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AUTHOR(S)

Gozie Offiah

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A Novel Study on the Functional Relevance of Junctional Adhesion Molecule-A in Breast Cancer

Gozie Offiah
B.Sc, MB, BCh, BAO (NUI) LRCSI/P, MRCSI

A thesis submitted to the Royal College of Surgeons in Ireland
for the degree of Doctor of Medicine (MD)

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Supervisor: Dr Ann Hopkins
Head of Department: Prof A.D.K. Hill

DECLARATION

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree MD is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed 

Dr. Gozie Offiah

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ABSTRACT

Breast cancer, a disease that arises from the epithelial cells within breast tissue, is a common illness that can affect all age groups. Each year it is diagnosed in an estimated 1 million women worldwide, and accounts for over 450,000 deaths. Despite advancements in breast cancer screening and treatment, breast cancer still remains one of the leading causes of female deaths worldwide.

Breast cancer is a heterogeneous disease encompassing many subtypes, which differ both in terms of their molecular backgrounds and clinical prognosis. Cancer initiation and progression is a multistep process involving dysregulations in cell adhesion, proliferation, survival and migration. Breast epithelial cells contain several multi-protein adhesion complexes at two principal sites, between neighbouring cells and between cells and their extracellular matrix. Adhesion proteins regulate a variety of cellular functions, and dysregulation of cellular adhesion has been implicated in the events that accompany cancer initiation and progression. Proteins of the intercellular tight junction have been found to be de-regulated in several human cancers including breast, and have recently been suggested as promising targets for cancer therapy. This thesis is focused on exploring the contribution of one tight junction protein, junctional adhesion molecule-A (JAM-A) to breast cancer progression.

In this thesis we used two isogenic breast cancer cell line models, HMT-3522 and Hs578T, both of which have "non-tumorigenic" and "tumorigenic" variants. We observed that tumorigenic HMT-3522 T4-2 cells had tighter junctions compared to non- tumorigenic cells, and that the tumorigenic variant of Hs578T cells expressed a higher level of JAM-A. JAM-A expression was also higher in primary breast cultures from tumour relative to non-tumour samples, and highest in aggressive high grade tumours. Interestingly, we detected high levels of soluble JAM-A in serum samples of breast cancer patients with benign disease compared to patients with invasive ductal carcinomas.

Functional inhibition of JAM-A was found to decrease protein levels of JAM-A in both non-tumorigenic and tumorigenic cells and to significantly reduce cell migration. In 3-dimensional cultures mimicking the *in vivo* microenvironment, JAM-A inhibition was found to decrease cell number and 3-dimensional spheroid formation and, for the first time, to partially normalise the abnormal/tumorigenic phenotype of invasive cells.

Taken together, our study provides novel evidence suggesting that JAM-A may be involved in breast cancer progression and have potential as a biomarker of disease progression or as a therapeutic target.

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ABBREVIATIONS

ATAC	Arimidex, Tamoxifen, Alone or in Combination trial
BSA	Bovine serum albumin
dH_2O	Distilled water
DMEM	Dublbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtOH	Absolute ethanol
EVOM®	Epithelial voltohmmeter
FBS	Foetal bovine serum
FITC	Fluoroscein isothiocyanate
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP	Horseradish peroxidise
KCl	Potassium chloride

MEBM	Mammary Epithelium Basal Medium
MTT	3-[4,5-dimethyl-2-yl]-2,5-diphenyl tetrazolium bromide
NaCl	Sodium chloride
NPI	Nottingham Prognostic Index
PBS	Phosphate buffered saline
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate (lauryl sulphate)
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TER	Transepithelial resistance
TJ	Tight junction
Tris	Trizma base
Tris-HCl	Tris hydrochloride
Tween-20	Polyoxyethylene-sorbitan monolaurate
TX-100	Triton-X 100
ZO-1	Zonula occludens protein-1

Chapter I

Introduction

1.1 Introduction to breast cancer

1.1.1 Structure of the breast

Mammary glands are modified sweat glands with a specialized function to produce milk. In the adult, the mature breast extends from the second ribs to the infra-mammary fold which is roughly at the level of the seventh rib. It extends from the lateral border of the sternum to the midaxillary line and projects into the axilla at the axillary tail of Spence (Monkhouse, 2007). The breast is located within the superficial fascia of the anterior thoracic wall and is made up of 15 to 20 lobes of glandular tissue (Bland and Copeland, 1998). Fibrous connective tissue forms the framework that supports the lobes and adipose tissue which fills the space between the lobes.

Each lobe of the mammary gland terminates in a lactiferous duct which opens onto the nipple. (Figure 1.1) These ducts have a sinus at the base beneath the areola called the lactiferous sinus. The lactiferous ducts are lined with stratified squamous epithelium. Myoepithelial cells of ectodermal origin lie within the epithelium between the surface epithelial cells and the basal lamina. Both the epithelial and myoepithelial cells of the breast duct lie on a basement membrane. Alterations in the basement membrane have been implicated in abnormal cell differentiation and metastasis (Kiosses et al., 2001, Antonelli *et al.*., 1991, Hewitt *et al.*., 1991).

The retromammary bursa contributes to the mobility of the breast on the thoracic wall. The bursa is a distinctive space identified surgically on the posterior aspect of the breast between the deep layer of the superficial fascia and deep investing fascia of the pectoralis

major muscle. Perpendicular to the superficial fascial layers of the dermis are the suspensory structures called Cooper's ligaments. These ligaments allow mobility of the breast and also provide support (Monkhouse, 2007).

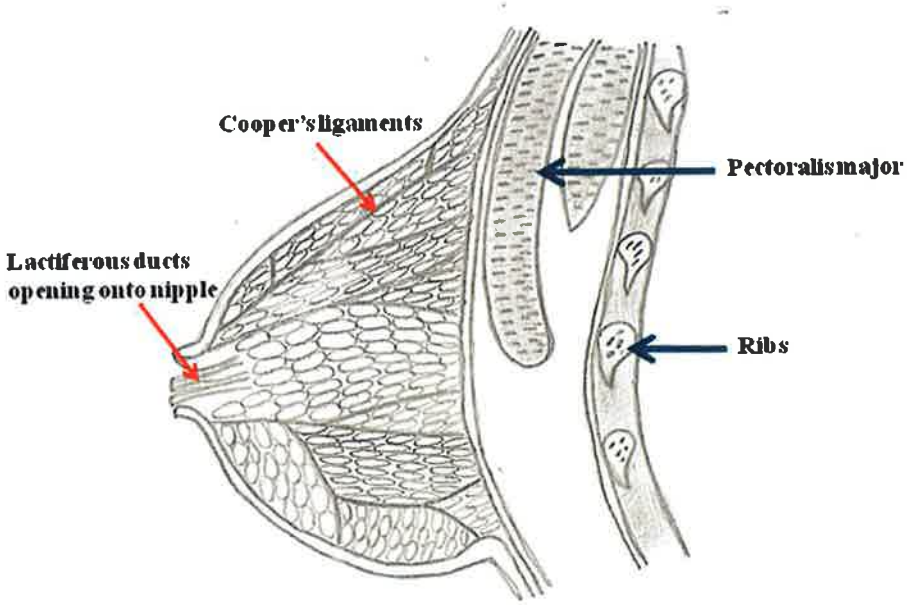


Figure 1.1: Structure of the breast showing the lobules and lactiferous sinuses terminating at the nipple. Picture modified from Master Medicine Clinical Anatomy by Stanley Monkhouse. Second Edition.

The epidermis of the nipple and areola are highly pigmented and covered by keratinised, stratified squamous epithelium. A nulliparous non-lactating breast weighs between 150 and 225g while the multiparous breast with pregnancy and lactation is usually larger (Bland and Copeland, 1998). With increasing age, the breasts usually undergo atrophy and thus become smaller.

The lymphatic drainage of the breast is of major significance to surgeons as this is a main route of cancer metastasis. The initial recognition of metastatic spread into internal mammary nodes as the primary route of dissemination is credited to the British surgeon R.S. Handley (Handley, 1975, Handley and Thackray, 1954). The majority of the breast is drained by the axillary nodes and the rest by the internal thoracic nodes and abdominal lymph nodes (Nathanson et al., 2001). Surgeons identify the axillary lymph nodes with respect to their relationship with the pectoralis minor muscle. Lymph nodes located lateral to the lower border of the pectoralis minor are referred to as level I nodes and include the external mammary, axillary vein and scapular lymph nodes. Lymph nodes posterior to the pectoralis minor are referred to as level II nodes, and include the central lymph node group and some of the subclavicular lymph nodes (Monkhouse, 2007). Finally, lymph nodes located superior to the upper border of the pectoralis minor are referred to as level III lymph nodes and include the subclavicular lymph node group. (Chevinsky et al., 1990, Danforth et al., 1986, Monkhouse, 2007) (Figure 1.2).

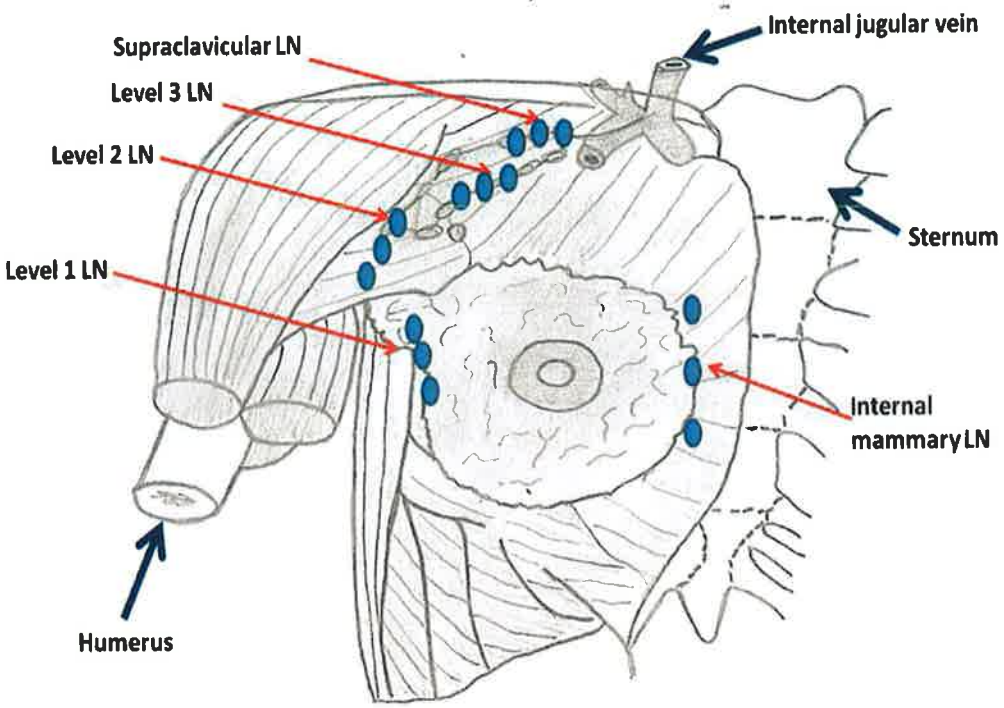


Figure 1.2: Structure of the breast illustrating the lymphatic drainage

Picture modified from Master Medicine Clinical Anatomy by Stanley Monkhouse. Second Edition.

