>L-Glutamine-2Mm (Gibco, UK)<br>1%v/v penicillin and streptomycin solution (Sigma, UK) |
| ZR 75 1 | RPMI-1640<br>(Gibco, UK) | Heat inactivated FCS-10%v/v (Sigma, UK)<br>L-Glutamine-2mM (Gibco, UK)<br>Sodium pyruvate-1mM (Sigma, UK)<br>1%v/v penicillin and streptomycin solution (Sigma, UK) |
| MCF | DMEM:F-12<br>(Gibco, UK) | Heat inactivated FCS-13%v/v (Sigma, UK)<br>L-Glutamine-2Mm (Gibco, UK)<br>1%v/v penicillin and streptomycin solution (Sigma, UK) |
| HUVECs | EBM-2<br>(Clonetics,<br>Lonza, UK) | Components of single quotes supplement pack (Clonetics, Lonza, UK) added to 500mls of media. Components consist of :<br>-Hydrocortisone-2mls<br>-Human fibroblast derived growth factor-B (hFGF-B) -0.5mls<br>-Vascular endothelial growth factor (VEGF) -0.5mls<br>-R3-insulin derived growth factor -1(R3-IGF-1) -0.5mls<br>-Ascorbic acid-0.5mls<br>-Heparin-0.5mls<br>-Human epidermal growth factor (hEGF)-0.5mls<br>-Foetal bovine serum-10mls<br>-Gentamycin and amphotericin(GA-1000) solution-0.5mls |

## ***2.3. Quality control steps:***

### *2.3.1 Viability testing of the cells:*

Cell cultures were routinely tested for the percentage of viable cells prior to utilizing them for experiments using the stain erythrosin-B. Erythrosin-B is a non-toxic and highly effective stain for viability testing particularly for cells in monolayer cultures. Erythrosin-B is a vital exclusion stain because it is

excluded by live cells which possess an intact plasma membrane. Viable cells, therefore, appear clear when stained with the dye. Erythrosin-B however, diffuses into dead cells which have lost the integrity of their plasma membrane and makes the cells appear pinkish red when viewed using a light or fluorescent microscope (Krause et al., 1984).

For viability testing, cell suspension was obtained as described in section 2.2.3. 100µl of the cell suspension was mixed with 100 µl of 0.8mg/ml working solution of erythrosine-B (Sigma, UK) in Hanks balanced solution (Sigma, UK). 10 µl of this mixture was placed on a haemocytometer slide and a cell count was obtained. The percentage of live and dead cells was noted and the cell suspension was used in experiments only if the percentage of viable cells was above 95%.

### *2.3.2. Testing for mycoplasma contamination:*

All the cell cultures were periodically tested for mycoplasma contamination by using the DNA stain Hoechst 33258 (Sigma). Hoechst 33258 stains nuclei of cells and mycoplasma and when viewed using a fluorescence microscope and UV filter, DNA excitation occurs at 360-365nm and the stained nuclei and mycoplasma fluoresce brightly. When cell cultures are stained with Hoechst 33258, clean cultures will demonstrate only clear, brightly fluorescent nuclei with uniformly black extra nuclear and intercellular background. Infected cultures on the other hand will display mycoplasma which appear as brightly fluorescent, extra nuclear and inter cellular bodies with uniform morphology (Chen, 1977).

To carry out testing for mycoplasma contamination, a cell suspension was obtained by trypsinisation as described in section 2.2.3. Cells were seeded at optimum seeding densities in 4-well chamber slides (Sigma, UK). Once the cultures reached a confluence of 60-70%, media was aspirated and the cells were washed with 3 changes of PBS. The cells were fixed at room temperature using 25% v/v glacial acetic acid in methanol for 10 min and washed again with 3 changes of PBS. Chamber slides were then placed in the dark and the cells incubated with a 0.05µg/ml solution of the stain Hoechst 33258 (Sigma) in FCS free media for 10 min. The cultures were then washed three times with sterile distilled water and the chambers were removed from the slide. The slide was mounted using Citifluor anti-fade mountant (Agar scientific) with a coverslip. The coverslip was sealed using clear nail varnish and the slide was viewed using an Axioplan Pol universal fluorescence microscope using a 40x objective and UV filter.

DNA excitation occurred at 360nm and emission at 490-500nm and in all the cultures tested. There was no evidence of mycoplasma contamination as evidenced by the presence of brightly fluorescent nuclei with no extra nuclear fluorescent bodies.

### *2.3.3 Karyotyping of the cells*

The endothelial cells and cancer cell lines utilized in this project were subjected to karyotyping prior to commencing this project with the help of colleagues in the genomic instability research group at the university. This process was performed to establish the karyotype of all the cells utilised for this project and

to ascertain that there was no modification of the cells from their standard reported karyotype. A detailed description of the procedure employed for karyotyping is beyond the scope of this thesis and can be found in standard text books [for example (Tirulinai, 2012)].

# Chapter 3: Optimization of rocking assay

## *3.1. Introduction*

During metastasis, cancer cells detach from the primary tumour mass, gain entry into the systemic circulation by a process referred to as intravasation, get carried via the blood stream to target organs where they adhere to and transmigrate across the endothelial cells lining the microvasculature of the target organs to form secondary tumours (as described in Chapter 1, sections 1.2.1-1.2.5). While cancer metastasis has been described as a cascade and cancer cells have to complete each step of the cascade in a sequential manner to be able to successfully metastasise, one of the key steps in cancer cell metastasis appears to be adhesion of cancer cells to endothelium and transendothelial migration. Early clinical studies by Tarin et al (1984) demonstrated that a major proportion of cancer cells which have reached the systemic circulation having successfully completed the preceding steps of the metastatic cascade fail to form metastatic tumours (Weiss, 2000). As described in section 1.2.5, whilst mechanical entrapment of cancer cells in the small-bore capillaries of target organs may contribute to the seeding of metastatic deposits, there is increasing evidence that cancer cells utilise highly selective, organ specific mechanisms to adhere to the endothelial cells lining the capillaries of the target organs and progress to transmigration and formation of secondary deposits. The behaviour of cancer cells during adhesion and transmigration is thought to be similar to the behaviour of leucocytes during inflammation as described in section 1.3. Adhesion and transendothelial migration in leucocytes

has been studied in great detail and is well understood. The analogous process in cancer cells however is still poorly understood.

Two adhesion assay systems, namely static adhesion assay systems and flow assay systems are traditionally utilised to study the process of adhesion and transendothelial migration in both cancer cells and leucocytes. Both assay systems utilise a monolayer of endothelial cells mimicking the capillary endothelium onto which the cancer cells or leucocytes are added. Whilst in the static assay system the adhesive interactions between the two cells are allowed to take place under static conditions, in the flow assay systems, the cancer cells or leucocytes are caused to sweep over the endothelial cell layer and the adhesive interactions occur under conditions of flow.

Static adhesion assay systems have the advantage of being simple to set up and easy to perform without the need of specialist equipment. However, their main disadvantage is that they do not mimic physiological conditions of dynamic flow which produces shear forces that are thought to be vital in up regulating important adhesion molecules. Flow assay systems have the advantage of closely mimicking physiological conditions and allow for precise control of the experimental variables. For example, in the study of leucocyte adhesion and transendothelial migration, flow assay systems can be precisely modulated to produce a shear stress in the range of 1-6 dyn/cm$^2$ which is believed to be the physiological range of shear stress for leucocyte recruitment at post capillary venules (Toetsch et al., 2009) . Despite this advantage however, flow assays require specialist equipment and hence can be expensive. They can also be time

consuming particularly when multiple replicates of the assay are required for obtaining quantitative data.

An alternative assay system to the static and flow assay system is a semi static adhesion assay system. In the semi static assay system, the cancer cells are laid on a monolayer of endothelial cells like the static adhesion assays, but conditions of flow are created by rocking the co culture of cells on a rocking platform. Preliminary experiments employing the semi static adhesion system were carried out initially by Valentiner et al to study the adhesion of breast cancer cell lines MCF 7, ZR 75-1 and DU 4475 to human umbilical vein cells and human mammary micro vascular endothelial cells by (Valentiner et al., 2005). Subsequently, the rocking adhesion assay system was further developed and optimised by Dr. Chloe Osborne one of the PhD students in our group (Osborne, 2004). In brief, the rocking adhesion assays optimised by Dr. Osborne consists of the following steps:

- A monolayer of endothelial cells is cultured on cover slips placed in 24 well plates. Prior to the assay, the endothelial cells are pre stimulated with TNFα for 2 h to up-regulate adhesion molecules.
- The cancer cells are labelled with the fluorescent dye, 8-hydroxypyrenetrisulphonic acid (PTS) to aid their differentiation from endothelial cells once adhesion had taken place. The cancer cells are then laid on the endothelial cell monolayers in the 24 well plates and the plates are rocked to and fro on a rocking platform placed in a 37$^{o}$C $CO_2$ incubator for 2 h.

- At the end of 2 h, the cover slips are washed to remove any non-adherent cancer cells, the cells are then fixed and the adherent cancer cells are counted using a fluorescence microscope.

This rocking adhesion assay system was utilised for many of the experiments described in this thesis because of the following advantages

- The assay is quick to set up and simple to perform.
- It facilitates multiple repetitions of the experiment and can be performed without the need of any specialist equipment.
- The adherent cancer cells can be visualised and counted to perform both qualitative and quantitative analysis of the results.

Prior to using the rocking adhesion assay for experiments described in this thesis, two main aspects of the assay were optimised. The optimisation experiments are described below.

### *3.1.1. Optimisation of rocking adhesion assay system for live cell imaging.*

In the original rocking adhesion assay described in section 3.1., rocking to bring about adhesion of cancer cells to endothelial cell monolayer was followed by fixing the cancer cells for the purposes of imaging. While fixation is widely utilised in both plant and animal biology, it is known to produce adverse effects on the cells including degradation of nucleic acids, bleaching of lipids and denaturation of proteins (Meade et al., 2010). In addition to this, experimental factors like choice of fixative, technique used and time of fixation all have an impact on the final results. Moreover, one of the aims of the work described in this thesis was to image the interactions between living cancer cells and

endothelial cells over timed assays using high resolution confocal microscopy. Since the initial development of the assay, a wide range of confocal microscope compatible cell culture devices that facilitate high resolution, live cell microscopy have become widely available. Hence the rocking adhesion assay system was developed to incorporate confocal microscope compatible dishes to enable live cell imaging. Experiments described here, were performed to determine whether these were suitable for use in the rocking adhesion assay

### *3.1.2. Determining the time of rocking to obtain optimum number of adherent cancer cells*

The second aspect of the assay system that needed further optimisation was the total period of rocking to be employed in order to produce maximum adhesion of cancer cells to the endothelial monolayer to facilitate both quantitative and qualitative analysis of the results. Previous assays, described in section 3.1., had employed an arbitrarily chosen time of 2 hours.

## ***3.2. Materials and methods.***

Components of the rocking adhesion assay system are illustrated in Figure 3.1

### *3.2.1 Optimisation of rocking adhesion assay for live cell imaging*

1. Prior to the assay, confocal microscope compatible ibidi µ-dishes (35mm, high, Thistle scientific, UK) were coated with 1 ml of 0.2% v/v bovine gelatine (Sigma, UK) in phosphate buffered saline for 30 min.

2. A cell suspension of HUVECs was obtained by trypsinisation as described in Chapter 2, section 2.2.3. The cells were seeded at a density of 1.5x $10^5$ to 2x$10^5$ cells /dish.

3. 2 ml of fresh, warmed, EBM-2 media with growth supplements (as detailed in Chapter 2, Table. 2.1.) was added to each dish and the dishes were incubated in a $37^0$C, 5% $CO_2$ incubator. Media was changed daily as described in Chapter. 2, section 2.2.2 until a confluent monolayer of endothelial cells with no gaps in the cell layer was obtained in each dish.

4. On the day of the assay, spent culture media was aspirated from ibidi dishes and 1ml of warmed, TNF-α in EBM-2 media at a concentration of 10 ng/ml was added to each dish. The dishes were incubated in a $37^0$C, 5% $CO_2$ incubator for 2 h.

5. At the end of 2 h, the TNF-α solution was replaced with 2 ml of fresh EBM-2 media/dish. The dishes were rocked on a rocking platform, (shown in Figure 3.2c) placed in a 370C, 5% CO2 incubator for 2 h. At the end of 2 h, the dishes were removed from the rocking platform and the cells were washed with 3 changes of PBS. 2 ml of fresh EMB-2 media with supplements was added to each dish and the dishes were imaged using Zeiss confocal microscope (458 nm laser, 475 LP filter and 10x plan-Neofluar objective) to assess the integrity of the endothelial monolayer. Each repeat of the assay was carried out by using 2 ibidi dishes and the assay was repeated 3 times.

6. The other component of the rocking adhesion assay namely the breast cancer epithelial cell lines were tested for the presence of surface HPA binding glycans. Cancer cell suspension was obtained as described in Chapter 2, section 2.2.3. 1 ml of cell suspension was placed in an ibidi dish and the cells were allowed to adhere to the culture surface for 12 hours. 10µg/ml of HPA FITC (Sigma Adrich, UK) was added and the cells were incubated for 2 h. The cells were then washed with 3 changes of PBS and imaged using Zeiss confocal microscope with 458 nm laser, 475 LP filter and 25x plan-Neofluar objective.

### *3.2.2 Determination of duration of rocking*

1. To determine the optimum duration of rocking, MCF-7 and ZR 75-1 cells were cultured to obtain confluent flasks of cells as described in section 2.2.3. and endothelial cells were cultured in ibidi dishes as described in section 3.2.1. ( Represented in figures 3.1a,b.)
2. Cancer cells were incubated overnight with a 10 mg/ml solution of the fluorescent dye 8-hydroxypyrenetrisulphonic acid (PTS) (Sigma, UK) in cell culture media.
3. To perform the assay, HUVEC cells in ibidi dishes were pre incubated with10 ng/ml of TNF-α in EBM-2 media.
4. The PTS solution was aspirated from the culture flasks containing cancer cells, cells were washed in 5 changes of PBS and trypsinised to obtain a cell suspension.

5. TNF-α solution was aspirated from ibidi dishes and replaced with 2 ml of fresh EBM-2 media. $2x10^5$ cancer cells were added to each dish and the dishes were rocked on the rocking platform (figure 3.1c), for a defined time period. The time points tested were 5 min, 15 min, 30 min, 45 min, 1 h, 1.5h and 2 h.
6. At the end of rocking for each time point, the dishes were washed with 3 changes of warmed PBS to remove any non- adherent cancer cells. The dishes were placed in an incubator after the addition of fresh EBM-2 media. The dishes were then removed one by one from the incubator to be imaged using Zeiss confocal microscope using a 10x plan-Neofluar objective and 458 nm laser and 475 LP filter. Imaging of all the dishes was completed within 30 min from the end of adhesion assays.
7. To determine the optimum duration of rocking 4 ibidi dishes were set up for each time point tested, 2 dishes for MCF 7 cells and 2, for ZR 75 1 cells. For a schematic representation, please see figure 3.2. The experiments were repeated three times for each time point of rocking tested. This gave a total of 6 dishes per time point for MCF 7 cells and 6 for ZR 75 1.
8. From each dish, 15 random images were captured giving a total of 90 images for each time point tested per cell line. The total number of adherent cancer cells in each of the 90 images was counted manually.

## ***3.3. Results:***

The result of the rocking adhesion assays with HUVECs alone, for optimisation to carry out live cell imaging (described in section 3.2.1), is shown in Figure 3.3. The endothelial monolayer is intact with no apparent detachment of HUVECs indicating that ibidi culture dishes form appropriate devices for the culture of HUVEC cells for rocking adhesion assay and HUVEC cells cultured in these dishes can withstand the shear forces produced during rocking with no loss of integrity of the endothelial monolayer.