1.1.2 Developmental physiology of the breast

In early foetal life, cords of epithelial cells form ducts that connect to the nipple and become surrounded by myoepithelial cells. These ducts undergo budding and by birth the mammary glands consists of a branching system of ducts that converge on the nipple. During childhood, isometric growth with proliferation of stromal tissue and elongation of the ducts in proportion to overall body growth is noted (Pusztaszeri, 2010). In females during puberty, the ovarian oestrogen surge promotes lengthening and branching of the ducts along with budding of the terminal ends and the deposition of increased fat and connective tissue.

During menarche, cyclical increases in oestrogen and progesterone cause ductal development and lobule formation. These hormones increase proliferation of connective tissue which replaces the adipose tissue thus providing support. The menstrual cycle is associated with a fluctuating volume of the breast with a pre-menstrual increase in size, density, nodularity and sensitivity of the breast (Bland and Copeland, 1998). This increase in volume is due to increased lobular size and not epithelial proliferation.

Increase in pregnancy hormones - oestrogen, progesterone and prolactin - lead to an increase in breast growth. The breasts enlarge as the ducts and lobules proliferate (Pike et al., 1993). Postpartum, there is a sudden drop in oestrogen and progesterone levels with the onset of lactation. Milk production and ejection is stimulated by the release of oxytocin from the posterior pituitary and prolactin from the anterior pituitary. Following weaning from breast feeding, the glands become inactive and retained milk leads to an increase in the inframammary pressure and subsequent alveolar rupture. The retained

secretory products of lactation undergo phagocytosis and the lobular structures undergo atrophy (Radisky and Hartmann, 2009).

At menopause, there is a decrease in the ovarian secretion of oestrogen and progesterone which results in progressive involution of the ductal and glandular components of the breast. The surrounding fibrous tissue matrix increases in density and the parenchyma of the breast is replaced with adipose and stromal tissue. The fat content and supporting stroma progressively decrease with age thus resulting in shrinkage of the breast and loss of lobular structure, density and contour. Hence, the persistence of breast lobules in the elderly with ER-positive cells with the potential for local oestrogen metabolism could result in ER-positive, slow-growing cancers of the breast (Walker and Martin, 2007).

While the processes mentioned above are mostly normal physiological events, very little is known about the correlation between lobular involution and the development of cancer. What is clear is that the breast serves as a target organ for a variety of hormones which either have active or passive roles in the physiology of the mammary gland. The main hormones that alter breast physiology are prolactin, oestrogen and progesterone. Oestrogen is known to promote the growth and development of the ductal system while progesterone stimulates lobular development (Pike et al., 1993, Mauvais-Jarvis et al., 1986). Any or all of these hormones could be implicated in the transition of a normal breast to a cancerous state, which will be discussed more fully in the next sections.

1.1.3 Pathology of breast cancer

Normal cell proliferation is controlled by growth-promoting proto-oncogenes and growth-inhibiting tumour suppressor genes. In most cases, normal cells divide as many times as needed and then stop. Carcinogenesis requires mutations in either (or both) oncogenes and tumour suppressor genes, along with subsequent interactions between defective genes and the breast microenvironment. Cells may become cancerous when mutations destroy their ability to stop dividing, resulting in abnormal growth and proliferation of cells (Lin et al., 2009).

The multistep model of carcinogenesis progresses from *initiation* to *promotion* to *conversion* and then *progression.* Initiation is irreversible and involves a direct carcinogen binding and damage to DNA. At the level of a mutation, there is usually no noticeable clinical change. Promotion occurs between initiation and premalignancy and tends to be reversible (Arpino et al., 2005). Here, the mutated cells are stimulated to grow and may be detected upon histological examination following biopsy. In conversion, there is uncontrolled growth and expansion of mutated cells with the clinical appearance of a benign tumour. Finally, there is the progression period between premalignancy and malignancy. Progression is generally irreversible, with complete loss of cellular control and the clinical appearance of invasion and metastasis (Evans and Manson, 2008, Missailidis, 2008). This sequence of events is the simplest and most logical paradigm of cancer progression, but not necessarily the route whereby all cancers progress. Therefore, the next section will explore further the different models involved in breast cancer progression.

1.2 Models of progression of breast cancer

As previously alluded to in the last section, breast cancer is a complex disease which likely initiates from genetic insults, with neoplastic conversion to invasive cancer occurring sometime during the pre invasive histological phases of usual hyperplasia, atypical hyperplasia and ductal carcinoma *in situ* (DCIS) (Hwang et al., 2004, Farabegoli et al., 2002, Holland et al., 1994, Tabar et al., 1999, Tabar et al., 1996).

Molecular analysis of invasive breast cancer and its precursors has furthered our understanding of breast cancer progression. Genomic data have supported several possible models of breast cancer progression. One hypothesis suggests that there exist genetically distinct subgroups of DCIS, only some of which ever have the potential to progress to invasion (Shackney and Silverman, 2003). An alternate de-differentiation theory proposes that DCIS progresses from lower to higher grade and then to invasive cancer with progressive accumulation of genomic changes (Farabegoli et al., 2002). The extent to which the genome is altered in DCIS indicates that genomic instability most likely precedes phenotypic evidence of invasion (Hwang et al., 2004). As mentioned previously, breast cancer is a heterogeneous disease and this heterogeneity suggests that malignant transformation is a dynamic process evolving through multiple multi-step pathway models. As well as genetic alterations, there are several other aetiological factors contributing to breast cancer and the next section will describe some of these factors.

1.3 Aetiological factors that contribute to breast cancer

Recent advances in technology and more importantly the awareness raised amongst women has led to earlier detection of breast cancer and in parallel a rising incidence. Several factors contribute to breast cancer, with age and gender being the main contributing factors. There is a rising incidence of breast cancer in women aged 45 to 75. Race and ethnicity have been implicated in breast cancer too, with the disease being much higher in westernised countries compared to Africa and Asia. Reproductive factors also play a major role in the development of breast cancer. The development and differentiation of the normal mammary gland in addition to growth and repression of breast cancer is influenced by the female hormone oestrogen working via its receptor (ER), as well as by a variety of polypeptide hormones and growth factors which interact with membrane receptors (Jemal et al., 2007). Thus, women with early menarche (before the age of 12) and late menopause (after the age of 55) have a higher risk of the disease (Bland and Copeland, 1998). Breast cancer risk is also higher in nulliparous women or those with a late first pregnancy.

Exogenous hormone therapies like oral contraceptives and Hormone Replacement Therapy (HRT) have also been implicated in the aetiology of breast cancer. Studies have shown that oral contraceptive pills provide a protective effect against breast cancer depending on time and length of use (Reeves et al., 2000). By the same token, several studies have disputed these findings stating that there is no effect on prognosis or survival with oral contraceptive use (Trivers et al., 2007, Wingo et al., 2007). Hormone replacement therapy has been proven to be protective in younger women and to increase the risk of breast

cancer in older women, depending on whether it is oestrogen-progestin combination or oestrogen only HRT (Howell and Evans, 2011). It has been suggested that the risk of breast cancer could be reduced by delaying the onset of regular ovulatory menstrual cycles and by minimizing the therapeutic use of oestrogens, and possibly of progestrogens, in postmenopausal women (Pike et al., 1993). It is known that the incidence of breast cancer increases with age and doubles every 10 years until the menopause when the rate of increase slows, supporting a link with hormonal status. Although very few cases of breast cancer occur in women in their teens or early 20s, it is the most commonly diagnosed cancer in women under 35.

Physical activity can also affect breast cancer risk through its effects on reproductive function. It is known that moderate physical activity at an early age decreases the frequency of ovulatory menstrual cycles, and moderate physical activity in young adults depresses luteal progesterone levels thus leading to the reduction of circulating hormones (Kelsey and Berkowitz, 1988).

Many other incidental factors, including radiation exposure, have also been associated with an increased risk of breast cancer. This was first observed among teenage girls exposed to radiation during the Second World War and also in patients who received recurrent radiation to the chest as a result of repeated X-rays for tuberculosis. They were noted to have a higher risk of breast cancer if first X-rayed between the ages of 10 and 14 years (McPherson et al., 2000). Other factors that may contribute to breast cancer include older age (Walker and Martin, 2007), obesity, diet, smoking and alcohol (Smith et al., 1994), family history and genetics.

In the majority of cases, cancer is a multifactorial disorder in which genetic and environmental factors interact to initiate carcinogenesis. Women with first-degree relatives who have had breast cancer are at an increased risk of developing the disease. The risk is more pronounced if more than one relative had breast cancer, if the breast cancer occurred before menopause or if it was bilateral. Other features suggestive of genetic predisposition to breast cancer include a family history of both breast and ovarian cancers (Tirona et al., 2010).

Mutations of *BRCA1* and *BRCA2* genes account for 5–10% of breast cancer cases, suggesting a genetic cancer syndrome. These are responsible for 80% of inherited breast cancers (Nathanson et al., 2001). The breast cancer gene 1 (BRCA 1) is localized on chromosome 17q12-q21, and was identified in the early 1990s using linkage analysis in site-specific families at high risk for breast cancer (Hall et al., 1992, Lalle et al., 1994). BRCA 1 confers an 85% risk by the age of 70 for the development of breast cancer.

Interestingly, BRCA1-related cancers often present at a lower stage and earlier age compared with sporadic cancers (Marcus et al., 1996). A second breast cancer gene, BRCA 2, was localized to 13q12-q13 chromosome also by linkage analysis. The BRCA 2 gene accounts for 35-40% of hereditary breast cancers with the risk of associated ovarian cancer being lower than the BRCA 1 carriers (Nelson et al., 2005). BRCA 2 related cancers tend to be more tubulolobular cancers than BRCA 1. More recently, another breast cancer gene not related to BRCA 1 or BRCA 2 has been identified; namely the BRCA 3 gene located on chromosome 8p12-22. In summary, the breast cancer genes confer a high risk of breast cancer in affected individuals with an increased risk of other associated cancers like ovarian cancer.

1.4 Incidence of Breast cancer

Worldwide, it is estimated that breast cancer is diagnosed in over 1 million women annually (Kasler et al., 2009); and accounts for over 450,000 deaths (Tirona et al., 2010). (Figure 1.3) Despite all the advances in breast cancer care, the incidence of the disease is rising with a remarkable decrease in mortality rates. This decrease in mortality in breast cancer has been attributed to improvements in screening techniques which permit earlier detection, surgical and radiotherapy interventions, better understanding of disease pathogenesis, and utilization of traditional chemotherapies in a more efficacious manner. However, while medical advances have significantly improved long term survival of women with early stage disease, the same is not true for women with advanced breast cancer.

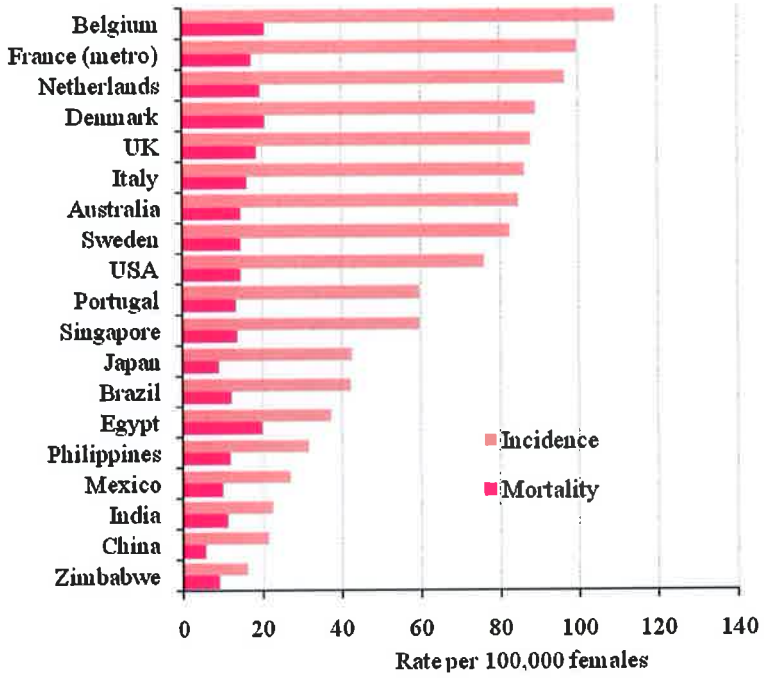


Figure 1.3: 2008 estimates of age standardised incidence and mortality rates of breast cancer. http://info.cancerresearchuk.org/cancerstats/types/breast/incidence

In the United States, breast cancer is considered to be of epidemic proportions with current estimates indicating that 1 in 8 women will develop breast cancer during their lifetime (National Cancer Institute www.cancer.gov). Figure 1.3 shows that breast cancer is of highest incidence in the United States and almost all of Europe. Closer to home, breast cancer accounts for 26% of all new cancer cases among women in Ireland annually (Jemal et al., 2008). In 2004, the National Cancer Registry recorded 2,285 new cases of breast cancer. (Figure 1.4) The overall annual breast cancer incidence is projected to increase to 4,700 cases by 2020, which represents a 105% increase with a predicted yearly mortality of over 600.

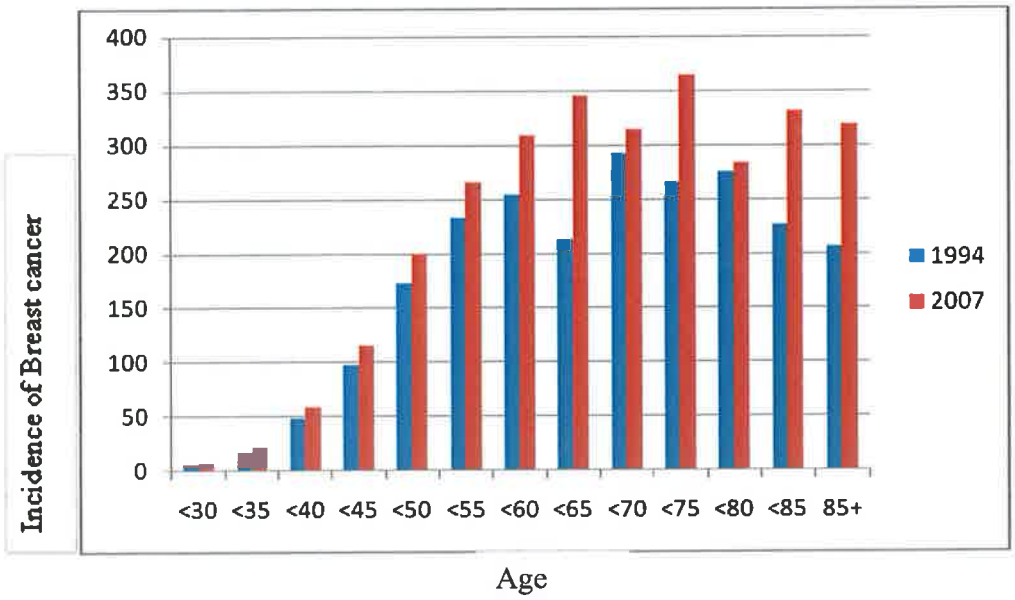


Figure 1.4: Age-specific incidence of breast cancer in Ireland

Figures from National Cancer Registry Ireland, report 2008

1.5 Classification of breast cancer

Breast cancer is a heterogeneous disease encompassing many subtypes, which differ both in terms of their molecular backgrounds and clinical prognosis. The clinical manifestations of breast cancer range from early stage localised tumours to advanced widely metastatic neoplasms, and can be classified by different schemata. The next few paragraphs will discuss the different classifications used in breast cancer.

1.5.1 Histological classification

Breast cancers are classified based on their histopathological appearance, which is the origin of much of the terminology. Most breast cancers are derived from the epithelium lining the ducts, and are classified as mammary ductal carcinomas. At its simplest, breast cancer is often considered as pre-invasive versus invasive categories. The two main pre-invasive breast cancers are known as ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). Carcinoma *in situ* is proliferation of cancer cells within the epithelial tissue without invasion of the surrounding tissue. *In situ* carcinoma of the breast was first recognized in the early 20th century and was identified morphologically as cells cytologically similar to invasive carcinoma (Bland and Copeland, 1998). DCIS is a heterogeneous group of non-invasive neoplastic growths with diverse morphology and risk of subsequent recurrence and invasive transformation. DCIS arises in the terminal ductal lobular units (TDLU) but also in extra-lobular ducts too. The histological diagnosis of DCIS or LCIS is based mainly on the histological pattern rather than its tissue of origin (Holland et al., 1994). Compared with LCIS, DCIS is generally more variable histologically and cytologically with a more pleomorphic nuclear morphology. LCIS,

which occurs in the breast lobules, is easy to diagnose as it is easily recognisable pathologically by the presence of populations of aberrant cells with small nuclei, while DCIS is a more heterogeneous condition and is diagnosed by exclusion of LCIS (Hanby and Hughes, 2008).

Invasive breast cancer has been sub-classified into invasive ductal breast cancer, invasive lobular breast cancer, inflammatory breast cancer and Paget's disease. Invasive ductal carcinoma (IDC) is the most common form of invasive breast cancer, accounting for around 85% of all cases. IDC is characterized by the presence of tumour cells outside the ductal-lobular units. Other histological types of invasive breast cancers include infiltrating ductal, medullary carcinoma, infiltrating lobular, invasive cribriform carcinoma, tubular carcinoma and mucinous carcinoma (Bland and Copeland, 1998). Invasive lobular carcinomas tend to be multifocal and bilateral. The cells invade as single cells in cords a pattern described as Indian filing and typically contain intracytoplasmic lumens. Invasive cribriform carcinomas form cribriform meshworks rather than distinct tubules. Invasive mucinous (colloid) carcinomas have abundant extracellular pools of mucin and have a very good prognosis (Hanagiri et al., 2010). Invasive medullary carcinomas are defined by surrounding lymphocytic response, circumscribed edge and abnormal looking cells.

Invasive cribriform carcinoma, tubular carcinoma and mucinous carcinoma are categorised into a special type as they have good prognosis even if they attain a large size. They account for about 20% of invasive breast cancers. Special type carcinomas make up about a third of the cancers picked up on screening. The non special type of ductal carcinomas represent three quarters of the invasive cancers and have no particular histological type to them (Sasaki and Tsuda, 2009).

1.5.2 Tumour Node Metastasis (TNM) classification

The TNM staging system is used to determine the anatomical extent of malignant disease on the basis of clinical (c TNM) and pathological (p TNM) criteria grouped under three broad headings: the primary tumour size (T stage), lymph-node involvement (N stage), and metastasis (M stage) (Arnone et al., 2010). The TNM classification is the single most useful prognostic indicator of disease stage and prognosis.

The TNM staging is further sub-divided into 3 groups. Stage 0, which is normally referred to as carcinoma *in situ*, is a pre-malignant disease and has excellent prognosis. Stages 1–3 are defined as early cancer and are usually potentially curable; while stage 4 is defined as advanced, metastatic cancer and incurable. This system was devised in 1942 by Pierre Denoix and is now used worldwide (Singletary and Connolly, 2006). Its use has enabled oncologists in all countries speak to each other in a common language.

Primary tumour (T):
TX: Primary tumour cannot be assessed
T0: No evidence of primary tumour
Tis: Carcinoma *in situ*
T1: Tumour 2.0 cm or less in greatest dimension
T1mic: Micro invasion 0.1 cm or less in greatest dimension T1a: Tumour more than 0.1 but not more than 0.5 cm in greatest dimension T1b: Tumour more than 0.5 cm but not more than 1.0 cm in greatest dimension T1c: Tumour more than 1.0 cm but not more than 2.0 cm in greatest dimension
T2: Tumour more than 2.0 cm but not more than 5.0 cm in greatest dimension
T3: Tumour more than 5.0 cm in greatest dimension
T4: Tumour of any size with direct extension to (a) chest wall or (b) skin,
T4a: Extension to chest wall T4b: Oedema (peau d'orange) or ulceration of the skin of the breast T4c: Both of the above (T4a and T4b)

T4d: Inflammatory carcinoma
Regional lymph nodes (N):
NX: Regional lymph nodes cannot be assessed N0: No regional lymph node metastasis N1: Metastasis to movable ipsilateral axillary lymph node(s) N2: Metastasis to ipsilateral axillary lymph node(s) fixed to each other or to other structures N3: Metastasis to ipsilateral internal mammary lymph node(s)
Distant metastasis (M):
MX: Presence of distant metastasis cannot be assessed M0: No distant metastasis M1: Distant metastasis present (metastasis to ipsilateral supraclavicular nodes)

Table 1: TNM staging of breast cancer showing the breakdown on the different categories

Information from the American Joint Committee on Cancer (AJCC). http://cancernet.nci.nih.gov

Another prognostic index used in breast cancer is the Nottingham Prognostic Index (NPI). The NPI was first developed in 1982 by Haybittle *et al.* for dividing patients into prognostic groups for appropriate management. It is based on tumour size, lymph node stage and pathological grade (Haybittle et al., 1982, Galea et al., 1992, Todd et al., 1987).