A sample photomicrograph of the HPA binding glycan staining with HPA TRITC is shown in figure 3.4. Abundant bright red fluorescent staining with HPA TRITC shows that MCF 7 cells synthesize high levels of HPA binding glycans.

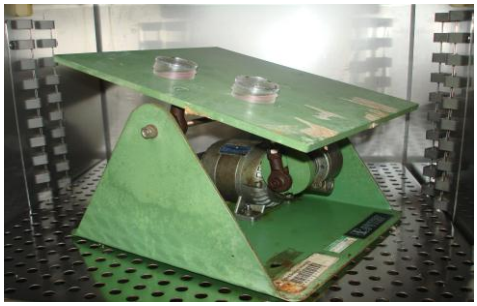
The results of rocking adhesion assay with MCF 7 cells over different time periods are shown in Table. 3.1 and represented graphically in Figure 3.7. Sample images depicting the adhesion of MCF 7 cells to endothelial monolayer over different time points is shown in figure 3.5.The total number of adherent cancer cells at the end of 5 min, and 15 min of rocking were 440 and 878 respectively. At the end of 30 min of rocking, there was a sharp increase in the number of adherent cancer cells with a total of 2524 cancer cells adhering to endothelial cell monolayer. At subsequent time points of 45 min, 1 h, 1 h30 min, there was a steady increase in the number of cancer cells with the time of rocking with a total number of adherent cancer cells being 2556, 2937 and 3689 at these time points respectively. There was no further significant increase in

the number of adherent cancer cells beyond 1 h30 min of rocking with the number of adherent cancer cells at the end of 2 h being 3706 in comparison to 3689 cancer cells at the end of 1 h30 min of rocking. In addition to this, continued rocking beyond 2 h resulted in the rocking platform getting overheated and resulted in the detachment of cancer cells.

The results of rocking adhesion assay with different periods of rocking for ZR 751 cells was similar to results obtained with MCF 7 cells. Sample images of adherent ZR 75 1 cancer cells are shown in figure 3.6. The results are shown in Table.3.2 and represented graphically in Figure 3.7. At the end of 5 min of rocking, there were 479 adherent cells gradually increasing to 838, 1921, 2335, 2392, 2827 and 2846 adherent cells at the end of 15 min, 30 min, 45 min, 1 h, 1 h30 min and 2 h respectively.

(a) Endothelial cells are grown in confocal microscope compatible dishes.

(b) Cancer cells grown in flasks are laid on the endothelial cells.



(c) The dishes are then rocked on a rocking platform to simulate blood flow.

Figure 3.1 Components of the rocking adhesion system: (a) Endothelial cells are cultured in confocal microscope compatible dishes. (b) Cancer cells are labelled with PTS and added to the endothelial cells. (c) The cells are co incubated on a rocking platform with constant rocking.


Figure 3.2.Schematic representation of experimental design of rocking adhesion assay: Each assay consisted of 2 ibidi dishes for the addition of MCF 7 cells and 2 for the addition of ZR 75 1 cells. The assay was repeated 3 times

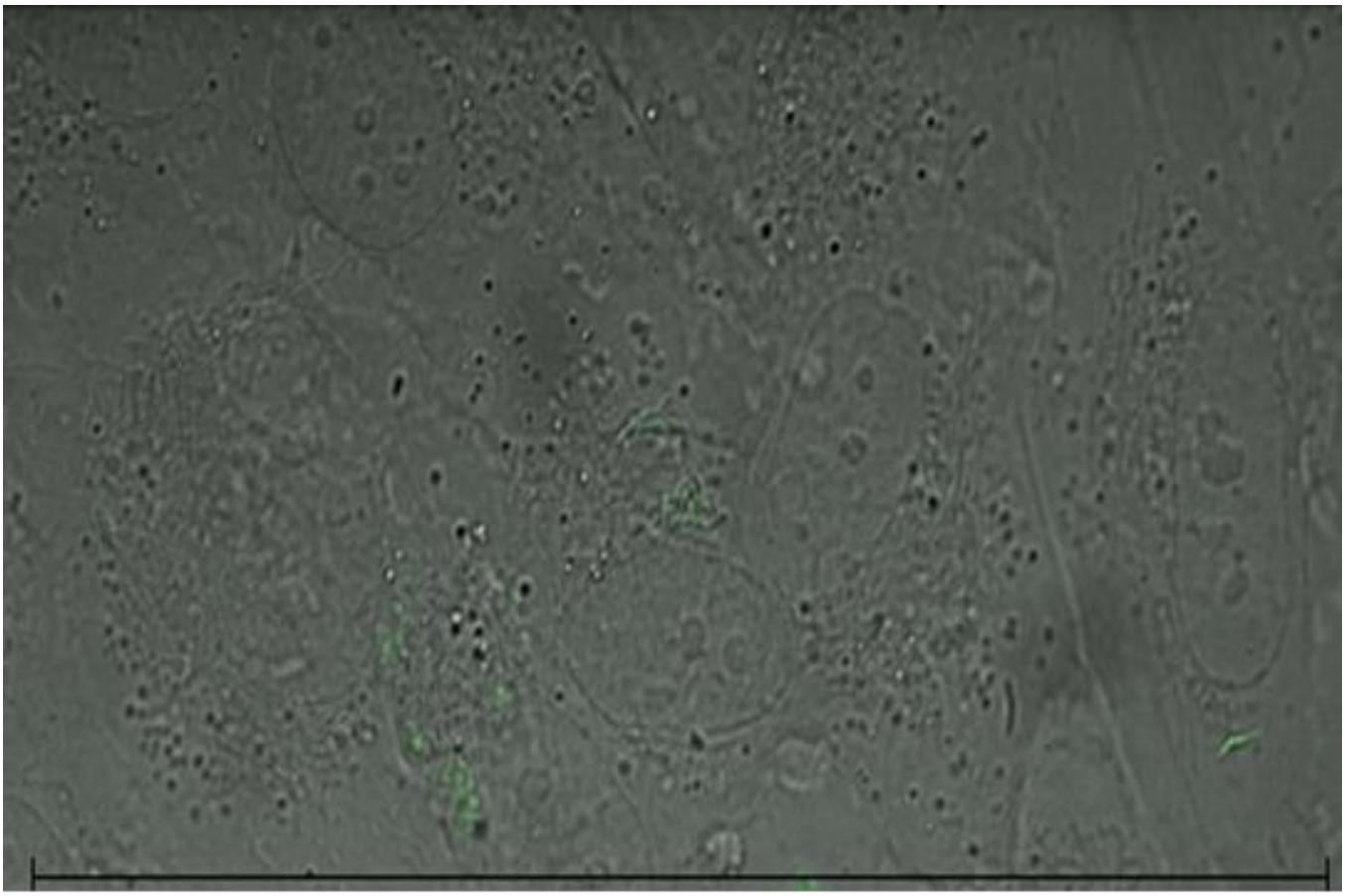



Figure 3.3. Photomicrograph showing monolayer of HUVECs after subjecting the monolayers to rocking assay. The monolayer is intact with no cell loss or detachment. Imaging carried out with Zeiss confocal microscope, using 458 nm laser, LP 475 filter and 25x objective.

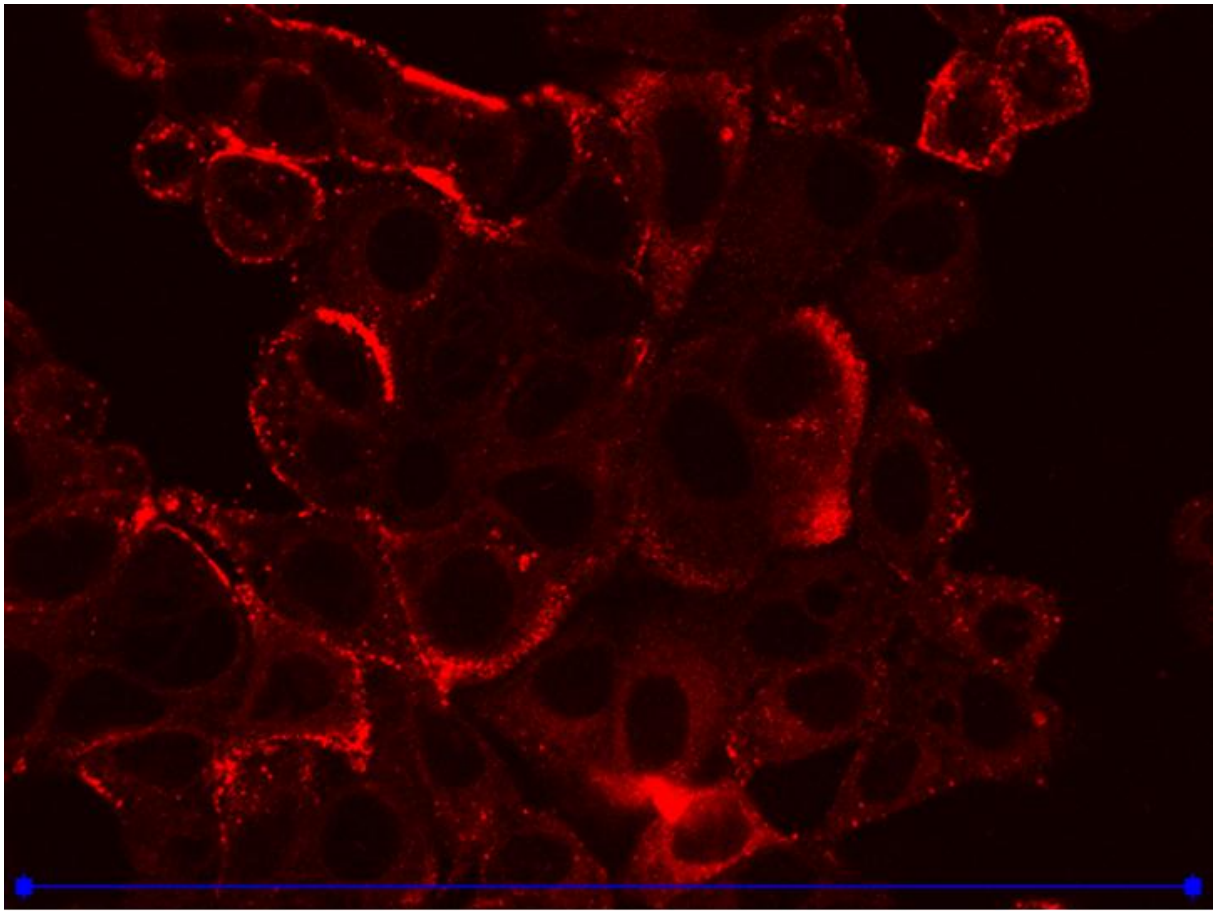



Figure 3.4. Photomicrograph of MCF 7 cells with mapping of surface HPA binding glycans using HPA TRITC: Abundant staining with HPA TRITC on the surface of MCF 7 cells demonstrates that MCF 7 cells synthesize high level of HPA binding glycans. Photomicrograph captured using Zeiss confocal microscope 543 nm laser, LP 560 filter and 63x objective.

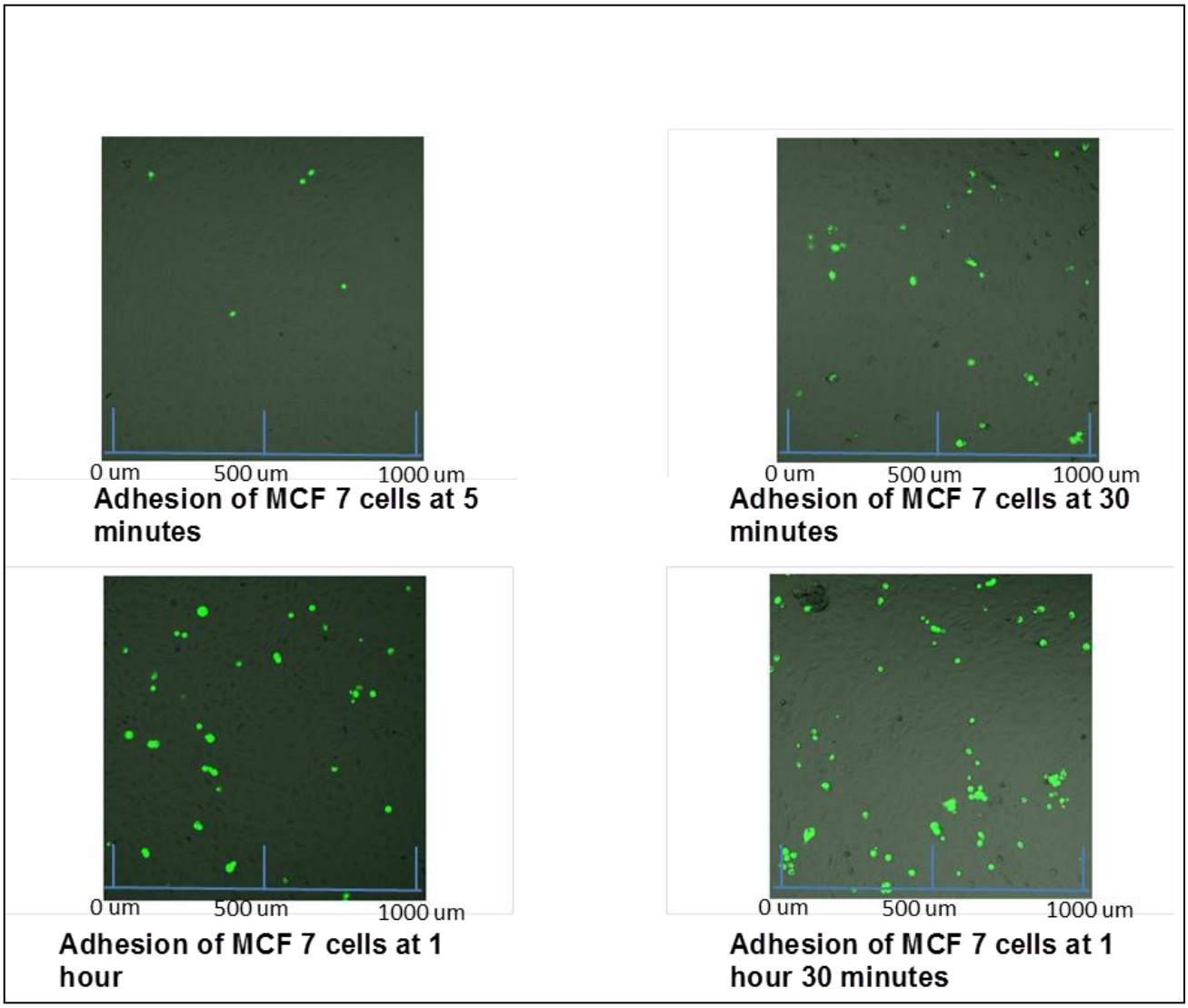



Figure 3.5: Results of the time series experiments for MCF 7: Showing minimal adhesion at 5 minutes and gradual increase in adhesion from 30 minutes to 1 hour 30 minutes. Imaging carried out with Zeiss confocal microscope, using 458 nm laser, LP 475 filter and 10x objective