Three prognostic groups are defined by the NPI, namely

1. <3.4 (Predicted 85% 5 year survival)
2. 3.41 to 5.4 (Predicted 70% 5 year survival)
3. >5.41 (Predicted 50% 5 year survival)

The prognostic index is calculated using the following equation:

(0.2 x largest diameter in cm) + Histological tumour grade + Nodal status

Tumour grade is defined as

1 = Grade I

2 = Grade II

3 = Grade III

Nodal status is defined as 1, 2 or 3 where

1 = (0 positive nodes)

2 = (1-3 positive nodes)

3 = (>3 positive nodes)

To date, TNM staging is the most widely used marker for classifying disease extent. However, both TNM staging and NPI are useful methods for prognostic indication. Generally, surgeons would employ the NPI along with TNM staging for clinical decision making.

1.5.3 Genetic / Hormonal classification

Recently, an adjunct classification method for breast cancer was introduced based on genomic technologies and the receptor and hormonal status. Cells have receptors on their surface, cytoplasmic structures and nucleus to which chemical messengers such as hormones bind, causing changes within the cell. Molecular markers like oestrogen receptor (ER), progesterone receptor (PR), and HER2/neu have proven useful in breast cancer prognostic classifications, diagnosis and treatment selection.

Characteristic patterns of gene expression were used to profile the ER and PR status of breast cancers, broadly separating tumours into ER+ and ER- tumours. The subsequent classification was then subdivided into 3 groups namely luminal, basal and HER2- positive types (Sorlie et al., 2001). Luminal breast cancers make up 40% of the ER positive cancers making them more responsive to hormonal treatments like tamoxifen and aromatase inhibitors with better prognosis (Sorlie et al., 2001). Luminal cancers are sub-divided into luminal A and luminal B cancers, with luminal A having the best 10 year survival rates of 90%.

ER-negative cancers include basal like tumours and HER2-positive tumours (Sorlie et al., 2001). Tumours outside this classification as they lack the receptors are referred to as

basal-like or triple negative cancers (De Brot et al., 2009). These tumours are more aggressive with poorer prognosis.

1.6 Diagnosis of breast cancer

Since the introduction of breast screening and increased breast awareness, the incidence of breast cancer has risen considerably as previously mentioned. Breast screening is the testing of otherwise healthy women for breast cancer in the hope of detecting a tumour at early stages to improve outcomes. Screening tests available are ultrasound, mammography, MRI and genetic screening. In Ireland, the government-funded BreastCheck programme was developed in 2000. BreastCheck is the Irish National Screening Breast Programme that provides breast screening mammograms for women aged 50 to 64 years on a two yearly basis. In the last 10 years, BreastCheck has detected over 4,000 breast cancers in over 300,000 women screened (BreastCheck, 2010). Breast screening was first introduced in the United States in 1990 and resulted in a decrease in annual mortality rates (Duffy et al., 2005).

Breast cancer is routinely diagnosed by triple assessment of clinical breast examination, mammography and fine needle aspiration cytology (FNAC). Mammography is used both as a screening and a diagnostic tool. Mammography is used to detect tumours located in areas of breast asymmetry, nipple discharge, skin retraction or axillary adenopathy. Mammography is not useful in the teenage population due to the high density of the breast but is indicated where a malignant process is suspected (Peer et al., 1996). Ultrasound has

been used since the early 1950s and is most helpful in the evaluation of dense breast tissue. It is useful in differentiating between cystic and solid lesions but has its shortfalls (Peer et al., 1996). Lesions less than 5 to 10 mm may not be visualised using ultrasound. Aspiration cytology involves the use of a fine needle and syringe to aspirate cells from a suspicious area. This is smeared on a glass slide, fixed and stained for cytological evaluation. Aspiration cytology also has its shortfalls, as specific histological diagnosis may be impossible because of the inability to maintain architectural patterns with aspiration (Hanby, 2005).

Other biopsy methods of diagnosis are also available including core biopsy using large bore needles. Core biopsy is more invasive than needle aspiration but has better accuracy. Excisional biopsy refers to the removal of all gross evidence of disease usually with a small rim of normal breast tissue, while incisional biopsy is done under local anaesthetic on lesions not amenable to excisional biopsy. Needle-guided biopsy precisely removes the lesion with little sacrifice of normal surrounding breast tissue. With needle core biopsy, a needle is introduced into the breast and directed towards the lesion with the aid of a mammogram film. Repeat mammography is performed with the needle secured to confirm the proximity of the needle to the suspicious area. The patient is then sent back to the operating room where the surgeon performs a biopsy of the area localised by the needle. The specimen is then x-rayed to confirm the presence of abnormal tissue.

1.7 Management of breast cancer

1.7.1 Surgery

The definitive management of breast cancer is surgery with or without chemotherapy or radiation. As mentioned previously, for early breast cancer (stage 0), with excellent prognosis, radical treatments are undertaken with an intention to cure. In the majority of cases, this treatment would involve either radiotherapy to the affected breast after conservative surgery (lumpectomy) or a mastectomy. Mastectomy is known to offer superior local control when there is extensive carcinoma *in situ* or multifocal invasion. Stage 1-3 disease has a progressively poorer prognosis and these patients usually undergo radical surgery with chemotherapy with or without radiation.

Radical surgery for breast cancer traditionally involved the excision of the whole breast and axillary lymph nodes. The extended radical mastectomy was a logical extension to the traditional radical mastectomy which achieved more lymphatic clearance by excision of the internal thoracic and supraclavicular nodes. These procedures were abandoned as morbidity increased with very little advantages in survival. The surgical approach now employed is total mastectomy and axillary clearance in which the pectoralis major is retained (Clarke et al., 2005, Tobias et al., 2006).

In the management of advanced breast cancer (stage 4), primary surgery is contraindicated if there is evidence of extensive skin involvement. In these circumstances, primary systemic therapy with chemotherapy or endocrine manipulations (or both) can bring

advanced local regional disease under control. This approach is also indicated when distant disease is found at presentation.

1.7.2 Adjuvant therapy

Adjuvant systemic therapy may considerably improve survival rates, but is associated with severe toxic side effects and is of major concern in patients with node-negative breast cancer where the pros and cons of adjuvant systemic therapy are always critically considered. There are three main classes of adjuvant therapies available; namely the hormone blocking agents like tamoxifen (which blocks oestrogen receptors) or the aromatase inhibitor anastrozole (which blocks the production of oestrogen); chemotherapy agents, with the most popular being cyclophosphamide, methotrexate and fluorouracil (CMF). Randomised trials of adjuvant therapy with CMF for 6 months have been shown to reduce death by 25% in premenopausal node- positive women (Drullinsky et al., 2010). By the same token, adjuvant tamoxifen given for 2-5 years has also been shown to reduce death from breast cancer in postmenopausal patients.

The use of adjuvant chemotherapy is based on predictions of tumour behaviour such as the Nottingham Prognostic Index, which gives a rating on how well treatment may work for an individual. Tumour size, histological grade and nodal status are also of importance in deciding on adjuvant chemotherapy. Patient age and menopausal status and hormone receptor status are also considered prior to hormonal manipulation and chemotherapy.

1.7.3 Endocrine therapy

In 1836, Sir Astley Cooper of St Bartholomew's Hospital in London observed that advanced breast cancer appeared to wax and wane during the course of a woman's

menstrual cycle. This led to speculations that there is a connection between the ovaries and the breast, unrelated to the nerve supply. The very first bilateral oophorectomy for treatment of advanced breast cancer was performed by Beatson in 1895 at Glasgow Cancer Hospital (Bland and Copeland, 1998). Hormonal manipulations are now commonly used in the management of women with advanced disease who have high oestrogen receptor expression in their tumours. Some of the manipulations include cessation of ovarian function by oophorectomy, radiation-induced ovarian ablation, down-regulation of the pituitary gland using luteinizing-hormone-releasing hormone (LHRH) analogues, or the use of anti-oestrogen drugs like tamoxifen in premenopausal women (Buzdar, 2009, Buzdar, 2003).

In post-menopausal women, hormonal manipulations are limited to the use of tamoxifen and aromatase inhibitors as previously mentioned. Tamoxifen is now the most successful and widely used endocrine therapy for the treatment of breast cancer. It is used as an adjuvant therapy either alone or following chemotherapy for early stage, hormone receptor-positive breast cancer in pre-and post menopausal women. Recent studies have shown the benefits of minimal surgery followed by tamoxifen to be best management for elderly patients with early disease (Rao et al., 2007). The anti-tumour effects of tamoxifen are believed to be mediated primarily through ER (Rivera-Guevara and Camacho, 2011).

Antagonism of ER is not the only adjuvant therapeutic option in ER-positive breast cancers. The aromatase enzyme is the rate limiting step in oestrogen biosynthesis that converts androstenedione to oestrone. In premenopausal women, the ovaries are the most important site of aromatase production. In the ovaries of premenopausal women, follicle-

stimulating hormone (FSH) stimulates the granulosa cell compartment to synthesise more aromatase, while luteinising hormone (LH) stimulates the theca cell compartment to synthesise the aromatase substrate androstenedione. Inhibition of ovarian aromatase in premenopausal women results in decreased oestradiol production which signals the pituitary to increase FSH and LH secretion (Bland and Copeland, 1998). In postmenopausal women, the precursor of oestrogen biosynthesis androstenedione is secreted by the adrenal gland. Thus, treatment options to reduce oestrogen levels include either blocking adrenal steroidogenesis and thus peripheral aromatase enzyme activity, or specifically blocking peripheral aromatase activity using aromatase inhibitor drugs. Aromatase inhibitors block oestrogen synthesis and are thus used for ER positive cancers in post menopausal women. The ATAC trial, a randomised double-blind study for treatment of post-menopausal women with early breast cancer with anastrozole alone or in combination with tamoxifen (Duffy et al., 2006), was the first to prove the benefit of adjuvant treatment of early oestrogen-receptor-positive breast cancer with an aromatase inhibitor. It was proved to be safe and effective over a long term (Cuzick et al., 2010).