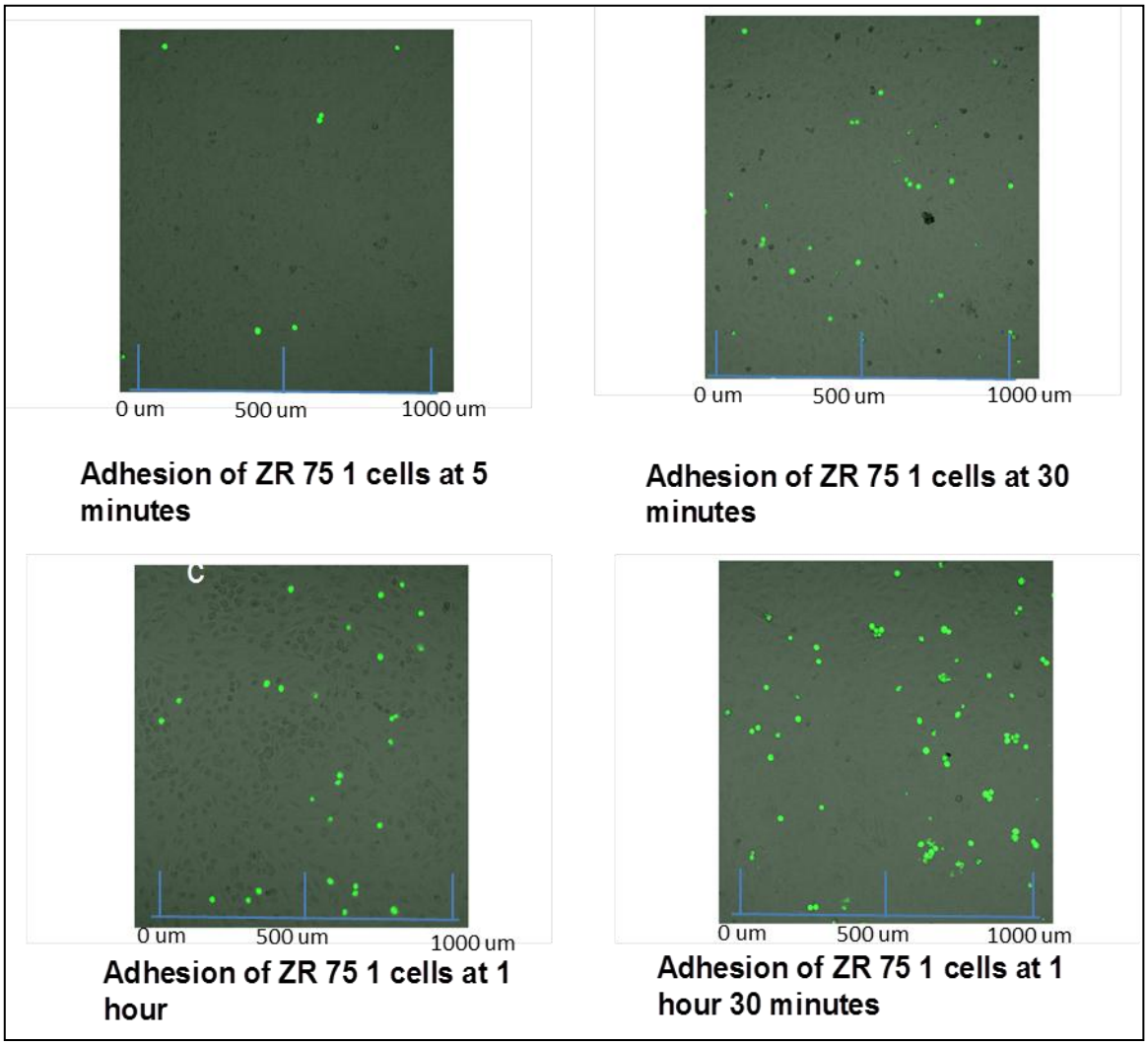



Figure 3.6: Results of the time series experiments for ZR 75 1: Showing minimal adhesion at 5 minutes and gradual increase in adhesion from 30 minutes to 1 hour 3 0 minutes. Imaging carried out with Zeiss confocal microscope, using 458 nm laser, LP 475 filter and 10x objective

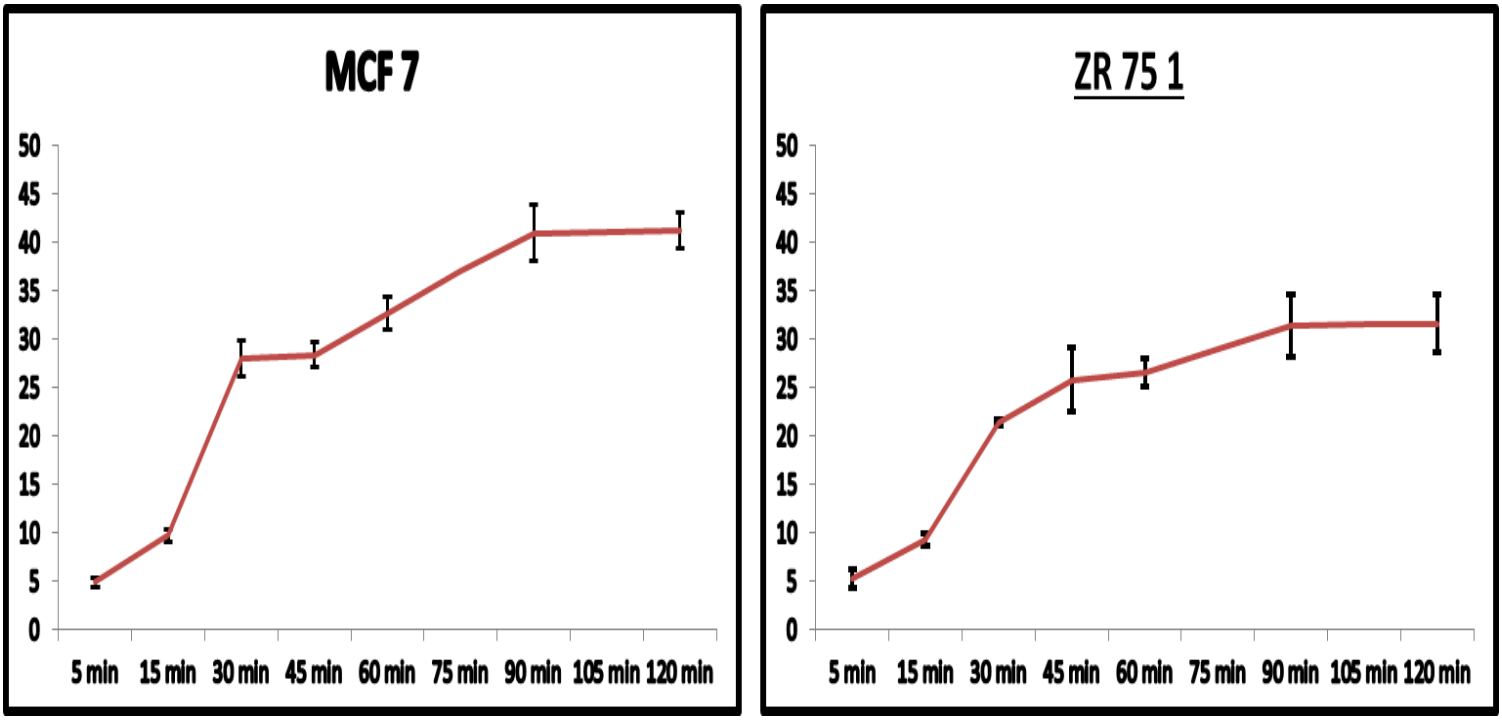



Figure 3.7. Graphical representation of the number of adherent MCF 7 and ZR 75 1 cells with various periods of rocking: The number of adherent cancer cells is minimal up to 15 minutes of rocking. From then on, there is a steady increase in the number of adherent cancer cells with rocking till the time period of 90 minutes. After this period, there is no further increase in the number of adherent cancer cells with duration of rocking.

**Table 3.1. Adhesion of MCF 7 cells to a monolayer of HUVECs over various time Points**

| Time points | 5 min | 15 min | 30 min | 45 min | 1 hour | 1.5 h | 2 h |
|---|---|---|---|---|---|---|---|
| Total | 440 | 878 | 2524 | 2556 | 2937 | 3689 | 3706 |
| Mean (n=90) | 5 | 10 | 28 | 28 | 33 | 41 | 41 |
| Standard deviation | 4.60 | 6.47 | 17.22 | 12.62 | 16.14 | 27.95 | 17.72 |
| SEM | 0.48 | 0.68 | 1.82 | 1.33 | 1.70 | 2.95 | 1.87 |

**Table 3.2. Adhesion of ZR 75 1cells to a monolayer of HUVECs over various time Points**

| Time points | 5 min | 15 min | 30 min | 45 min | 1 hour | 1.5 h | 2 h |
|---|---|---|---|---|---|---|---|
| Total | 479 | 838 | 1921 | 2325 | 2392 | 2827 | 2846 |
| Mean (n=90) | 5 | 9 | 21 | 26 | 27 | 31 | 32 |
| Standard deviation | 4.24 | 8.85 | 17.19 | 10.85 | 10.19 | 16.33 | 9.94 |
| SEM | 0.45 | 0.93 | 1.81 | 1.14 | 1.07 | 1.72 | 1.05 |

## ***3.4. Summary of main findings***

From the experiments described in this chapter, it can be concluded that

- Rocking adhesion assay system is a useful and reliable technique for the live cell imaging of adhesive interactions between cancer cells and endothelial cells.
- Adhesive interactions between the cancer cells begins at an early time point between 5 min -15 min of rocking and then steadily increases, plateauing after 1h 30 min. Beyond 1 h30 min of rocking, there is no increase in the number of adherent cancer cells. Moreover, continued rocking then results in the detachment of the endothelial cells from the culture surface. Hence 1h30 minute was used as the duration of rocking for all subsequent experiments.

# Chapter 4:

# Rocking Adhesion Assay

## *4.1. Introduction*

Metastasis, the process by which cancers spread from the primary organ of their origin to various target organs in the body is the main cause of cancer related deaths. Metastasis is a complex, multistep process brought about by the interplay of a variety of diverse and complex factors. Of particular relevance is the abnormal cellular glycosylation of cell surface glycoproteins in cancers. A wide array of aberrant glycosylation changes have been described as a prominent feature of several cancers (for a detailed review, please refer to (Brooks et al., 2008a) and to Chapter 1 sections 1.7. Many of these abnormal glycosylation changes are associated with increased aggressiveness and metastatic capability of cancers. One such aberrant glycosylation change consisting of exposure of terminal and sub-terminal GalNAc residues in breast cancer that is recognised specifically by the lectin HPA is the focus of this research project.

The fascinating background research pertaining to HPA binding glycans, their link to aggressiveness and metastatic capability in breast cancers and early studies to investigate the role of HPA binding glycans in metastatic cascade has been described in detail in chapter 1 (sections 1.8.1-1.8.4) and has led to and is being continued through the work described in this thesis. To summarise, exposure of HPA binding glycans in breast cancer specimens was shown to be associated with shorter disease free interval and poor prognosis in patients for the first time by Leathem and Brooks in 1987 (Leathem and Brooks, 1987). Further research by the same group also showed that the presence of HPA

binding glycans in primary breast cancer also correlated with the presence of lymph node metastases and was a significant, independent prognostic indicator in breast cancer (Brooks and Leathem, 1991, Brooks et al., 1993). These early findings were further substantiated by various other researchers both for breast cancer [for example (Fenlon et al., 1987, Fukutomi et al., 1989)] and for several other cancers [for example Schumacher and Adam for colon cancer (Schumacher and Adam, 1997). For a detailed review please see, Chapter 1 section 1.8. The reported association between exposure of HPA binding glycans and metastasis led to further research to evaluate the role of HPA binding glycans in the various steps of the metastatic cascade in breast cancers, the first attempt being a matrigel invasion assay to ascertain the role of HPA binding glycans in the adhesion to and invasion through basement membrane. This study failed to find evidence that HPA binding glycans played a role in the adhesion of breast cancer cells to basement membrane and their subsequent invasion through the basement membrane (Brooks and Hall, 2002). A further, very limited pilot study by Valentiner et al investigated the role of HPA binding glycans in the adhesion of breast cancer cells to endothelial cells during the adhesion and transendothelial migration step of the metastatic cascade. Results from this study suggested that HPA binding glycans could be involved in the adhesion of breast cancer cells to vascular endothelium during the adhesion and transendothelial migration step of the metastatic cascade (Valentiner et al., 2005). This chapter describes further research leading on from this pilot study and the study by Dr. Lomax- Browne (described in Chapter 1, section 1.8.5.)

The experiments described in this chapter utilise three breast cancer epithelial cell lines of progressively diminishing aggressiveness and HPA binding profiles; namely MCF 7, ZR 75 1 and BT474 in a rocking adhesion assay system (whose optimisation is described in chapter 3) to investigate whether HPA binding glycans are involved in the adhesion of breast cancer cells to endothelial cell monolayer. The evidence corroborating the 3 cell lines used here as representative of clinical breast cancer specimens and hence validating their use in the rocking adhesion assay system have been described in chapter 1, section 1.8.5. The rationale behind using the semistatic assay system instead of the flow assay systems and the static assay system is given in chapter 3.

For the rocking adhesion assays described in this chapter, the lectin HPA was utilised as the competitive inhibitor to block the HPA binding glycans on the surface of the breast cancer cell lines. The alternative agents for this purpose are the monosaccharide GalNAc or GalNAc linked to human serum albumin (GalNAc-HSA). In optimisation experiments of the rocking assay performed by Dr. Osborne, one of the previous PhD students in our group, GalNAc, when used as a competitive inhibitor was found to have an adverse effect on the endothelial cells and was found to cause their detachment from the tissue culture surface. GalNAc-HSA, even though it did not have any adverse effect on the endothelial monolayer was found to be too expensive. Hence, HPA was chosen as the competitive inhibitor of choice as it caused no adverse effect on the endothelial mono layer and was a cost effective option (Osborne, 2004).

### *4.1.1. Rocking adhesion assay with peanut agglutinin (PNA) and concanavalin A (Con A) pre-incubation.*

When performing rocking adhesion assays utilizing HPA as a competitive inhibitor, it was vital to ensure that the any inhibition in adhesion of breast cancer cells to endothelial cells, if it were to be observed, was due to the HPA binding glycans actually being functionally involved in cancer cell-endothelial cell adhesion and not an inhibitory effect due to a steric effect of the bulky HPA molecule physically interfering with other cell-cell interactions. As a control set of experiments, and in order to rule this out, rocking adhesion assays were also repeated with two other lectins, peanut agglutinin (PNA) and Concanavalin A (Con A) which, like HPA, are reported to preferentially bind abnormal glycans in breast cancer, but to glycans distinct from the GalNAc-glycans recognized by HPA and of interest in this thesis. Peanut agglutinin is a tetrameric protein extracted from peanut (*Arachis hypogaea*) and exhibits preferential binding specificity to galactosyl(β1-3)N-acetyl galactosamine (Gal(β1-3)GalNAc), D-galactose and T antigen, Gal(β1→3) GalNAc-Ser/Thr (Lotan et al., 1975, Banerjee et al., 1994).

PNA has been shown to bind normal breast epithelial cells and epithelial cells in breast cancers, particularly in well differentiated adenocarcinomas (Newman et al., 1979). PNA is also believed to be a marker of tumour progression and differentiation with a sequential loss of PNA binding being demonstrated during progression from normal mammary cells to pre-neoplastic cells to mammary carcinomas. In addition to this, an inverse relationship has also been

found between tumorigenicity and PNA binding (Rak et al., 1992). In a study comparing PNA binding profiles of primary adenocarcinomas of breast, stomach and colon with their respective lymph node metastatic deposits, PNA binding was present in less than 5% of the primary tumours. Interestingly however, more than 75% of the cells in the metastatic deposits derived from these primary tumours showed positive binding to PNA. It was therefore deduced that a PNA binding, abnormal glycosylation change has facilitated the progression from primary tumour to metastasis and hence, PNA binding was proposed as a marker of metastatic potential in adenocarcinomas including breast carcinomas (Kahn and Baumal, 1985). Of particular relevance to this thesis, in studies involving breast cancer epithelial cell lines, PNA has been shown to bind the highly metastatic MCF 7 cell lines and in a way that is proportional to the binding with HPA (Schumacher et al., 1995) and hence PNA has been used as one of the test lectins in the experiments described below.