1.7.4 Radiation treatment

Radiation therapy was first introduced into clinical medicine in the early 1900s soon after the discovery of x-rays in 1895 by Roentgen following the observation of the apparent biological damaging effects of x-rays. The use of radiotherapy is effective in the treatment of breast cancer and may be used as an alternative treatment to surgery. Combining conservative surgery with radiotherapy of the affected breast gives results comparable to radical surgery. Radiation is usually delivered as external beam radiotherapy; however brachytherapy (internal beam) is also available but less popular.

1.8 Breast cancer microenvironment

Metastatic breast cancer remains an incurable disease despite ongoing therapeutic advances. Thus, a greater understanding of tumour biology is required for the development of targeted therapies for advanced / metastatic breast cancer. As mentioned in section 1.1, the majority of breast cancers are epithelial carcinomas which originate in the milk ducts (Sainsbury et al., 2000). These cells are strongly influenced by their microenvironment. Thus the next section of the thesis will look at cells of the breast microenvironment in the hope of further understanding the process involved in epithelial carcinogenesis.

Epithelial cells, fibroblasts, myoepithelial cells and leucocytes are some of the cells that make up the breast microenvironment. (Figure 1.5) Epithelia are tissues composed of one or more layers of cells which line the cavities and surfaces of structures throughout the body. Many of the glands in the body are formed from epithelial tissue. Epithelial cells function in secretion, absorption, protection, transcellular transport, sensation detection, and selective permeability of cells. Myoepithelial cells on the other hand are found in glandular epithelium such as sweat glands, mammary glands and salivary glands. They are localized between luminal epithelial cells and the stroma, which ideally positions them to communicate with both compartments. (Figure 1.5) Myoepithelial cells have been implicated in maintaining epithelial cell polarization (Runswick et al., 2001).

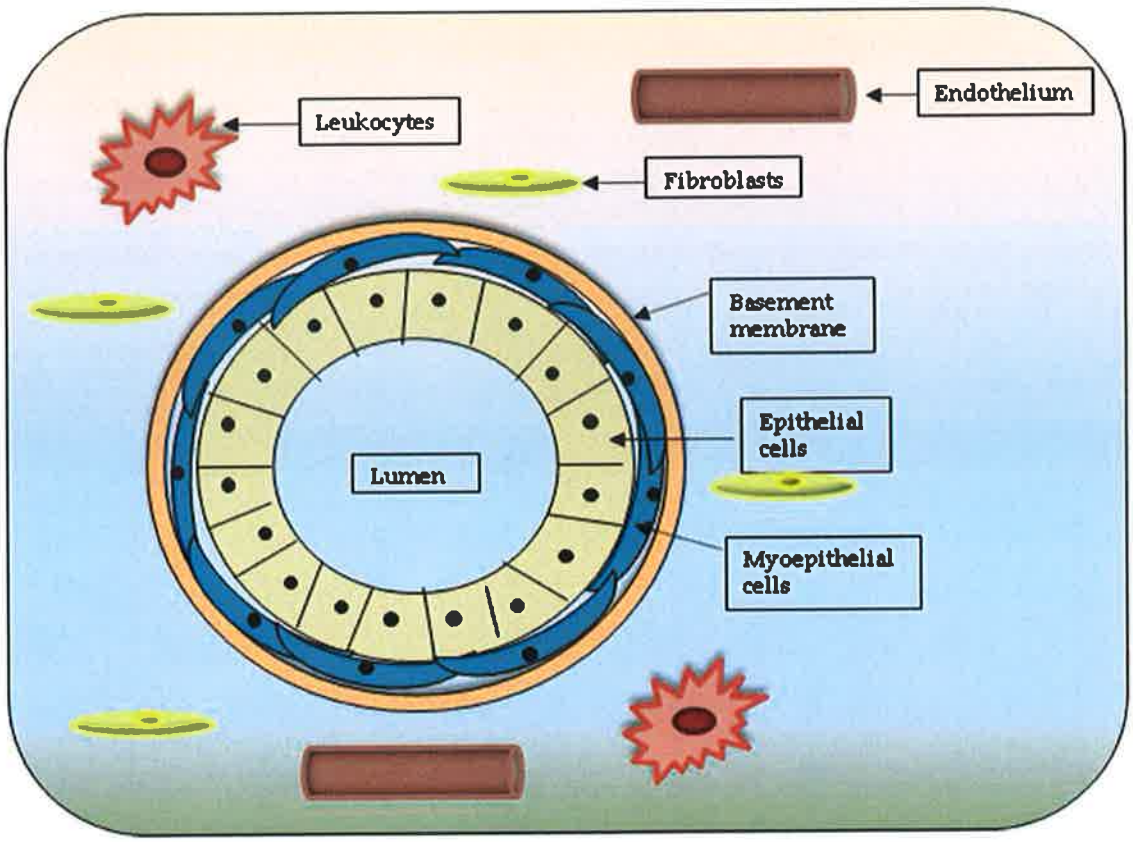


Figure 1.5: Diagram of a breast duct depicting location of epithelial and myoepithelial cells and other cells of the breast microenvironment.

Epithelial polarity is also created and maintained by adherent junctions which form between cells. Cell-cell junctions are traditionally divided into three functional categories namely adhering junctions (which mechanically hold cells together), impermeable junctions (which hold cells together and also seal the space between them), and communicating junctions (which mediate the passage of small molecules from one interacting cell to another). Tight junctions (TJ) are the main impermeable junctions, while gap junctions make up the majority of the communicating junctions. (Figure 1.6) In light

of accumulating evidence that TJs play a role in controlling breast cancer progression (Brennan et al., 2010), this thesis focuses mainly on TJs as will be outlined in due course.

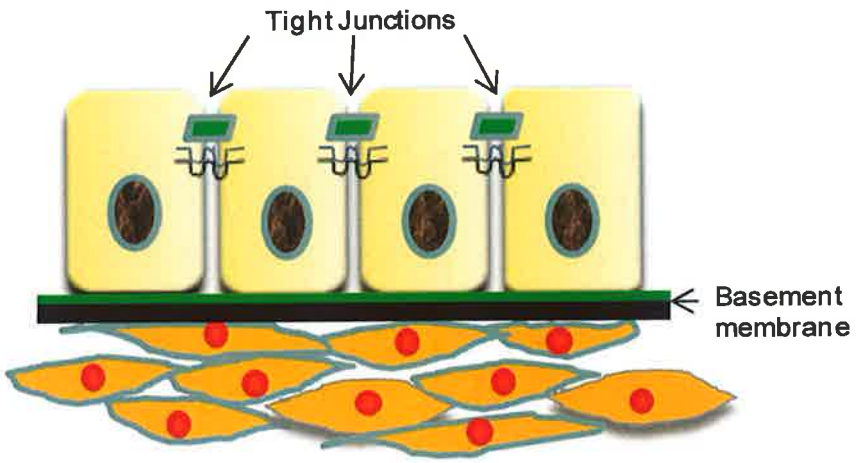


Figure 1.6: Epithelial cells showing the location of tight junctions (TJ). TJ are the apical-most component of intercellular junctional complexes. Together with adherens junctions, desmosomes and gap junctions, tight junctions mediate adhesion and communication between adjoining cells.

Cell-cell junctions are not the only type of multi-protein adhesion complex that regulate the polarity of epithelial sheets. A second type of junctional complex, cell-matrix adhesion complexes are foci of cellular attachment to the extracellular matrix. They regulate cell migration, cell survival, cell differentiation and cell proliferation (Lock et al., 2008). Interactions between cells and the extracellular matrix are critical to the development and function of multicellular organisms. Accordingly, cell-matrix adhesion molecules regulate a variety of functions including signal transduction, cell growth, differentiation, site-

specific gene expression, morphogenesis, immunologic function, cell motility, wound healing and inflammation (Okegawa et al., 2004). The extracellular matrix (ECM) is the defining feature of connective tissue. It usually provides structural support to the cells. Extracellular matrix includes the interstitial matrix and the basement membrane. Interstitial matrix is present between various cells within the intercellular spaces, while basement membranes are sheet-like depositions of ECM on which various epithelial cells rest. (Figure 1.6)

Although carcinoma progression is a multi-step process involving dysregulation in diverse processes including cell proliferation and survival, we are most interested in the contribution of altered adhesion to this process. Maintenance of apico–basal polarity in normal breast epithelial acini requires proper cell-cell and cell-extracellular matrix signalling; aberrations in which can disrupt tissue architecture and initiate tumour formation (Itoh et al., 2007). The next section will discuss the role of adhesion molecules in cancer cell and invasion and tumour progression.

1.9 Cancer progression and invasion

The focus of this thesis is on the potential role of adhesion proteins in cancer initiation and progression. Epithelial polarity is regulated by adhesion complexes at the cell-cell and cell-matrix interfaces. Tight junction proteins control the cellular processes that regulate polarity, differentiation and migration (Brennan et al., 2010), thus alterations in the adhesion properties of neoplastic cells could lead to the development and metastasis of

cancer. Loss of intercellular adhesion and the desquamation of cells can allow malignant cells to escape from their site of origin, degrade the extracellular matrix, and acquire a more motile and invasive phenotype, and finally, invade and metastasize (Okegawa et al., 2002, Okegawa et al., 2004). The invasion and metastasis of cancer is a complex process including changes in cell adhesion and motility. This allows tumour cells to invade and migrate through the extracellular matrix. The survival of normal epithelial cells critically depends on cell–cell and cell–matrix contact. Without these contacts epithelial cells die via the controlled process of apoptosis (Frisch et al., 1996) or by the process of anoikis, which is programmed cell death induced by anchorage-dependent cells detaching from the surrounding extracellular matrix (ECM) (Frisch and Francis, 1994).

Cancer cell invasion involves the breaching of tissue barriers by cancer cells, and in breast cancer; invasion at the molecular level requires the coordinated efforts of numerous processes within the cancer cell and its surroundings (Mc Sherry et al., 2007). The acquisition of invasive properties by cancer cells thus represents a crucial step in cancer progression (Thiery and Sastre-Garau, 1995).