Concanavalin A (Con A) is a lectin derived from the jack bean (*Canavalia ensiformis*) and specifically binds the α- mannose and glucose residues and the trimannose core of complex glycoproteins (Bhattacharyya and Brewer, 1988, Naismith and Field, 1996). Haemadsorption studies utilising Con A coated erythrocytes with breast cancer cells have shown that Con A demonstrates strong reactivity to malignant breast epithelial cells. In contrast, no reactivity is observed in normal breast epithelial cells. This reactivity to malignant epithelial cells is seen both in cells derived from solid breast tumours as well as

malignant breast cancer cell lines including MCF 7 (Voyles et al., 1978). Furthermore, in a prospective, double blinded study on breast cancer specimens of patients operated for breast cancer, Con A reactivity was associated with early recurrence of tumours. In addition to this, Con A, like HPA was found to be an independent prognostic factor in breast cancer (Furmanski et al., 1981). This finding along with the fact that Con A binds to malignant breast cancer cell lines like MCF 7, one of the principal cell lines used in all the experiments described in this thesis (Voyles et al., 1978, Schumacher et al., 1995) prompted the use of Con A in the experiments described below.

## *4.2. Materials and Methods*

### *4.2.1. Rocking adhesion assay with HPA inhibition*

The rocking adhesion assay optimised in the experiments described in chapter 3 was employed in these investigations.

1. Confluent monolayers of HUVEC cells were cultured on ibidi dishes, as described in section 3.2.1 steps 1-3.
2. Cancer cells in were pre incubated with 10mg/ml solution of HPTS in tissue culture media overnight as described in chapter 3 section 3.2.2
3. On the day of assay, the cancer cells were washed with 5 changes of fresh, warmed PBS.
4. The cells were then trypsinised and resuspended in the appropriate culture media to obtain a cell suspension as described in chapter 2 section 2.2.3

5. The cell suspension was then split into test and control groups. The cells in the test group were pre incubating with 10µg/ml solution of HPA (Sigma Aldrich, UK) in tissue culture medium in a $37^0$c, 5% $CO_2$ incubator for 2 hours to achieve binding of HPA to the cells. The control cell suspension was similarly incubated tissue culture medium without the addition of HPA.
6. At the same time, HUVEC cell monolayers were prepared for the assay by removing the cell culture media from the dishes and pre-incubating the cells with 10ng/ml solution of TNF-α in EBM-2 media 2 hours, as described in chapter 3 section 3.2.1
7. At the end of two hours, the test and control cancer cell suspensions were removed from the incubator, centrifuged at 1200rpm for 10 minutes and resuspended in fresh tissue culture medium. The process was repeated thrice to remove any non-adherent HPA from the solution.
8. TNF-α solution from each ibidi dishes was aspirated and replaced with 2mls of fresh EBM-2 medium.
9. $2x10^5$ cancer cells from the test group and control group were added to the respective ibidi dishes and the dishes rocked on a rocking platform placed in a $37^0$c, 5% $CO_2$ incubator and rocked for 2 hours.
10. At the end of 2 hours, rocking was stopped and the cells were washed with 3 changes of warmed PBS to remove any non-adherent cancer cells. 2mls of fresh warmed EBM-2 medium was then added to each

dish and the dishes were imaged with confocal microscope using a 10x plan-Neofluar objective and 458 nm laser.

11. Each experimental group consisted of two test dishes (to which cancer cell pre-incubated with HPA were added) and two control dishes (to which cancer cells without HPA pre-incubation were added). 15 images from random fields of view were captured from each dish and the experiments were repeated 3 times for each cancer cell line tested. This gave a total number of 90 images each for the test and control groups per cancer cell line tested.
12. The number of adherent cancer cells in each image was counted manually and total number of adherent cancer cells from all the 90 images for both the test and the control groups was calculated.
13. The results were then statistically analysed using Mann Whitney test.

### *4.2.2. Rocking adhesion assays with PNA and Con A pre-incubation*

Rocking adhesion assays with PNA and Con A as competitive inhibitors was carried out as described in the section 4.2.1 described above but with the following modifications:

1. Each cancer cell line was split into four experimental groups: control group with no lectin pre-incubation, and HPA, PNA and Con A groups - pre-incubated with 10µg/ml solutions of HPA, PNA and Con A (all from Sigma, UK) respectively.

2. Since no significant increase in the number of adherent cancer cells was found beyond 1hour 30 minutes of rocking (as described in Chapter 3), assays were carried out for a duration of 1 hour and 30 minutes.
3. Each experiment consisted of 4 dishes, one each for control (no lectin), HPA, PNA and Con A groups. The experiments were repeated thrice and 20 images were captured for each dish giving a total of 60 images per group for each cell line tested. The number of adherent cancer cells in each image was manually counted to obtain the total number of adherent cancer cells from all the 60 images. For example, the control group for MCF 7 consisted of 60 images and the total number of adherent cancer cells for the control group was a sum of all the adherent cancer cells in the 60 images.

## ***4.3. Results:***

### *4.3.1. Rocking adhesion assay with HPA pre-incubation*

Sample images of the cancer cells adherent to the endothelial cell monolayer post rocking adhesion assay in the control group (without HPA inhibition) and in the test group (with HPA inhibition) for MCF 7, ZR 75 1 and BT 474 cell lines is shown in  shown in figure 4.1.

For rocking adhesion assays with MCF 7 cells, in the control group (without HPA pre incubation), the total number of adherent cancer cells was 3706 (mean-41) .There was a significant reduction in the number of adherent cancer cells in the test group (with HPA), that was easily noticeable by eye, as shown in Figures 4.1 a (control group) and b (test group), with total

number of adherent cancer cells being 1868 (mean-21). This difference was statistically significant ($p<0.00001$). The results are summarised in the Table 1 and represented graphically in Figure 4.2.

Similar results were also obtained for rocking adhesion assays with ZR 75 1 cells. The total number of adherent cancer cells for ZR 75 1 was 2846 for the control group (without HPA) (mean-32) and 1800 for the test group (with HPA) (mean-20). The difference in the adhesion was statistically significant ($p<0.0001$). The sample images for the control and test groups for rocking adhesion assays with ZR 75 1 cells is shown in Figure 4.1 c and d, summarized in Table 2 and represented graphically in Figure 4.3.

The total number of adherent cancer cells for adhesion assays with BT474 cells in the control group was 2058 (mean 23). The total number of adherent cancer cells in the test group for BT 474 cells was 1971 (mean 22). The difference in the number of adherent cancer cells was not statistically significant ($p>0.2587$). The sample images for rocking adhesion assays with BT 474 cells is shown in the Figure 4.1 e and f for the control and test groups respectively. The results of the assay are summarized in Table 3 and represented graphically in Figure 4.3

### *4.3.2. Results of rocking adhesion assay with HPA, PNA and Con A pre incubation*

The results for the rocking adhesion assay with MCF 7 cells is shown in Table 4 and represented graphically in the 4.4. The total number of adherent cancer cells in the control group was 3019 (mean 50). The number

of adherent cancer cells with HPA, PNA and Con A inhibitions were 1610 (mean 27), 2858 (mean 48) and 2893 (mean 48) cells respectively. The inhibition in adhesion with HPA pre incubation was statistically significant ($p < 0.0001$). The results are consistent with the results of the rocking adhesion assay described in 4.3.1. There was no significant inhibition in adhesion with the other test lectins with p value for PNA group being 0.1681 and for Con A being 0.3805.

The rocking adhesion assay results with ZR 75 1 cells is shown in the Table 5 and represented graphically in the Figure 4.5. The total number of adherent cancer cells in the control group was 1380 (mean 23). The number of adherent cancer cells with HPA, PNA and Con A pre incubation were 763 (mean 13), 1348 (mean 22) and 1418 (mean 24) respectively. The total number of adherent ZR 75 1cancer cells in the control group (without lectin) was fewer than in the analogous control group (without lectin) for MCF 7 cells. There was significant inhibition in the adhesion with HPA pre incubation of both MCF7 and ZR 75 1 cancer cells. Both these findings were consistent with the results of the rocking adhesion assay results described in section 4.3.1. There was no statistically significant inhibition of adhesion in the presence of PNA ($p = 0.1110$) The number of adherent cancer cells with Con A pre incubation was greater than in the control (no lectin) group but this difference did not reach statistical significance ($p = 0.6936$).

The results of rocking assay with BT 474 are shown in Table 6 and represented graphically in the Figure 4.6. There were 1343 cancer cells in the control group (mean 22). There were 1281 (mean 21), 1325 (mean 22) and 1323 (mean 22) cells adherent with HPA, PNA and Con A pre incubation respectively. None of the results were statistically significant with p value for HPA group being 0.7447, for PNA group being 0.9101 and for Con A group being 0.7328.

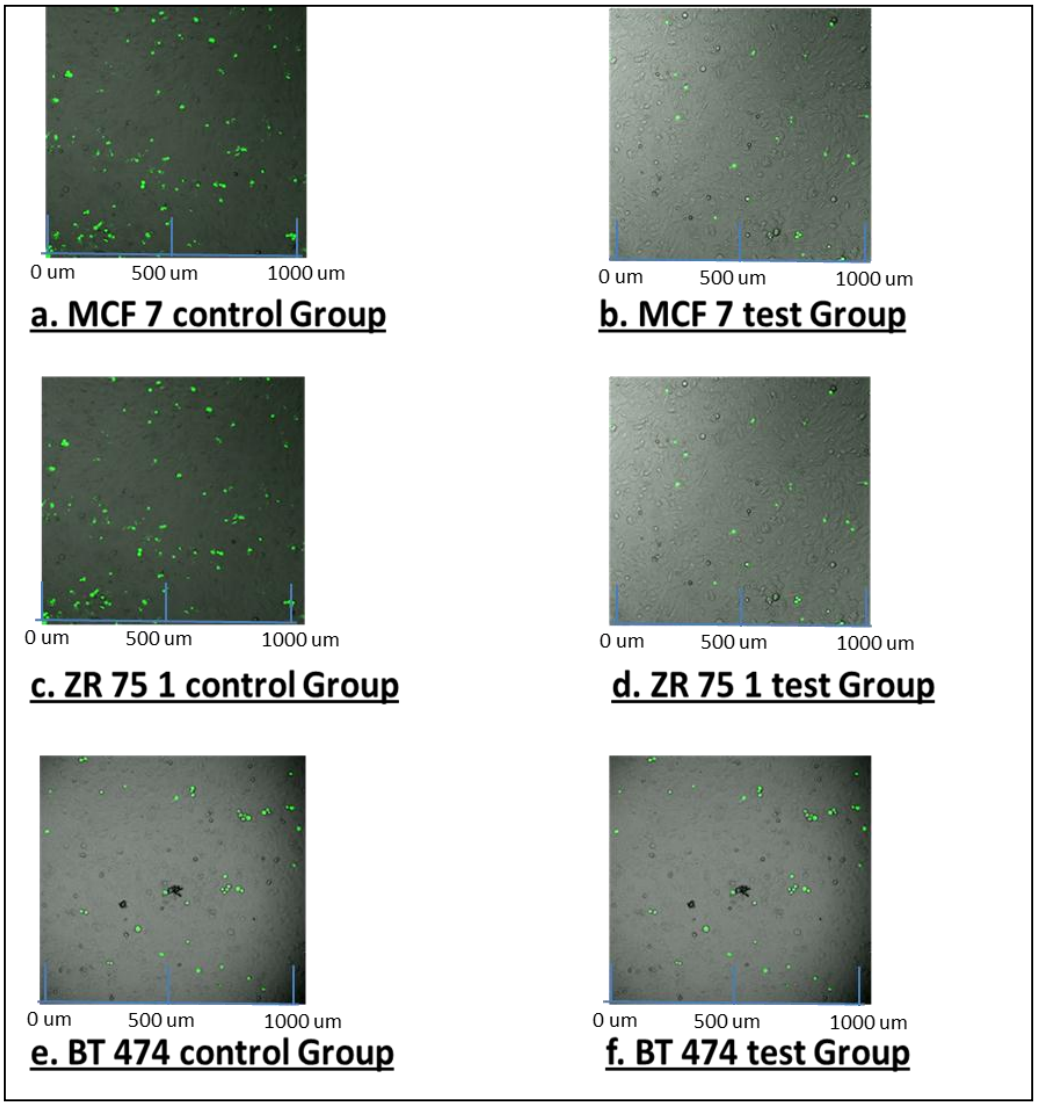



Figure 4.1.Sample images from rocking adhesion assays with and without blocking by HPA in MCF 7, ZR 75 1 and BT 474 cell lines: The cancer cells appear green and endothelial cells appear as grey background. Figures a and b show adhesion in control and test groups respectively for MCF 7ells, figures c and d show adhesion in control and test groups respectively for ZR 75 1 cells and Figures e and f show results for control and test groups for BT 474 cells respectively. Imaging carried out with Zeiss confocal microscope, using 458 nm laser, LP 475 filter and 10x objective

**Table 1.Adhesion of MCF 7 cells to HUVECs monolayer in the presence and absence of HPA.**

| | Control group | Test group |
|---|---|---|
| **Total number of adherent cancer cells** | 3706 | 1868 |
| **Mean** | 41 | 21 |
| **Standard deviation** | 17.72 | 7.41 |
| **Standard error of mean** | 1.87 | 0.78 |
| **Two-tailed P value** | **< 0.0001** | |
| **Significance** | **Significant** | |

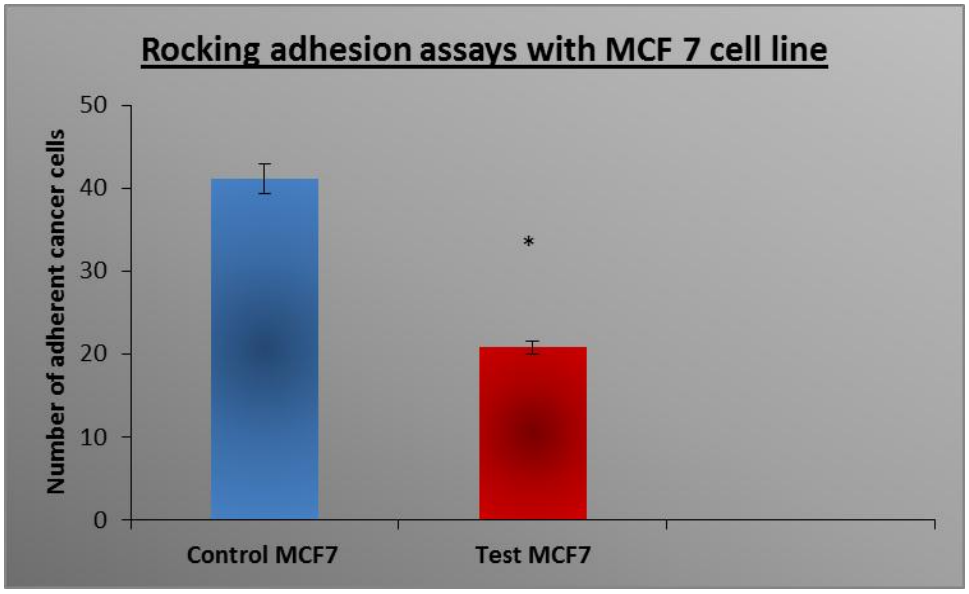



Figure.4.2. Adhesion of MCF 7 cells to HUVECs monolayer in the control and test groups. The control group without HPA inhibition is shown in blue and the test group with HPA inhibition is shown in red. Significant inhibition of adhesion is seen in the test group compared to the control group.

## Table 2.Adhesion of ZR 75 1 cells to HUVECs monolayer in the presence and absence of HPA inhibition

| | Control group | Test group |
|---|---|---|
| **Total number of adherent cancer cells** | 2846 | 1800 |
| **Mean** | 32 | 20 |
| **Standard deviation** | 9.94 | 6.12 |
| **Standard error of mean** | 1.05 | 0.64 |
| **Two-tailed P value** | **< 0.0001** | |
| **Significance** | **Significant** | |

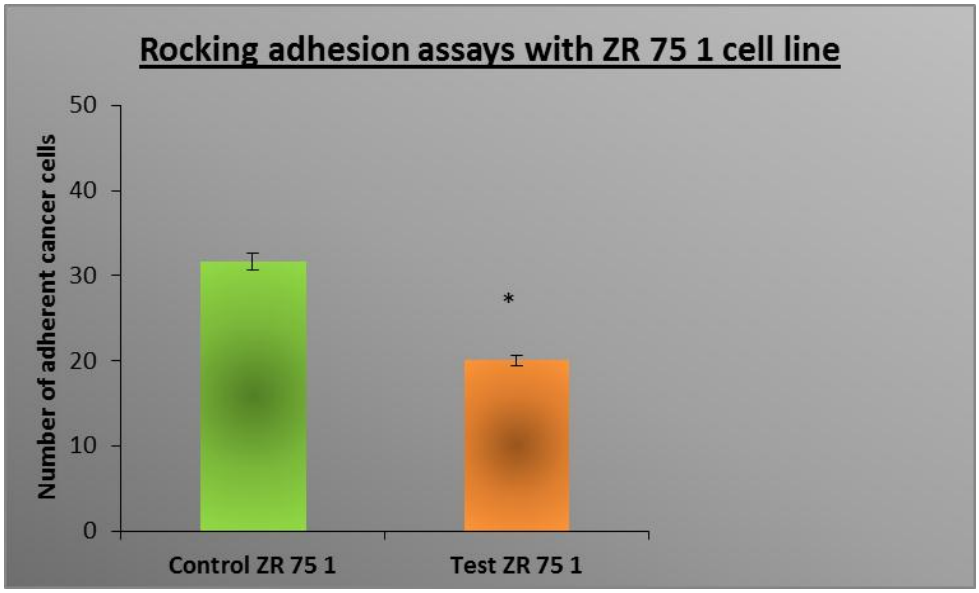



Figure.4.3. Adhesion of ZR 75 1 cells to HUVECs monolayer in the control and test groups. The control group without HPA pre incubation is shown in green and the test group with HPA inhibition is shown in yellow. Significant inhibition of adhesion is seen in the test group compared to the control group

## Table 3.Adhesion of BT 474 cells to HUVECs monolayer in the presence and absence of HPA.

| | Control group | Test group |
|---|---|---|
| **Total number of adherent cancer cells** | 2058 | 1971 |
| **Mean** | 23 | 22 |
| **Standard deviation** | 10.05 | 10.71 |
| **Standard error of mean** | 1.06 | 1.13 |
| **p value** | **> 0.2587** | |
| **Significance** | **Not significant** | |

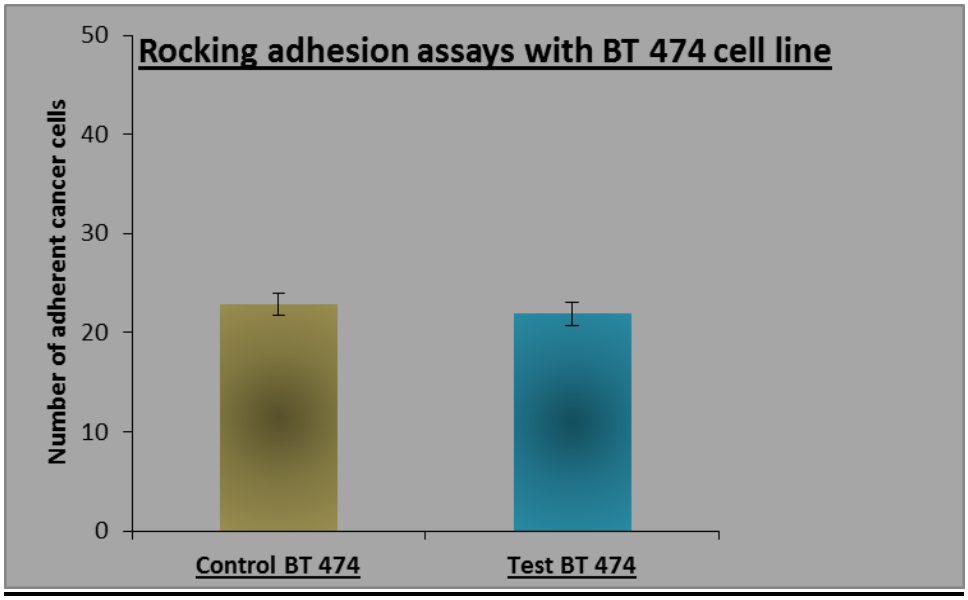



Figure.4.3. Adhesion of BT 474 cells to HUVECs monolayer in the control and test groups. The control group without HPA pre incubation is shown in green and the test group with HPA pre incubation is shown in blue. Minimal inhibition of adhesion is seen in the test group compared to the control group and this is not statistically significant

## Table 4.Adhesion of MCF 7 cells to HUVECs monolayer with HPA, PNA and Con A inhibition

| | Control | HPA | PNA | Con A |
|---|---|---|---|---|
| **Total number of adherent cancer cells** | 3019 | 1610 | 2858 | 2893 |
| **Mean** | 50 | 27 | 48 | 48 |
| **Standard deviation** | 15.72 | 9.01 | 20.04 | 20.81 |
| **Standard error of mean** | 2.03 | 1.16 | 2.59 | 2.69 |
| **p value** | N/A | **< 0.0001** | **= 0.1681** | **= 0.3805** |
| **Significance** | N/A | Significant | Not significant | |

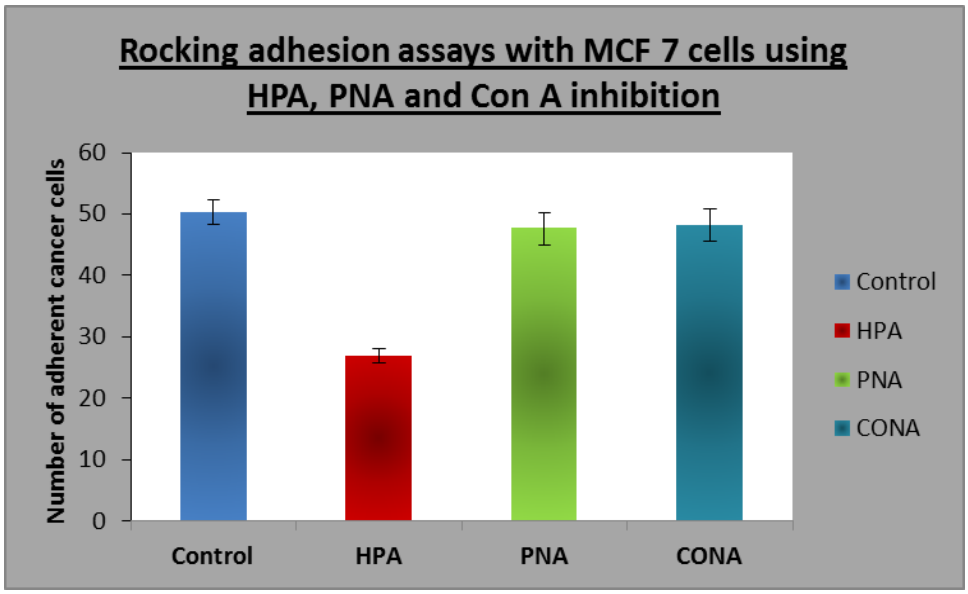



Figure.4.4. Adhesion of MCF 7 cells to HUVECs monolayer with HPA, PNA and Con A inhibition. The control group without HPA pre incubation is shown in sky blue. HPA group is shown in red, PNA group in green and Con A group is shown in light blue. There is a statistically significant inhibition of adhesion with HPA pre incubation ($p<0.001$) but no inhibition of adhesion is seen with the other two lectins.

## Table 5.Adhesion of ZR 75 1 cells to HUVECs monolayer with HPA, PNA and Con A inhibition

| | Control | HPA | PNA | Con A |
|---|---|---|---|---|
| **Total number of adherent cancer cells** | 1380 | 763 | 1348 | 1418 |
| **Mean** | 23 | 13 | 22 | 24 |
| **Standard deviation** | 8.04 | 7.44 | 15.56 | 12.12 |
| **Standard error of mean** | 1.04 | 0.96 | 2.01 | 1.56 |
| **p value** | N/A | **< 0.0001** | **= 0.1110** | **= 0.6936** |
| **Significance** | N/A | Significant | Not significant | |

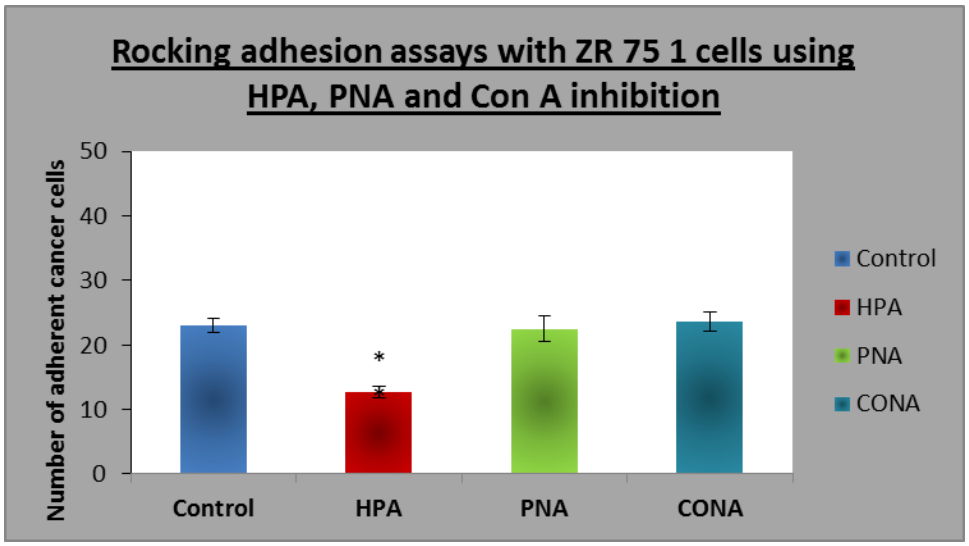



Figure.4.5. Adhesion of ZR 75 1 cells to HUVECs monolayer with HPA, PNA and Con A inhibition. The control group without HPA pre incubation is shown in sky blue. HPA group is shown in red, PNA group in green and Con A group is shown in light blue. There is a statistically significant inhibition of adhesion with HPA pre incubation ($p<0.001$) but no inhibition of adhesion is seen with the other two lectins.

## Table 6.Adhesion of BT 474 cells to HUVECs monolayer with HPA, PNA and Con A pre incubation

| | Control | HPA | PNA | Con A |
|---|---|---|---|---|
| **Total number of adherent cancer cells** | 1343 | 1281 | 1325 | 1323 |
| **Mean** | 22 | 21 | 22 | 22 |
| **Standard deviation** | 11.28 | 9.23 | 10.12 | 12.14 |
| **Standard error of mean** | 1.46 | 1.19 | 1.31 | 1.57 |
| **p value** | N/A | = **0.7447** | **= 0.9101** | = **0.7328** |
| **Significance** | N/A | Not significant | | |

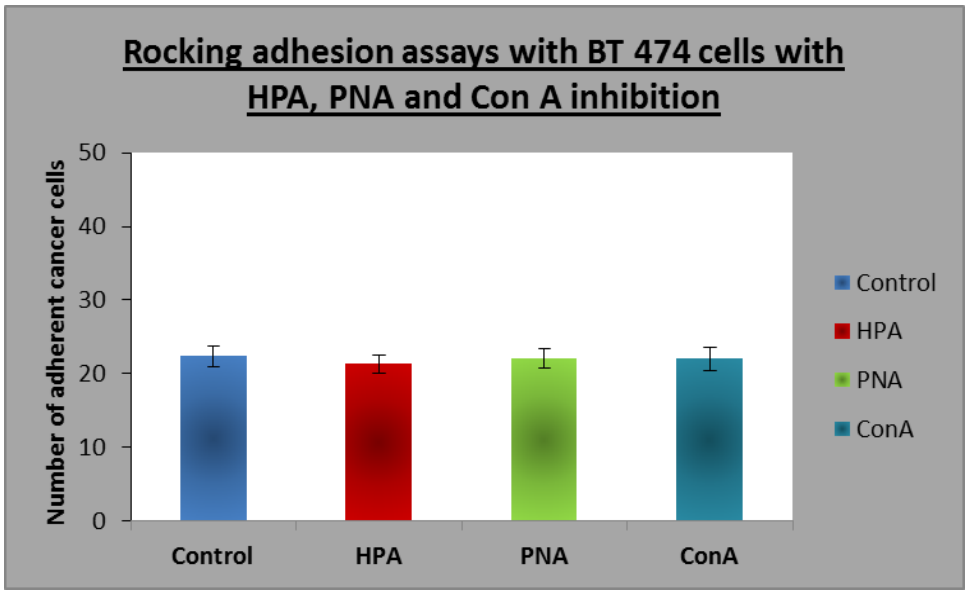



Figure.4.6. Adhesion of BT 474 cells to HUVECs monolayer with HPA, PNA and Con A pre incubation. The control group without HPA pre incubation is shown in sky blue. HPA group is shown in red, PNA group in green and Con A group is shown in light blue. There no inhibition in adhesion with any of the 3 lectins.

## ***4.4. Summary of main findings***

- The results from the experiments described in this chapter are consistent with HPA binding glycans playing a functional role in the adhesion of cancer cells to endothelium. The number of cells adherent to the endothelial monolayers was consistent with both the derivation of the cells and their HPA-binding characteristics. Thus, those derived from metastatic sources, MCF7 and ZR 75, exhibited greater adhesiveness to endothelial cells than those derived from non-metastatic primary tumor, BT 474. The MCF 7 cell line, labels most strongly with HPA, also exhibits the maximum number of adherent cancer cells. This was followed by ZR 75 17 which exhibit slightly less HPA binding, and finally by BT474 which is very weakly HPA binding.
- There was a significant decrease in the adhesion of cancer cells to the endothelial cell monolayer when HPA binding glycans were blocked by pre-incubation of the cells with the lectin in the MCF 7 and ZR 75 1 cell lines. No statistically significant inhibition in adhesion was found in BT 474 cells. Thus, physically blocking exposure of HPA binding glycans has an inhibitory effect on the binding of the metastatic breast cancer cells to the endothelial cells, consistent with the HPA-binding glycans being functionally implicated in this cell-cell adhesive interaction during adhesion and transendothelial step of metastatic cascade.

- Significant inhibition of adhesion was demonstrated with HPA pre incubation with HPA in the two metastatic cancer cell lines tested. However, no inhibition in adhesion was demonstrated with BT 474 cell line. There was also no inhibition demonstrated in all the three cell lines with PNA and ConA inhibition. These results are therefore consistent with HPA binding glycans playing a functional and specific role in the adhesion of breast cancer cells to endothelial cells during adhesion and transendothelial migration.

# Chapter 5:

# Post adhesive behavior of cancer cells

## *5.1. Introduction*

Adhesion of metastatic cancer cells in the circulation to the vascular endothelium and their subsequent transmigration across the endothelial layer has been shown to be a crucial and limiting step in the metastatic cascade (Tarin et al., 1984). Mechanisms utilised by metastasising cancer cells during adhesion and transendothelial migration are thought to be similar to leucocyte adhesion and transendothelial migration during an inflammatory response as described in section 1.3 . Unlike adhesion and transendothelial migration in leucocytes, which has been extensively researched at the molecular level and is well reviewed [e.g. (Barreiro and Sanchez-Madrid, 2009, Carlos and Harlan, 1994)] the analogous process in cancer cells is still poorly understood.