1.10 Introduction to tight junctions

As mentioned already, this thesis focuses on the cell-cell junctions known as tight junctions, which are emerging as potentially important players in cancer initiation and progression (Brennan et al., 2010). Tight junctions are areas along the lateral membranes of adjacent epithelial cells where the membranes join together to form a virtually-impermeable barrier to fluid (Okegawa et al., 2004). They are however, selectively permeable to solutes in a highly-regulated fashion. Tight junctions are formed by a network of protein strands that continue around the entire circumference of each cell. Each of these strands is composed of transmembrane proteins that bind to similar proteins on adjacent cells, thereby sealing the space between their plasma membranes (Madara, 1998). The efficiency of tight junctions in preventing ion passage increases exponentially with the number of strands present. These associate with different peripheral membrane proteins located on the intracellular side of plasma membrane which anchor the strands to the actin cytoskeleton. Thus, tight junctions ultimately join together the cytoskeletons of adjacent cells. (Brennan et al., 2010, Martin and Jiang, 2001)

Tight junctions perform at least three vital roles within the cell. Firstly, they hold cells together; secondly, they prevent the free diffusion of integral membrane proteins between the apical and basolateral membranes of the cell (thus preserving the specialized functions of each surface). Finally, they regulate the passage of molecules and ions through the space between cells allowing movement via diffusion or active transport (Brandner, 2009, Kirschner et al., 2010).

Loss of cell-cell adhesion at tight junctions leads to loss of tissue architecture and promotes matrix remodelling (Singh et al., 2010). A growing body of evidence suggests that alterations in the adhesion properties of neoplastic cells play a pivotal role in the development and progression of cancer (Okegawa et al., 2004).

1.11 Junctional Adhesion Molecule–A

Junctional adhesion molecule-A (JAM-A) is one of the key transmembrane tight junctions proteins. It is a glycosylated 43 kDa protein expressed by many different cell types including epithelial cells, endothelial cells and leukocytes. (Figure 1.7)

The JAM family comprises a small subgroup within the immunoglobulin superfamily of proteins, and consists of five members (JAM-A, -B, -C, -4, -L). JAM-A interacts with the PDZ proteins AF6, Par-3, CASK, MUPP1, and ZO-1 (Ebnet et al., 2000, Severson et al., 2009b). The PDZ domain is made up of 80-90 amino acids and is located on many signalling proteins. In general, PDZ proteins are scaffold proteins which anchor transmembrane proteins like JAMs to the cytoskeleton while organising signalling comlexes (Harris and Lim, 2001). JAM-A is highly expressed at the tight junctions of mammary epithelium. It is known to function in the recruitment of specific protein complexes to sites of cell-cell adhesion, playing an important role in cell polarity and the assembly of multi-protein interacting networks (Ebnet et al., 2004).

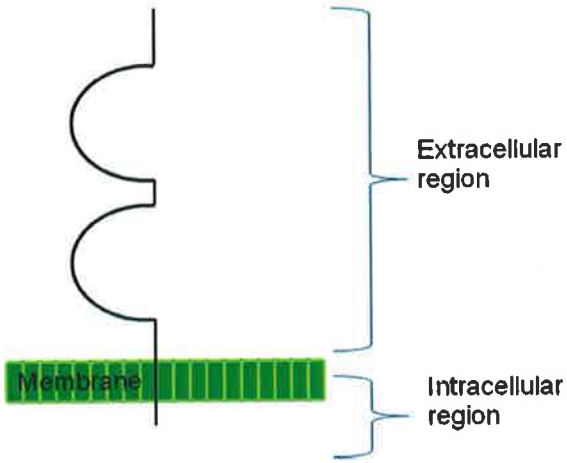


Figure 1.7: Structure of Junctional Adhesion Molecule A showing its extracellular region with two Ig-like domains, a single transmembrane domain, and a short intracellular tail with a PDZ binding motif through which JAM-A interacts with the PDZ proteins.

JAM-A proteins regulate many physiological adhesive processes including intercellular junction assembly, cell morphology, and leukocyte migration (Mandell et al., 2005, Martin-Padura et al., 1998). Since its discovery in 1998 (Martin-Padura et al., 1998) much has also come to be known about its involvement in pathophysiological states such as inflammation. In the colonic mucosa, JAM-A knockdown mice were observed to develop enhanced permeability and inflammation (Laukoetter et al., 2007). In cardiovascular disease, JAM-A controls proliferation and migration of smooth muscle cells (Azari et al., 2010). There is also accumulating evidence to suggest that JAM-A dysregulation could play a role in certain cancers including prostate and pancreatic islet cell cancers (Murakami et al., 2010, Wang et al., 2009, Fuse et al., 2007). However, little is known about the

contribution of JAM-A to breast cancer. To date, only 3 studies have been published regarding JAM-A and breast cancer. In 2008 and 2009, Naik *et al.* (Naik et al., 2008) and McSherry *et al.* (McSherry et al., 2009) respectively published data following work on tissue microarrays, breast cell lines and primary cultures. In 2010, Gotte *et al.* (Gotte et al., 2010) published work on micro RNA145, JAM-A and breast cancer invasiveness. These studies will be discussed in detail in the body of the thesis. Since loss of tissue architecture and cell polarity (as controlled by adhesion proteins including JAM-A) is a hallmark of breast cancer progression, it is reasonable to speculate that disruption of JAM-A proteins may be involved in disease progression. Thus we speculate that JAM-A may be an important regulator of (and target for) signals that regulate breast cancer cell migration and invasion.

1.12 Current knowledge regarding TJ proteins in other diseases.

Tight junctions as discussed above are essential for numerous cellular processes. Dysregulation of tight junctions and thus loss of polarity is known to lead to cancer dissemination, migration and progression (Escudero-Esparza et al., 2011, Martin et al., 2010, Martin et al., 2011). Alterations in JAM family members have been implicated in several cancers including pancreatic cancer (Murakami et al., 2010), glioma (Tenan et al., 2009), melanoma (Fuse et al., 2007), endometrial cancer (Koshiba et al., 2009), renal cell cancer (Gutwein et al., 2009), lung cancer (Santoso et al., 2005) and of course breast cancer of interest in our study. Despite, these cancer associations with JAM proteins, there

are other conditions in which JAMs have been implicated; as discussed in the next paragraph.

Inflammation is a physical condition and a biological response to injury within the body. JAM family members are known to play a major role in inflammation. Inflammatory bowel disease is a process involving inflammation of the bowel wall thus leading to disruption of the mucosal barriers. Tight junctions proteins including JAMs are known to regulate the passage of ions through epithelial barriers and to play a major role in paracellular permeability and neutrophilic transmigration (Kucharzik et al., 2001, Vetrano and Danese, 2009). A recent study showed that JAM-A (-/-) mice had increased intestinal permeability thus leading to the hypothesis that JAM-A is essential for maintaining intestinal integrity and permeability (Vetrano et al., 2008).

Acute pancreatitis involving acute inflammation of the pancreas leading to immense tissue injury and necrosis has also been linked to JAM. JAM family members including JAM-C are involved in leukocyte transendothelial migration and recruited to sites of inflammation like in cases of acute pancreatitis (Vonlaufen et al., 2006), acute meningitis (Del Maschio et al., 1999) and acute pulmonary inflammation (Aurrand-Lions et al., 2005). An inhibitory antibody to JAM-C was noted to block the influx of leucocytes within the pancreas in acute pancreatitis (Vonlaufen et al., 2006) and within the alveoli in acute pulmonary inflammation (Aurrand-Lions et al., 2005) thus preventing tissue injury.

JAM-A is present on the intimal surface of smooth muscle cells of the coronary arteries in patients with coronary artery disease, and plays a role in the formation of atherosclerotic plaques (Azari et al., 2010). Furthermore, several studies have shown that both JAM-A and

JAM-C are needed for the growth of the plaques and leukocyte adhesion respectively with subsequent migration of the inflamed smooth muscle cells on the vessel wall both on patient with peripheral vascular disease and on animal models of atherosclerosis (Azari et al., 2010, Shagdarsuren et al., 2009, Cavusoglu et al., 2007, Babinska et al., 2007). This series of events thus lead to atherosclerosis within vessels which predisposes to conditions like myocardial infarctions and cerebrovascular events.

JAM-C levels have been shown to be elevated on synovial fibroblasts of murine experimental arthritis; with antagonism of JAM-C reducing severity of inflammation (Palmer et al., 2007). JAM-C is also noted to be expressed on synovial fibroblasts of both osteoarthritis and rheumatoid arthritis patients, with JAM-C-dependent adhesion of myeloid cells to these fibroblasts (Rabquer et al., 2008). High levels of JAM-A have also been described in systemic sclerosis, an inflammatory condition affecting the skin compared to patients with normal dermal fibroblasts (Hou et al., 2009).

Taken together, it is clear that JAM family members play a crucial role in many disease processes. Since relatively little is known about its role in breast cancer, this makes it important protein to further investigate in breast cancer.

Chapter II

Material and Methods

2.1 Cell culture

2.1.1 Breast cell lines

All breast cell lines were of epithelial origin and derived from human subjects. In addition, primary breast cell cultures were also generated from breast cancer patients in Beaumont Hospital, following ethical approval from Beaumont Hospital research ethics committee and informed consent of patients.

The isogenic HMT-3522 breast cell line series of S1 and T4-2 cells (Briand and Lykkesfeldt, 2001) was kindly donated by Prof Mina Bissell of Lawrence Berkeley National Laboratory, CA, USA; while the Hs578T cell line series of Hs578T- P and Hs578T-i(8) (Hughes et al., 2008) were kindly donated by Dr. Susan McDonnell, University College Dublin, Ireland.

2.1.2 Cell culture environment

Cell manipulations were carried out in a laminar airflow hood. Cells were regularly tested for mycoplasma infection. All cells were grown as adherent monolayers on tissue culture plates and cultured to approximately 80% confluence before sub cultivation.

2.1.3 Culturing of cells from cryo-storage

Cell culture media for use were warmed to 37°C. Cells were retrieved from liquid nitrogen and thawed at 37°C. They were then pipetted quickly into a container with the pre-warmed media, re-suspended and centrifuged at 1200 x g for 3minutes to pellet the cells. The

supernatant containing DMSO (dimethylsulphoxide) was removed by pipetting, and cells were re-suspended in fresh cell culture media in a tissue culture flask (3mls / T25-cm^2, 10mls / T75-cm^2). Flasks were then kept in a humidified incubator at 37°C with 5% CO_2. Cells were monitored regularly by phase contrast microsopy.

2.1.4 Cell culture medium

A comprehensive list of supplements added to the media is listed in Appendix A. The HMT-3522 cell line series were grown in DMEM/Hams F12 medium while the Hs578T cell line series were grown in DMEM. The primary breast cells were grown in MEBM (Mammary Epithelium Basal Medium).

2.1.5 Sub culturing and counting of cells

Media was removed from flasks by pipetting. Cells were then washed with autoclaved PBS to remove traces of media with serum. 2ml of 0.05% trypsin-EDTA was added into the cells and incubated for approximately 5 minutes.