The main steps of leucocyte adhesion and transendothelial migration during inflammation can be briefly summarised as below (for an in depth description of the same, please refer to sections 1.4.) The first step involves the recruitment of leucocytes from the blood stream. Leucocytes initially tether and then roll along the activated vascular endothelium. The process of tethering and rolling is mediated through selectin-carbohydrate mediated interactions (Crockett-Torabi, 1998, Symon and Wardlaw, 1996). A comprehensive description of structure and function of selectins, selectin ligands and their function is given in section 1.3.11 and section 1.4.1.

Selectin mediated rolling results in activation of integrins and causes firm adhesion of the leucocytes to the endothelial surface (Gahmberg et al., 1997). Integrins, their ligands and their combined role in firm adhesion of leucocytes is

described in Chapter 1 sections 1.3.12-13 and 1.4.2-1.4.3. Adherent leucocytes then crawl along the endothelial layer until they reach the intercellular junctions. The endothelial cells then retract and leucocytes use diapedesis to migrate out of the blood vessel. This mode of transmigration in between the retracted endothelial cells is referred to as para cellular migration. In addition to para cellular migration, some leucocytes pass directly through the endothelial cells and this process is called as trans cellular migration. Paracellular and transcellular migration have been shown to be mediated by several molecules, including ICAMs, VCAMs, PECAMs and JAMS (Nourshargh et al., 2006, Rahman and Fazal, 2009, Wittchen, 2009).

There are several similarities between leucocyte adhesion and transendothelial migration and those of cancer cells. There are also some important differences. The similarities and differences between the two processes has been well reviewed by Strell and Entschladen (Strell and Entschladen, 2008) and can be summarised as follows.

Like leucocytes, selectins appear to be the main mediators of rolling in cancer cells (Hanley et al., 2006). The role of selectins, however, is not limited to rolling alone and selectins have been shown to mediate cancer cell diapedesis across endothelial layer (Tremblay et al., 2008). In addition to selectins, other mediators have been implicated in mediating cancer cell rolling. For example, N-cadherins were found to mediated MDA-MB-468 cells on pulmonary vascular endothelium (Strell et al., 2007).

Like leucocytes, firm adhesion in cancer cells has been shown to be mediated by integrins [for e.g.(Garofalo et al., 1995)]. Other mediators have also been implicated in firm adhesion of cancer cells to endothelial layer. Galectin and Thomsen-Freidenreich antigen (TF or T antigen) mediated interactions have been shown to result in adhesion of breast and prostate cancer cell adhesion to endothelium (Glinsky et al., 2001). Like selectins, the role of integrins in cancer cell adhesion is not limited to firm adhesion alone. αvβ3 integrins have been implicated in mediating transmigration of melanoma cells across endothelium (Voura et al., 2001).

Unlike transendothelial migration of leucocytes, which doesn't permanently damage vascular endothelial layer integrity (Huang et al., 1988), cancer cell transmigration has been shown to result in irreversible damage to the integrity of the endothelial monolayer (Heyder et al., 2002).

Hence, the main aim of the experiments carried out in this chapter was to study the post adhesive behaviour of the cancer cells, utilising the approaches of live cell imaging and high resolution imaging with scanning electron microscopy.

For live cell imaging of post adhesive behaviour of cancer cells, an innovative smartslide system called the Wafergen smartslide system (Wafergen Biosystems, UK) was incorporated in to the rocking adhesion assay system. The Wafergen smartslide system consists of a 6 well, confocal microscope compatible well plate with a lid. The base and the lid possess independent temperature controls and the base, in addition, also possesses microinjection ports on the side for gas flow. The smartslide system can be directly mounted

onto the confocal microscope and when connected to its base unit for temperature control and a gas supply, functions as a micro incubator system and allows long term imaging of live cells. The system in illustrated in figure 5.1

## ***5.2. Materials and methods***

### *5.2.1. 24 hour live cell imaging with Wafergen smartslide system*

1. Monolayer cultures of HUVEC cells were established in one well of a 6 well smartslide (for details of endothelial cell culture please refer to Chapter 2).
2. MCF 7 cells labelled with HPTS were placed on the endothelial cell monolayer and rocking adhesion assay was carried out as described in Chapter, section 3.2.2.
3. Post rocking, the endothelial cells were washed with 3 changes of PBS and 5mls of fresh warmed EBM 2 media was added to the cells. The smartslide was placed on its compatible holder on the confocal microscope. The slide was then connected to the base unit of the smartslide system to maintain the temperature of the base plate at $37^0$c. The temperature of the lid was set at $38^0$c to prevent condensation during prolonged imaging. Through the micro injection port at the side, 5% $O_2$ air mixture was continuously passed in to the micro incubating system.

4. Optimum field of view were selected with sufficient number of adherent cancer cells without overcrowding and imaging was carried (458 laser, 10x plan-Neofluar objective).
5. The microscope was set to capture 1 image every 5 minutes and the imaging was carried out for a period of 24 hours.
6. After the imaging, the images were collated in to a movie by utilising LSM image browser and Microsoft movie making software.

### *5.2.2. Scanning electron Microscopy (SEM)*

1. The endothelial cells were cultured on plastic coverslips placed at the bottom of 24 well plates.
2. The rocking adhesion assay was carried out using MCF 7 cells without HPTS pre labeling as described in section 3.2.2
3. Post adhesion, the 24 well plates were incubated in a $37^0$c, 5% $CO_2$ incubator.
4. The plates were removed from the incubator at the end of 5 1 hour, 2 hours, 6 hours, 12 hours and 24 hours post adhesion and processed for imaging for SEM as described below
5. The cells were fixed with 2% glutaraldehyde (TAAB, U.K) + 2% paraformaldehyde (Agar Scientific) in 0.1M sodium cacodylate (Agar Scientific) at pH 7.4 for 1 hour
6. The cells were then washed three times and soaked with 1% osmium tetroxide (Agar Scientific) for 1 hour.

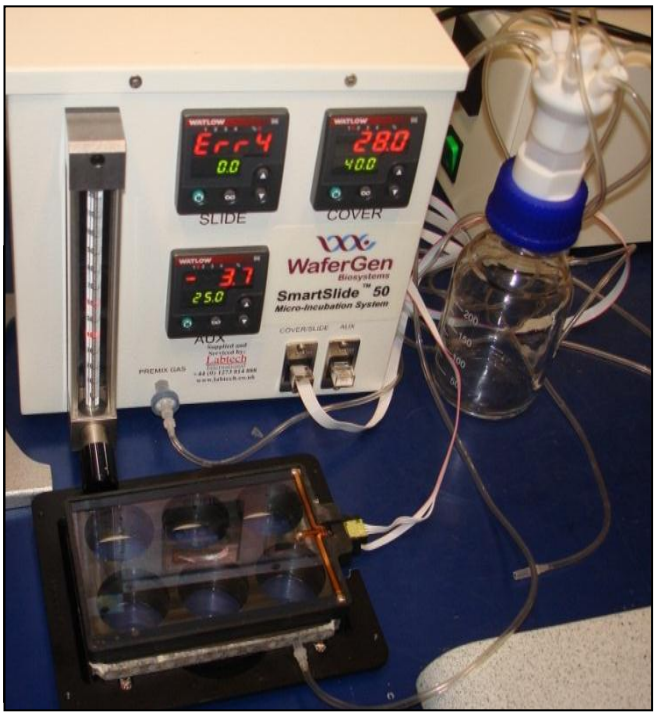
7. The cells were washed 3 times and then dehydrated for 20 minutes each with 20%, 30% 50%, 70%, 90% and 100% ethanol alcohol solutions.
8. Further dehydration was carried out by placing 100% ethanol dried over anhydrous sodium sulphate on the cell layer till critical point drying was carried out.
9. At the end of dehydration process, the coverslips were mounted on stubs with adhesive and critical point dried using a Samidry critical point dryer.
10. The cells were sputter coated with gold using a conductive metallic sputter coater (Agar auto sputter coater) and imaged using Hitachi 3400N scanning electron microscope.
11. The entire process of fixation and imaging with scanning electron microscopy was performed with the help and guidance of Mr. Barry Martin, Laboratory Manager.

## ***5.3. Results***

The components of the Wafergen smartslide system are shown in Figure 5.1. The results of the 24 imaging series utilising Wafergen smartslide system are shown in the attached movies 1 and 2 and still images are given in Figure 5.2. The adherent cancer cells are in constant crawling motion in the time period between 1 hour to 6 hours. From 6 hours post adhesion, the endothelial cells begin to retract and the cancer cells settle in the gaps between these endothelial cells. From 10-12 hour onwards, the cancer cells

settled in the gap between endothelial cells begin to divide to form colonies of cancer cells.

Similar results are also seen when high resolution imaging is carried out using scanning electron microscopy. Figure 5.3 shows adherent cancer cells 1 hour post adhesion. Cancer cells 2 hour post adhesion are shown in Figure 5.4. The cancer cells put out finger-like projections which might aid in crawling. Figures 5.5 shows retracted endothelial cells and cancer cells settling in the gap between the endothelial cell monolayer 6 hours post adhesion. Figures 5.6 shows cancer cells settled in the gap between endothelial cells dividing. Figure 5.7 shows trans cellular migration of cancer cells through endothelial cells.



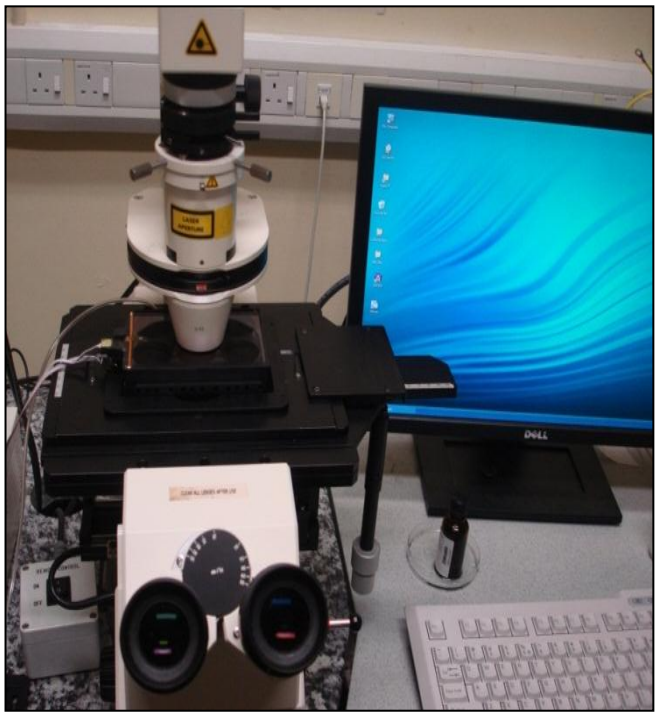



Figure.5.1. The Wafergen smartslide system: The system consists of a smartslide which has in built temperature control and inlet for CO2. The smartslide is also compatible with confocal microscopy thereby allowing continuous imaging of live cells

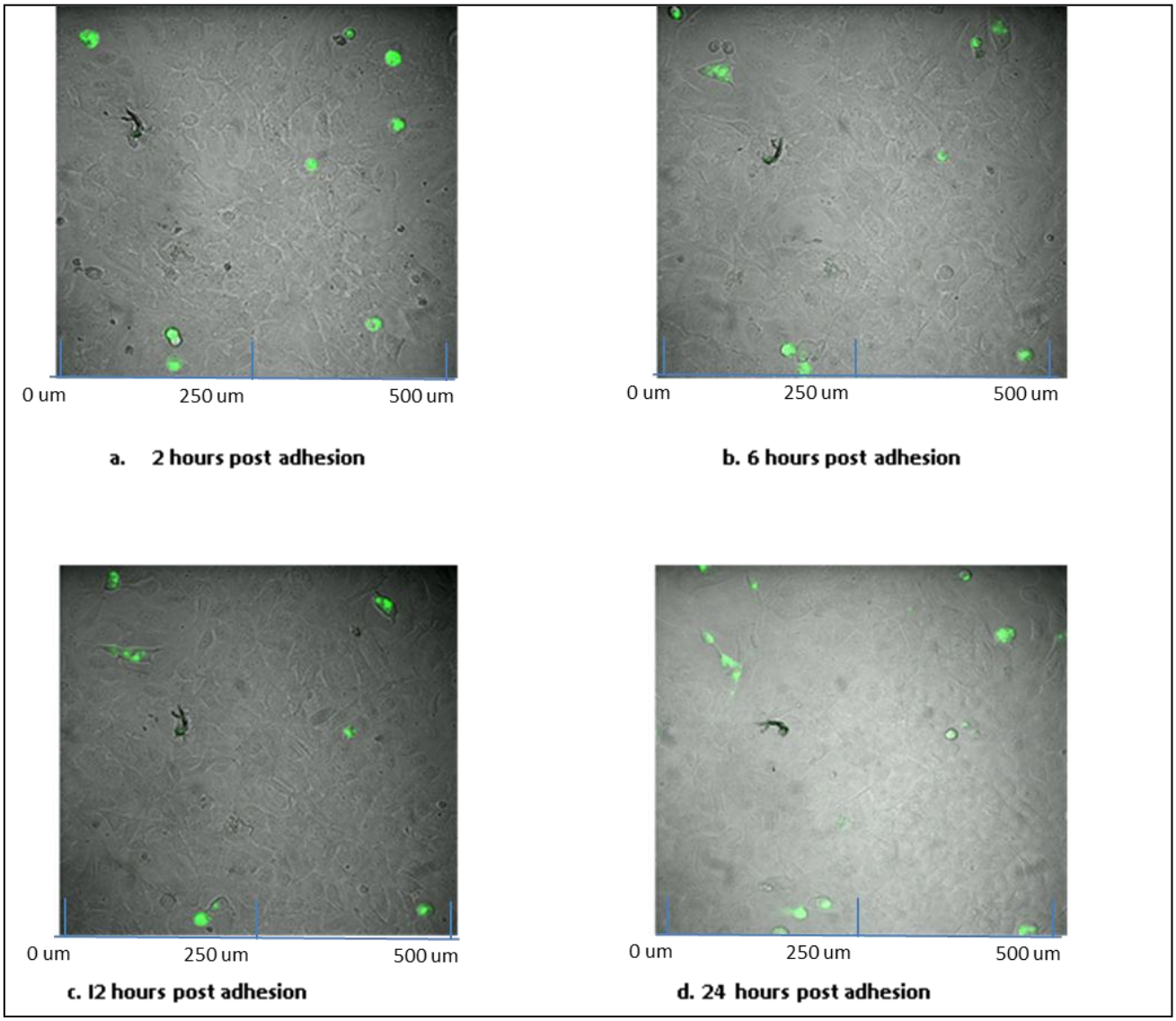



Figure.5.2. Static images of adherent MCF 7 cells at different time points post adhesion Figures a and b. In the initial period, the cancer cells adhere on the endothelial layer and flatten out. Figures c and d. 12-24 hours post adhesion the endothelial cells form a clear zone around the cancer cells and the cancer cells settle in this area of clearance and divide. Imaging carried out with Zeiss confocal microscope, using 458 nm laser, LP 475 filter and 25x objective