Cells were allowed to detach as monitored by light microscopy. When fully detached, in the case of the HMT-3522 cell line series and primary breast cultures, 2ml of 1X soybean trypsin inhibitor was added to the cells to deactivate the trypsin. The mixture was collected in a 15ml conical tube and centrifuged at 1200xg for 3 minutes. The supernatant was removed and the cell pellet re-suspended in a small volume of fresh media. In the case of the Hs578T cell line series, trypsin was inactivated using 3mls of serum-containing medium, and cells were spun down and resuspended as described above.

An aliquot of diluted single cell suspension was counted using a haemocytometer under a phase contrast microscope. Cells were counted in four quadrants and the mean value

multiplied by the dilution factor and then by 10^4, which accounts for the volume of the haemocytometer. Thus, number of cells per ml was estimated. The number of cells needed per flask was then seeded into a new tissue culture flask. Appendix A shows seeding densities per tissue culture dish/flask.

2.1.6 Preparation of cell stocks

Cell stocks were prepared by trypsinizing cells for 3 minutes. Once fully detached, trypsin inhibitor or serum-containing media was added to each flask, and the contents centrifuged at 1200xg for 3 minutes. The supernatant was removed, the cell pellet re-suspended in 3ml media containing 5% cryoprotective agent (DMSO) and 10% serum. One ml aliquots were quickly pipetted into cryovials and frozen using a cryo 1°C freezing container for 24 hours in the -80°C freezer. The vials were then removed and stored under liquid nitrogen.

2.1.7 Splitting and passaging of cells

HMT3522 S1 cells were split (subcultured) once their colonies formed rounded islands with smooth edges; usually 6-10 days after plating and at approximately 60% confluence. At passage numbers in excess of 34, S1 cells were plated directly onto plastic. If lower than passage 34, S1 cells were plated onto Vitrogen (collagen 1) -coated plates. HMT3522 T4-2 cells were split at 80% confluence and plated onto Vitrogen (collagen 1) -coated plastic surfaces.

2.1.8 Generation of primary breast cell cultures

Tumour and non-tumour primary cell cultures were generated from lumpectomy or mastectomy specimens obtained from breast cancer patients following ethical approval from Beaumont Hospital research ethics committee and informed consent of patients with

a positive diagnosis of breast cancer. Tumour tissue samples were cut from the centre of resected tumours by a pathologist, while the normal (non-tumour) tissue samples were cut away from the tumour margins. Samples were transported to the lab in a 10X antibiotic mix of 0.5mg/ml penicillin, 0.5mg/ml streptomycin and 0.10mg/ml neomycin. The samples were then minced under sterile conditions using previously prepared digestion media. (Appendix A).

The minced tissue was then collected in a tube containing 5mls of digestion medium and allowed to digest at 37°C on an orbital shaker at 200rpm speed for 2 hours. The digested tissue was left to settle in the tube for up to 5 minutes; the supernatant was collected and spun down at 1500 rpm for 3 minutes. The supernatant was discarded and cells were re-suspended in digestion medium and spun down at 1500 rpm for 3 minutes. This was repeated twice and the pellet was re-suspended in MEGM media (Lonza, Appendix A). The mixture was centrifuged at 1500 rpm for 3 minutes and then plated in MEGM medium onto a T25 flask.

2.2 Functional assays

2.2.1 MTT proliferation assay

MTT is a yellow water-soluble tetrazolium dye that is reduced by the mitochondria in viable cells to form a water-insoluble purple formazan product. The amount of formazan can be determined by solubilising it in DMSO and measuring the absorbance using spectrophotometry. A comparison of different cell lines over time is then used to estimate viability and proliferation.

Harvested cells were re-suspended to give $1x10^4$cells per ml. A 200µL volume of cell suspension was added to triplicate wells of five 96-well plates. On days 2 and 5 (HMT-3522 cell line series) or days 2 and 4 (Hs578T cells), the existing media was removed and replaced with 180 µL of fresh media. 20 µL volume of sterile-filtered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock of 5 mg/ml in PBS was added to each well and the plates incubated in the dark for four hours at 37°C. Following incubation, media was removed from all the wells. 200 µL of DMSO was added and mixed by pipette to solubilise the products. Absorbance was measured at 550 nm in a plate reader. Graphs were plotted to determine rates of cell proliferation over time, with increasing optical density being directly proportional to the number of viable cells.

2.2.2 Cyquant proliferation assay

The CyQUANT™ proliferation assay is a sensitive method used to determine the density of cells in culture by measuring the cellular DNA content. Cells are lysed by the addition

of CyQUANT-GR dye buffer which leads to fluorescence enhancement upon binding to cellular nucleic acids. The fluorescence is then measured directly by spectrophotometry.

Harvested cells were re-suspended to give $1x10^4$cells per ml. A 200µL volume of cell suspension was added to triplicate wells of five 96-well plates. On days 2 and 5 (HMT-3522 cell line series) or days 2 and 4 (Hs578T cells), each microplate was gently inverted and blotted onto paper to remove media. The plates were frozen and stored at -80°C. Plates were thawed at room temperature and 200µl per well of CYQUANT GR dye was added, mixed gently and incubated for five minutes at room temperature protected from light. Fluorescence emission was read on a plate reader at 480 nm maximum excitation and 520 nm maximum emissions. To evaluate the number of cells per fluorescence reading, a standard curve was set up by re-suspending frozen pellets with known amount of cells and plating serial dilutions ranging from 50 to 50,000 cells in a total volume of 200 µL of CYQUANT GR dye. Results were analysed by converting the fluorescence units to the number of cells using linear regression analysis.

2.2.3 Transepithelial Resistance Assay

Transepithelial resistance (TER) is a measure of tight junction integrity, or gate function; with higher TER values indicating an electrically-sealed monolayer of cells. S1 and T4-2 cells were trypsinised and 1 x 10^5 cells/ml suspensions were prepared. Polyester Transwell filters of 0.33 cm^2 area and 0.4 µm pore size were placed in sterile 24 well plates. Cells were seeded in 250µl of media in the apical chamber of the filter. In the basolateral (lower) compartment, 750µl of media only was added. Filters were then placed in the incubator at 37°C.

At the desired time points, cells were taken out of the incubator 5 minutes before reading and left in the fume hood. The EndOhm voltometer chamber was filled with 3ml of ethanol to sterilise 10 minutes before use. Ethanol was removed and rinsed twice with 3mls of dH_2O and then finally with 3mls of media. The chamber was then filled with 1ml of media. Each filter was lifted carefully from the 24 well plate and placed into the EndOhm to take readings.

2.2.4 Fluorosceinated (FITC)-dextran permeability assay

Transepithelial transport of the small marker molecule FITC-dextran was also used as a functional indicator of tight junction gate function; with tightly-sealed cells having a low transepithelial permeability across the paracellular pathway. Confluent S1 and T4-2 cells on $0.33cm^2$ polyester Transwell filters were washed twice with Hank's balanced salt solution containing calcium and magnesium (HBSS) and equilibrated at 37°C for 10 minutes in HBSS. 150 µL and 1ml of HBSS were added to the apical and basolateral chambers respectively. 15µL of FITC-dextran stock (final concentration of 1mg/ml) was added to the apical compartment at time zero. 50 µL samples were removed from the basolateral compartment into a 96 well plate at 30 minute intervals over two hours. During the two hour intervals, the plate was placed in an incubator at 37°C with gentle shaking. The apical compartment was replenished with the equivalent sampled volumes of 37°C HBSS at each time point. The fluorescence intensity was measured in a fluorescent plate reader at 485 nm excitation and 530 nm emission. Tracer concentrations were determined from a standard curve of FITC-dextran stock diluted in HBSS. Fluorescence was plotted against time to estimate the FITC-dextran flux rate in [FD-3] ng/well

2.2.5 Fence function assay

Tight junction fence function, or the ability to restrict intra-membranous diffusion of lipids, was assessed by microscopic observation of the distribution of a fluorescent lipid BODIPY-FL-C5 sphingomyelin. BODIPY-FL-C5 sphingomyelin and defatted BSA were used to prepare sphingomyelin/BSA complexes (5 nmol/ml) in P buffer (Appendix A). Polarized cells grown to confluence on Transwell filters as before were washed twice in pre chilled P buffer. 0.5 ml of 5 μM sphingomyelin/BSA complex was added to the apical compartment while 1 ml P buffer was added to the basal compartment and incubated for 10 minutes on ice. Filters were washed three times in cold P buffer. One filter was processed immediately for confocal microscopy while the other filter was washed extensively in P buffer and left on ice in P buffer containing BSA for one hour before fixing and staining for confocal microscopy.

2.2.6 3-Dimensional (3D) cell culture assay (On top cultures)

To determine the 3-dimensional polarization of cells in response to various conditions, cells were grown under 3-dimensional conditions using an extracellular matrix gel essentially as described (Debnath et al., 2003).

Growth factor reduced Matrigel™ was thawed on ice and 40 μL was used to coat each chamber of an eight well chamber slide. The Matrigel™ was allowed to solidify for 20 minutes at 37°C. Cells were harvested and re-suspended at $2.5x10^4$ cells/ml in complete media. 200 μL volume of cell suspension and 200 μL of 10% Matrigel™ in media were added to the chambers. Cells were fed with 5% Matrigel™ every four days. 3D cultures were allowed to form spheroids over a 14 day period. After 14 days in culture, cells were fixed, stained and analysed as described in Section 2.3.2

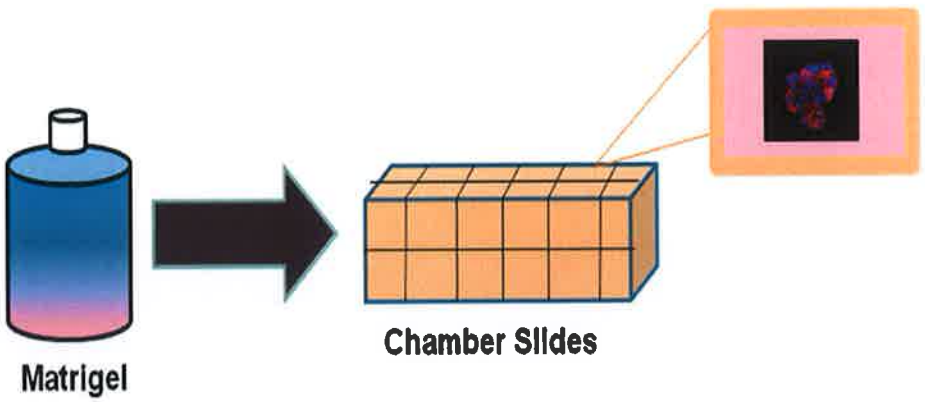


Figure 2.1: Schematic representation of 3D cell cultures on Matrigel™

Cells are grown in a specialised 8 well chamber slides embedded in Matrigel™ for 14 days prior to harvesting. Cells grow in clusters as shown in picture forming 3-dimensional structures. Matrigel™ must be thawed in ice to prevent solidification at room temperature.