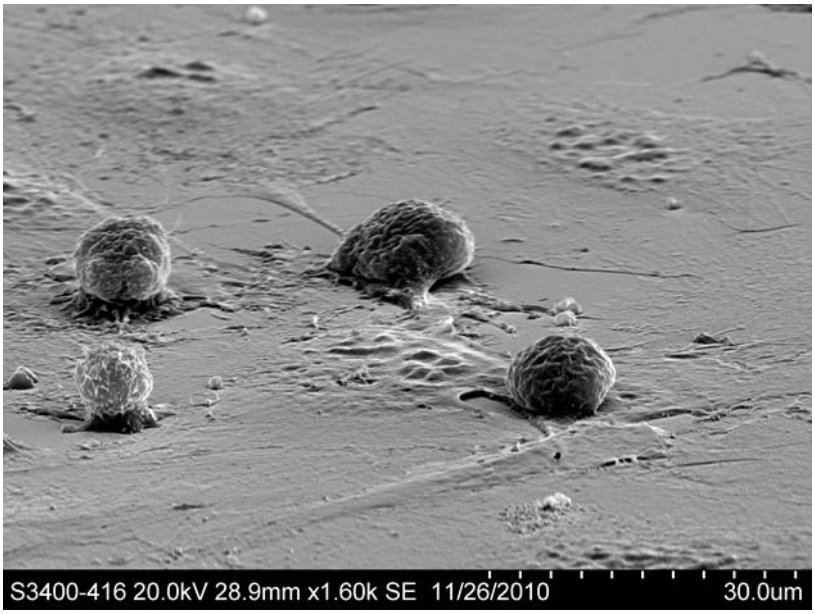



Figure.5.3. 1 hour post adhesion. The cancer cells are found adherent to endothelial monolayer and show a rounded appearance. Magnification x1.6k with 75 degree tilt

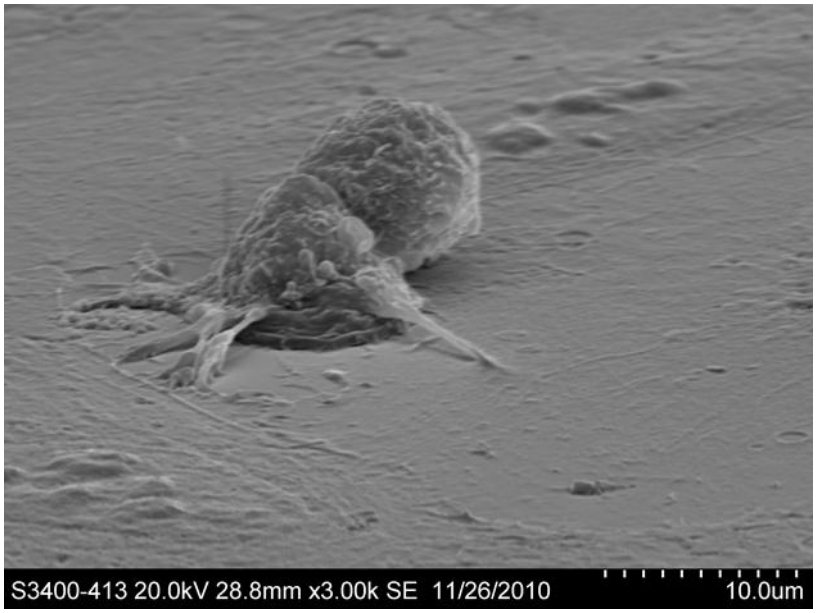



Figure 5.4. 2 hours post adhesion: Cancer cells extending processes which appear to be helping with crawling along the endothelial monolayer. Magnification x3.00k with 75 degree tilt

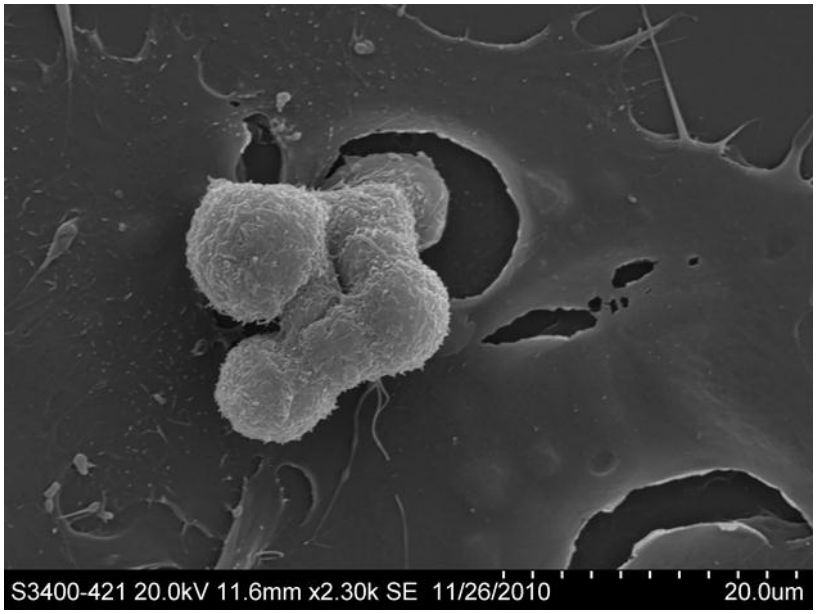



Figure 5.5. 6 hours post adhesion. The cancer cells are shown to be migrating in the gap between endothelial cells. Magnification x2.30k


Figure 5.6. 12 hours post adhesion. The cancer cells are shown to be migrating in the gap between endothelial cells. The endothelial cells are present in a confluent monolayer and a clear zone is present just around the cancer cell. Magnification x1.80K

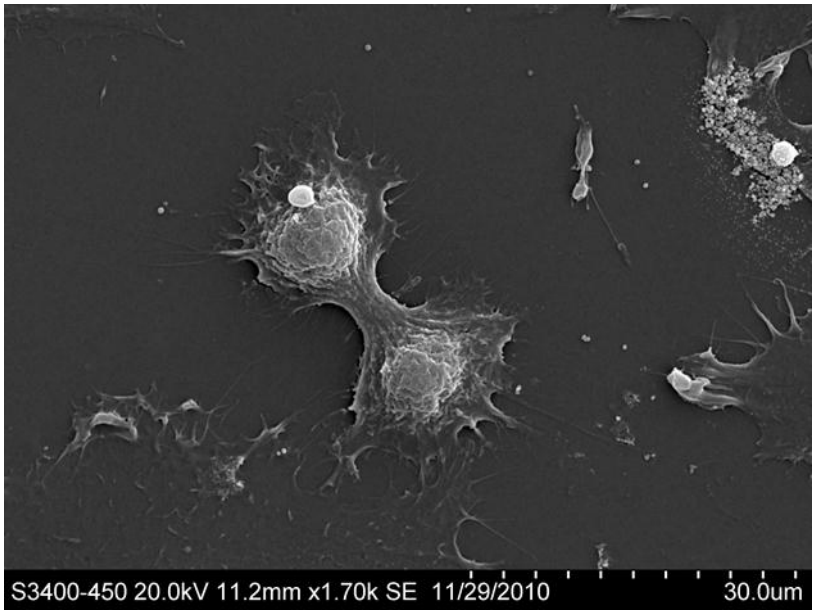



Figure 5.7. 12 hours post adhesion: Cancer cells are shown in cell division. Magnification x1.70k

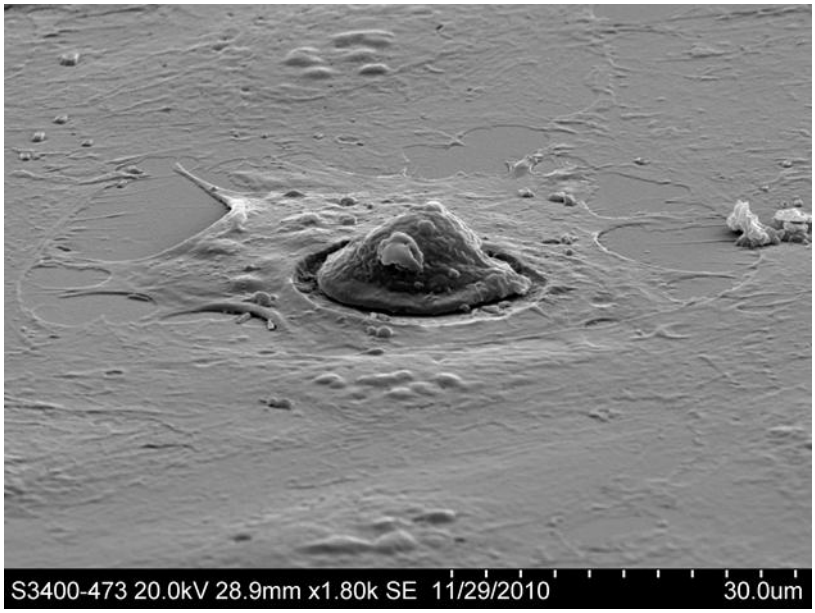



Figure 5.8. Transcellular migration: Cancer cell migrating through endothelial cell. Magnification x1.80k

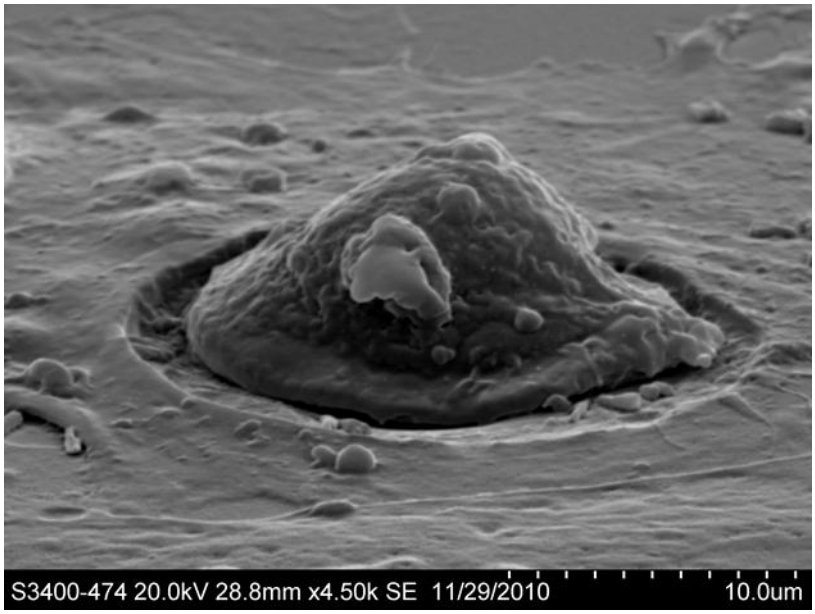



Figure 5.9.Transcellular migration A higher resolution image showing transcellular migration. Magnification x4.50k

## ***5.4. Summary of main findings.***

From experiments described in the above sections, it can be visualised that post adhesion, cancer cells crawl along the endothelial cell monolayers. The endothelial cells retract to let the cancer cells pass through the endothelial monolayer and the cancer cells divide to form colonies of cancer cells. During transmigration, the majority of the cancer cells utilise para cellular migration even though, transcellular migration was also observed. This behaviour of cancer cells appears to be similar to the behaviour of leucocytes during inflammation described in section 1.4.4 . However, further quantitative data with confocal microscopy and scanning electron microscopy is needed to ascertain this.

# Chapter 6:

# Discussion

## *6.1: Introduction*

As described in chapter 1, section 1.2, cancer metastasis, the dissemination of cancers from the primary organ of their origin to other organs in the body is the most important cause of cancer related deaths. Metastasis is brought about by a series of highly co-ordinated and sequential set of events and is often termed a cascade. The steps of the metastatic cascade include angiogenesis, detachment of the tumour cells from the main tumour mass, intravasation, dissemination of the cancer cells via the blood stream to various organs, adhesion and trans endothelial migration in the target organs and finally the formation of metastatic tumour foci in the target organs (Chambers et al., 2002, Brooks et al., 2010). It has been long recognised that adhesion and transendothelial migration step of the metastatic cascade is one of the limiting and key steps of the metastatic cascade (Tarin et al., 1984). Hence this step of the metastatic cascade was the focus of the experiments described in this thesis and was studied in a model of breast cancer metastasis.

Abnormal glycosylation of proteins on the surface of cancer cells is reported to be a salient feature of several cancers, as described in section 1.7. and such changes have been linked to enhanced metastatic capability and hence poorer patient prognosis in these cancers (Brooks et al., 2008a). Among the abnormal glycosylation changes associated with cancers, glycans with terminal and sub terminal GalNAc residues specifically recognised by the lectin HPA are of particular interest. Described as a marker of poor prognosis for the first time in breast cancers by Leathem and Brooks in 1987 (Leathem and Brooks, 1987),

the presence of HPA binding glycans were subsequently found to be associated with metastatic capability and poor prognosis in several other cancers including cancers of oesophagus, stomach, colon and lung (Fukutomi et al., 1989, Alam et al., 1990, Kakeji et al., 1991, Noguchi et al., 1993, Thomas et al., 1993, Yoshida et al., 1993, Schumacher and Adam, 1997, Chen et al., 2007, Fenlon et al., 1987). In addition to this, in breast cancers, a vital and significant link was found between the presence of HPA binding glycans on cells of the primary tumour and lymph node metastasis – thus, with a physical manifestation of metastatic competence (Brooks and Leathem, 1991, Brooks et al., 1993). As a result of this strong association between HPA binding glycans and metastasis, investigations to link HPA binding glycans to a specific step of the metastatic cascade were embarked upon. The first in the series of studies searching for a link between HPA binding glycans and a specific step of the metastatic cascade was an investigation by Brooks and Hall (Brooks and Hall, 2002). This study investigated whether HPA binding glycans were involved cancer cell adhesion to, and invasion of, basement membrane and was carried out by using a matrigel invasion assay. This study found no link between HPA binding glycans and adhesion of cancer cells to basement membrane.

A very limited pilot study was then undertaken to investigate the role of HPA binding glycans in the adhesion of cancer cells to vascular endothelium. In this study, HPA binding glycan inhibition was found to significantly reduce the adhesion of breast cancer cells to the endothelial cell layer thereby providing tantalising evidence that HPA binding glycans present on cancer cell surface

might contribute to adhesion of the cancer cells to vascular endothelial cells (Valentiner et al., 2005). Furthering this early work, the role of HPA binding glycans in endothelial adhesion and transendothelial migration was chosen as the main focus for further investigations through experiments described in this thesis.

To bring about the adhesion of the cancer cells to endothelial monolayers, a semi static rocking adhesion assay system previously developed in house was employed. However, since it was not previously optimised for live cell imaging which formed an essential component of several experiments in this project, the assay system needed to be further developed and optimised to facilitate live cell imaging.

The key aims of the project, as already stated in chapter 1, section 1.9, were therefore

- To develop and optimise rocking adhesion assay system to incorporate and enable live cell imaging
- To investigate whether HPA binding glycans play a functional role in the adhesion of breast cancer cells to endothelial cells
- To study the post adhesive behaviour of cancer cells with the aid of confocal laser microscopy and scanning electron microscopy

## ***6.2. Summary of the key findings:***

### *6.2.1 Optimization of rocking adhesion assay system for live cell imaging*

(described in Chapter 3):

- Trial rocking adhesion assays with HUVEC cell monolayers cultured on confocal microscope compatible dishes, on imaging, displayed viable cells at the end of rocking assays with no apparent loss of integrity of the endothelial monolayer or detachment of the cells from the culture surface. This indicated that live HUVEC cells cultured on confocal microscope compatible dishes could withstand the shear forces produced during rocking adhesion assays could be incorporated in the assay systems.
- The viability of the cells and integrity of the monolayers was not lost for the entire duration of imaging.
- Adhesion assays with MCF 7 and ZR 75 1 cells to investigate the optimum time of rocking to be employed to obtain the maximum number of adherent cancer cells for quantitative assays showed that adhesion begins at early time points (5-15 min), increases progressively with the duration of rocking to a maximum of 1 hour 30 minutes of rocking and then plateaus up to a period of rocking of 2 hours. Hence all subsequent rocking adhesion assays were carried out with 1hour 30 minutes – 2hours of rocking

The rocking adhesion assay system was employed in all of the experiments described in this thesis to produce the adhesion of cancer cells to endothelial

monolayers. Traditionally, there are two types of in vitro adhesion assays systems - namely static adhesion assays, where leucocytes or cancer cells are placed on endothelial layer and adhesive interactions take place under static conditions, and flow assays where leucocytes or cancer cells are perfused over the endothelial cell layers and adhesive interactions take place under conditions of flow. Static adhesion assays are simple to set up and easy to perform and require no specialist equipment. Hence they are ideal in experiments requiring greater throughput, for example when quantitative data is required (Butler et al., 2009) and have been used extensively in studying adhesive interactions both in leucocytes [for e.g. (Bevilacqua et al., 1985, Gimbrone et al., 1984) ] and in cancer cells [for e.g. (Dejana et al., 1988, Heimburg et al., 2006). However, static adhesion assays do not provide conditions of flow which produce shear forces across the endothelial layer. Shear forces produced by conditions of flow have been shown to up-regulate vital adhesion molecules like ICAM-1 and hence leucocytes adhesion to endothelial cells (Nagel et al., 1994, Morigi et al., 1995). Flow assays closely mimic physiological conditions and in addition, possess the advantage of providing precise control of experimental conditions like flow rate and shear forces across the endothelial layer (Morigi et al., 1995) but tend to be expensive, time consuming, technically complex and require specialist cell culture and microscopy equipment.

The rocking adhesion assay system used in this project has the advantage of the static assay system in that it is easy to set up and allows multiple repetitions of experiments for quantitative analysis but provides the conditions of flow

required for up-regulation of adhesion molecules like the flow assays. However, its main disadvantage is that experimental conditions like flow rate and shear forces cannot be controlled and also the electrical components of the rocking platform tend to heat up on prolonged use which has a potential to change experimental conditions.

The experiments to assess the number of adherent cancer cells in relation to the time described in chapter 3 section 3.2.2 of rocking showed a gradual increase in the number of adherent cancer cells with duration of rocking up to 1 hour 30 minutes. In previously reported flow assay experiments to assess the expression of adhesion molecules in response to laminar flow, a time dependant and gradual increase in the expression of ICAM-1 was seen in HUVEC cell monolayers exposed to shear forces compared to HUVECs in static conditions. In addition to this, there was up-regulation of leucocyte adhesion in the cells subjected to laminar flow compared to those in static conditions (Morigi et al., 1995). It is therefore likely that in the rocking adhesion assays, with continued rocking, there was gradual up-regulation and recruitment of adhesion molecules and hence produced progressive increase in the number of adherent cancer cells with relation to time. Saturation of all the adhesive molecules with progressive increase in cancer cell adhesion with time is likely to have resulted in the plateau reached after 1 hour 30 minutes of rocking.

***6.2.2. Rocking adhesion assays to investigate the role of HPA binding glycans in the adhesion of cancer cells to endothelial cell monolayer*** (described in Chapter 4):

- Three breast cancer cell lines: MCF 7 cells, derived from metastatic breast cancer and with a high surface HPA binding glycan profile, ZR 75 1 cell line, representing intermediate aggressiveness and HPA binding glycan profile, and BT 474 cell line representing primary, non-metastatic breast cancer with minimal surface HPA binding glycans as (described in chapter 2, section 2.1) were employed in the rocking adhesion assays.
- Rocking adhesion assays using MCF 7 and ZR 75 1 cell lines with and without HPA inhibition showed that there was significant reduction in the adhesion of the cancer cells to endothelial monolayer when HPA binding GalNAc –glycans were masked. No difference in adhesion was found in the poorly HPA-binding BT474 cells with or without HPA inhibition.
- The number of adherent cancer cells and hence the degree of inhibition of adhesion appeared to be directly proportional to the HPA binding glycan profile of the cancer cells, being greatest in MCF 7 cells.
- Control experiments involving pre-inhibition with the lectins PNA and Con A showed no decrease in the adhesion of the cancer cells to the endothelial monolayer with either of these lectins.

The cell lines utilised in the rocking adhesion assays were chosen to represent a spectrum of malignant disease presentation. Thus, BT 474 cell lines derived from primary ductal carcinoma represent non metastatic, primary breast carcinoma cells (Lasfargues et al., 1978), ZR 75 1 cells derived from malignant

ascites secondary to ductal carcinoma of the breast (Engel et al., 1978) represent cells of intermediate aggressiveness and MCF 7 cells derived from malignant pleural effusion secondary to infiltrating ductal carcinoma of breast (Soule et al., 1973) represent aggressive, metastatic breast cancer cells. The cancer cell lines also have well established HPA binding profiles with MCF 7 cells possessing the maximum, ZR 75 1 with intermediate and BT 474 with minimal surface GalNAc-glycans, and hence HPA binding profiles.

Rocking adhesion assays with HPA inhibition showed a significant decrease in the adhesion of cancer cells to endothelial cells when performed using MCF 7 and ZR 75 1 cell lines. No significant reduction in adhesion of poorly HPA-binding BT 474 cells was seen. These are novel findings and provide evidence that HPA binding glycans may play a functional role in the adhesion of breast cancer cells to endothelial cells during metastasis. These findings are entirely consistent with, and further reinforce, preliminary data reported by Valentiner et al (Valentiner et al., 2005). Moreover, the number of adherent cancer cells and the inhibition in adhesion was greatest in MCF 7 cells which possess the maximum amount of surface HPA binding glycans of the three cell lines and least in BT 474 cells.

Adhesion assays carried out in the presence or absence of PNA and Con A binding confirm that the inhibition in adhesion seen in the presence of HPA binding is a specific phenomenon, unlikely to be secondary to steric binding. This may suggest that HPA binding glycans could be recognising and specifically binding a putative receptor on the endothelial cell surface. Since,

the exact identity of HPA binding glycans on cancer cells remain incompletely characterised despite exhaustive research (Dwek et al., 2001, Saint-Guirons et al., 2007), the identification of this putative receptor might aid in the understanding of this complex process.

Finally, there is a need to link the findings from the rocking adhesion assay with HPA inhibition described in this thesis to the broader perspective of adhesion and transendothelial migration step of the metastatic cascade and its similarity to leucocyte adhesion and transendothelial migration.

Several new advances have been made in the study of breast cancer cell metastasis. Endothelial E selectin has been shown to be involved in breast cancer cell transmigration through its ligand CD44v4 (Zen et al., 2008). In addition to this selectin ligand Sialyl- Lewis x antigen has been shown to drive metastasis in ER positive breast cancers and with ER positive breast cancers with high expression of sialyl Lewis x correlated with metastasis (Julien et al., 2011). However, the role played by HPA binding glycans in adhesion and transendothelial migration is still unclear Hence, it is impossible to define the exact role of HPA binding glycans in this intricate and likely to be highly specialised set of events without further research.

Protein elution from HPA affinity chromatography and subsequent characterisation of these proteins by proteomic analysis has revealed that HPA binding proteins include molecules like integrin α6, integrin α5 and annexins, which are involved in cell adhesion and migration, filament proteins like α-tubulin, β-tubulin, cytokeratins and actin which are involved in remodelling,

and HSP-70, HSP-90, HSP-96 and TNFR-1 which are involved in antiapoptotic pathways. While several of these molecules have been associated with cancer and might be subject to abnormal glycosylation and hence recognised by HPA, the exact mechanism of binding of these complexes to HPA is still unclear. Some of these molecules might bind HPA directly through the lectin-glycan interactions described earlier, while others might bind HPA indirectly by forming complexes with HPA binding glycoproteins, for example, by binding O-GlcNAc glycoproteins in the cytoplasm (Saint-Guirons et al., 2007).

### *6.2.3. Study of the behaviour of the cancer cells post adhesion* (described in chapter 5)

- Study of post adhesive behaviour of cancer cells by live cell imaging using the Wafergen smartslide system showed that in the initial phases of the imaging (up to 6 hours post adhesion) the adherent cancer cells crawled on the endothelial monolayer. The endothelial cells in the vicinity of the adherent cancer cells then retracted forming a zone of clearance around the cancer cells. The individual cancer cells then flattened out in this zone and proceeded to divide forming a new colony of cancer cells.
- This sequence of events follows a pattern similar that reported to the post adhesive behaviour of leucocytes during the inflammatory response as described in section 1.3 and 1.4
- Higher resolution microscopy of the same process using scanning electron microscopy produced images consistent with

those obtained using confocal microscopy of live cells. In the early stages of the adhesion (1-2 hours post adhesion) cancer cells bearing finger-like projections were imaged. In the intermediate time points (6-12 hours), areas of clearance apparently resulting from endothelial cell retraction were seen. In the later stages dividing cancer cells were seen nestled in the areas cleared by the endothelial cells, and cancer cell transmigration through endothelial cells was also captured.

- Adhesion and transendothelial migration in cancer cells is thought to involve mechanisms similar to leucocyte adhesion transendothelial migration and is therefore often termed 'leukocyte mimicry' (Barthel et al., 2007, Albini, 1998). The understanding of the process of adhesion and transendothelial migration in cancer cells is still limited, but according to available evidence, there are several similarities and certain, vital differences in comparison to leucocyte adhesion and transendothelial migration and these are highlighted below.

### *6.2.4. Selectin mediated rolling:*

Like leucocytes, cancer cells appear to utilise selectin mediated rolling. Unlike leucocytes, cancer cells lack the expression of selectins and instead, express selectin ligands. Expression of selectin ligands has been associated with enhanced metastatic capability (Kannagi et al., 2004, Strell and Entschladen, 2008), thereby indicating that they might be facilitating cancer cell adhesion to

endothelial cells. For example, E selectin ligands, which are vital for E-selectin mediated binding to endothelial cells, have been shown to be expressed in metastatic prostate carcinoma cells (Dimitroff et al., 2005). In addition to expressing E-selectin ligands, cancer cells have also been found to possess the ability to induce endothelial E-selectin expression in endothelial cells. (Kannagi et al., 2004) and might aid E-selectin mediated adhesion of cancer cells to endothelium. In colon cancer cells slow, intermediate and fast rolling have been found to be mediated by E-, P- and L-selectins respectively (Hanley et al., 2006). P selectin binding has been shown to produce integrin activation in colon carcinoma cells and this process is in keeping with activation of integrins produced by selectins in leucocyte adhesion (Reyes-Reyes et al., 2006). However, unlike leucocyte adhesion, where selectins alone mediate rolling, in cancers, selectins have been shown to mediate the diapedesis of cancer cells through endothelial layer (Tremblay et al., 2008). Furthermore, mediators other than selectins have also been implicated in mediating rolling of cancer cells. For example, rolling in MDA-MB-468 breast carcinoma cell lines have been shown to be mediated by N- cadherin (Strell et al., 2007). Hence, it can be deduced that though tumour cells appear to utilise selectin mediated rolling similar to leucocytes, the role of selectins in the adhesion and transendothelial migration in cancers might not be limited to rolling alone and might be involved in other aspects of adhesion and transendothelial migration. In addition to this, mediators other than selectins might also play a role in tumour cell rolling.

Due to the nature of rocking adhesion assay system, imaging of adhesive behaviour could only be performed in the later stages of cancer cell adhesion i.e. once firm adhesion of cancer cells to endothelial surface had occurred and flow adhesion assays with direct video–microscopic imaging would be required to image the initial events in cancer cell adhesion.

### *6.2.5. Integrin mediated firm adhesion*

The function of integrins in mediating firm adhesion and in the subsequent crawling of leukocytes on endothelial cell monolayers is well described (Barreiro and Sanchez-Madrid, 2009).

There is evidence that cancer cells might utilise other members of the integrin family for this process. For example, the integrin very late antigen 4 (VLA-4) was shown preferentially cause the binding of melanoma cells to VCAM-1 (which is the ligand for VLA-4 on activated endothelial cells) and this caused significant augmentation in the formation of metastatic colonies in nude mice. (Garofalo et al., 1995). However, like selectins being implicated in rolling, the role of integrins is not restricted to firm adhesion alone in cancers. αvβ3 integrins have been shown to mediate the transendothelial migration of melanoma cells by their interaction with the adhesion molecule L1 (Voura et al., 2001). Of particular interest to this thesis, in addition to integrins, Thomsen-Freidenreich antigen and galectin mediated adhesion has been shown to be involved in the adhesion of breast and prostate cancer cells to the vascular endothelium (Glinsky et al., 2001).

The experiments described in chapter 4 indicate that HPA binding glycans may be involved in mediating adhesion of cancer cells to endothelial cells. However, the exact mechanism of adhesion is as yet unexplored  still largely unknown. It remains to be seen therefore, whether like T antigen, whether binding is being mediated by a specific adhesion molecule interacting with HPA binding glycans on cancer cell surface. Characterisation of the putative HPA binding glycan receptor on endothelial cells would shed light on this

### *6.2.6. Transendothelial migration*

Leucocytes utilise two methods to transmigrate across the vascular endothelium- paracellular migration where in the leucocytes pass between two endothelial cells and trans cellular migration in which leucocytes pass through the endothelial cells. For an in depth description of leucocyte migration and the mediators of trans and para cellular migration, please refer to chapter 1 section 1.3 and 1.4

From the results of chapter 5, it is clear that cancer cells also appear to utilise similar modes of migration as leucocytes i.e. post adhesion the cancer cells crawl along the endothelial cell surface, the endothelial cells then retract and cancer cells move in to this retracted area and divide (please refer to Figures and Movies 1, and 2). In addition to this, in the SEM images, trans cellular migration was also noted.

It is well known that after leucocyte transmigration, there is no loss in the integrity of the endothelial monolayer (Huang et al., 1988). However, in our imaging experiments, the endothelial retraction appeared to be followed by

permanent damage to the endothelial monolayer layer integrity. Similar findings were reported in a study of transmigration of human invasive bladder carcinoma cells across a monolayer of HUVECs. Real time confocal laser microscopy imaging demonstrated that post transmigration, there appeared to be permanent loss of endothelial monolayer integrity (Heyder et al., 2002). Hence, it can be inferred that unlike the physiological process of leucocyte transmigration, where the endothelial monolayer is not permanently damaged, the pathological process of tumour cell transmigration appears to cause irreversible damage of endothelial monolayer integrity.

In conclusion, even though leucocyte adhesion and transmigration is thought to be a paradigm for adhesion and transendothelial migration during metastasis and to a considerable extent, there are similarities, there appear to be several differences. Adhesion and transendothelial migration of cancer cells is not as well understood as leucocyte adhesion and transendothelial migration. However, from the available evidence, the role of the key mediators appears to be not restricted to a particular step of the adhesion and transendothelial migration process and the changes produced due to cancer cell adhesion and transendothelial migration are more destructive and permanent.

## ***6.3. Future Work***

- The results described in this thesis indicate that a potential functional role of HPA binding glycans in cancer cell-endothelial cell interactions may warrant further investigation.
- Owing to the technical complexity of glycan analysis, one way forward may be to attempt to identify, isolate and characterise the putative endothelial cell receptor recognised by cancer cell GalNAc-glycans.
- Though the role of HPA binding glycans during cancer cell-endothelial cell adhesion was investigated in the studies described in this thesis, the potential role of HPA binding glycans during transendothelial migration still has not been studied. This could be addressed using trans well migration assays and by mapping surface HPA binding glycans with fluorescent labels during various stages of adhesion and transendothelial migration.
- Imaging the ashesion assays has revealed several interesting observations. However, these studies need to be extended to obtain more quantitative and qualitative data, particularly using high resolution imaging with SEM, by using flow assays in a three-dimensional model, in animal studies and real time imaging modalities

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