2.2.7 Scratch wounding migration assay

The migratory capacities of cells in response to various conditions were assessed using scratch wound migration assays. Cells were grown to confluence in triplicate wells of a 24-well plate. Media was removed and a scratch wound made in the confluent sheet of cells using a sterile p10 pipette tip. The wounded monolayers were washed in PBS and serum-free media was added to the cells and incubated at 37°C. At time points 0, 2, 4, 6, 8, 24

hours, cells were photographed at the same point of the well. Scion Image software was used to measure closure of the wound over time by averaging six individual measurements of wound size for each wound at each time point. For inhibitory antibody assays, scratches were performed on cell monolayers pre-incubated for two hours with 5 μg/ml mouse anti-human JAM-A antibody (J104) or isotype-matched IgG1 as control.

2.2.8 ELISA assay

A 96-well microplate was coated with 100μl per well of 0.5mg/ml of capture anti-JAM-A antibody overnight at 4°C. The next day each well was washed 3X with 300μl of wash buffer (0.05% Tween 20 in TBS, pH 7.2-7.4). Wells were blocked by adding 300μl of blocking buffer (2% BSA in wash buffer) and incubating at room temperature for 1 hour. Wells were washed with 300μl of wash buffer three times. 100 μl of standards or sample in sample dilution buffer (0.1% BSA in wash buffer) was added to each well and incubated for 2 hours. 100μl of 1 μg/ml biotinylated rabbit anti JAM-A polyclonal antibody was added to each well and incubated for 1 hour. 100μl of streptavidin-HRP was added to each well and incubated for 1 hour. 200μl of substrate solution (0.1 mg/ml of Tetremethylbenzine in 0.05 M Na_2HPO_4 and 0.025M acidic acid, 0.0023% H_2O_2) was added and incubated for 20 minutes, before adding 50μl stop solution (2N H_2SO_4) to each well. The optical density was determined by reading the microplate at 450nm.

Pre-operative bloods were collected from breast cancer patients following ethical approval from Beaumont Hospital research ethics committee and informed consent of patients with a positive diagnosis of breast cancer. Bloods were collected from patients on admission for surgical procedure. Following collection, bloods were centrifuged at 4°C, 1500 rpm for 10 mins and the supernatant / serum aliquoted and stored at -80°C for later use.

2.3 Immunofluorescence

2.3.1 Immunofluorescence staining of 2-dimensional (2D) cultures

Cells were seeded at different concentrations (2 x 10^4 cells/well - 5 x 10^4 cells/well) on coverslips which had been previously sterilised in 70% ethanol and washed with sterile PBS followed by culture medium. Confluent cells were fixed in either 100% ice-cold ethanol for 20 minutes at -20°C or with 3.7% paraformaldehyde (pH 7.4) for 20 minutes at room temperature. Cells were washed three times in PBS. Cells fixed with paraformaldehyde were permeabilised with 0.5% Triton X-100 for 30 minutes at room temperature. Cells were washed three times in PBS, then blocked in 5% normal goat serum (in PBS) for one hour at room temperature or overnight at 4°C. Coverslips were transferred onto a humidity chamber where they were washed three times in PBS. Primary antibody in blocking buffer was applied for one hour at room temperature (Appendix A - Antibody concentrations). Cells were washed three times in PBS, and secondary antibody applied for one hour at room temperature. To stain F-actin, Alexa Fluor Phalloidin (0.3U) was incorporated into the secondary antibody solution. Cells were washed three times in PBS and then incubated at room temperature for 10 minutes with 4', 6-diamidino-2-phenyl-indole (DAPI; 1mg/ml) in PBS. Cells were washed three times in PBS and coverslips mounted onto slides with p-phenylenediamine hydrochloride: PBS: glycerol (Appendix A). Coverslips were sealed with nail polish and examined on a Zeiss LSM 510 Meta confocal microscope.

2.3.2 Immunofluorescence staining of 3-dimensional (3D) cultures

Media was aspirated from each well of the chamber slide and cells immediately fixed with 3.7% paraformaldehyde for 20 minutes at room temperature. Wells were permeabilised with PBS containing 0.5% Triton X-100 for 10 minutes at 4°C. This was followed by a wash with PBS/Glycine (Appendix A) for 10 minutes at room temperature. Wells were incubated with 200 µl/well of IF buffer (Appendix A) plus 10% normal goat serum for 60 minutes at room temperature, and incubated with a 1:200 dilution of primary antibody in block solution overnight at 4°C. Wells were washed three times for 20 minutes each with IF buffer at room temperature with gentle rocking. This was followed by incubation with fluorescent secondary antibody in IF buffer containing 10% goat serum for 15 minutes at room temperature. Wells were washed once for 20 minutes at room temperature and subsequently with PBS X3 for 10 minutes each, and then incubated with 0.5 ng/ml DAPI for 15 minutes at room temperature. Following one wash with PBS for five minutes at room temperature, slides were mounted in anti-fade reagent p-phenylenediamine hydrochloride: PBS: glycerol (Appendix A) and allowed to dry overnight at room temperature. Chamber slides were examined using an LSM510 Confocal Microscope.

2.4 Protein biochemistry

2.4.1 Whole-cell lysate preparation

Confluent cells on dishes were washed 1X in PBS for 10 minutes, then subsequently washed 2X in ice-cold PBS at 4°C for 10 minutes each, and scraped in ice-cold Relax

buffer (Appendix A) containing 1% Triton X-100, protease inhibitors and phosphatase I and II inhibitor cocktails. Lysates were dounced 20X and centrifuged at 1500xg at 4°C for five minutes. Supernatants were quantified for protein content with a bicinchoninic acid assay (BCA).

2.4.3 Protein quantification

A standard curve was obtained by serially diluting 5 mg/ml bovine serum albumin in lysis buffer. 10 µL of each standard curve dilution and 10 µL of the unknown sample were added to different wells of a 96 well plate. BCA reagents A and B were mixed 1:50 according to be manufacturer's instructions. 150 µL of the working solution was added to each well. This was incubated for 30 minutes at 37°C and read at 550 nm on a plate reader. Linear regression analysis of the BSA standard curve was used to calculate the unknown protein concentrations in each sample.

Samples	*Concentration of BSA*
1	5mg/ml
2	2.5mg/ml
3	1.25mg/ml
4	0.625mg/ml
5	0.3125mg/ml
6	0.156mg/ml
7	0.078mg/ml
8	0mg/ml

Table 2.1: Concentration of standards. Standards were used to calculate the unknown proteins concentrations. Samples were prepared in lysis buffer.

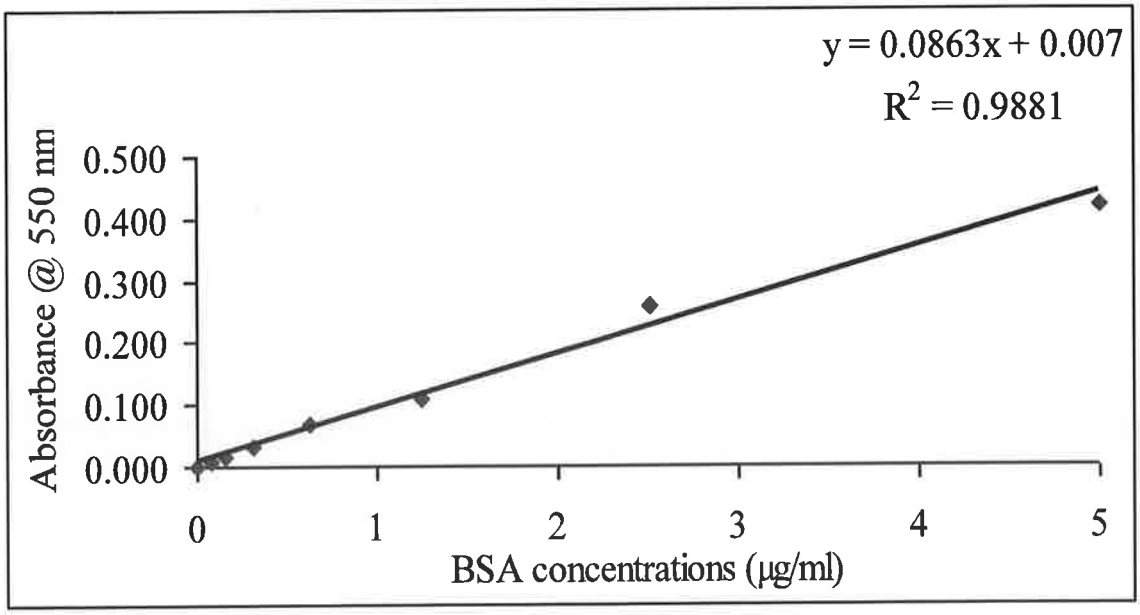


Table 2.2: Example of a standard curve derived using the BCA protein assay

2.4.4 Immunoprecipitation

Cells were washed 1X with warm PBS and placed at 4°C for 10 minutes. Working on ice for all subsequent steps, cells were washed 2X for 10 minutes with ice-cold PBS (placed at 4°C during incubations). Cells were scraped in ice-cold Relax buffer (Appendix A) containing 1% Triton X-100, protease inhibitors and phosphatase I and II inhibitor cocktails. Cells were then dounced 20 times with a dounce homogenizer and the nuclei removed by low speed centrifugation (1500 x g for five minutes at 4°C). 50µl of Protein G sepharose was used for each sample. Prior to addition of the sample to the sepharose beads, the Protein G sepharose beads were washed x 3 with lysis buffer to remove preservatives and spun at 3000 rpm for 30 seconds each. Post-nuclear lysates were incubated for one hour at 4°C with 50µl of Protein G sepharose. The lysate was incubated for 1 hour at 4°C with 5 µg antibody or isotype-matched control IgG antibody after the pre clear was spun gently at 4°C for one hour. The bound antibody was retrieved with 20 - 50 µL Protein A/G sepharose for three hours at 4°C. The beads were washed 4X with lysis buffer for 10 minutes each. 2X Laemelli sample buffer was added to the sample and boiled. Electrophoresis and western blot analysis was subsequently performed as per normal protocols.

2.4.5 SDS-PAGE and western blotting analysis

Different gel concentrations were made up (Appendix A) and equivalent concentrations of proteins were loaded in each lane. Gels were run at 40 milliamps constant current per gel and transferred onto a 0.2µm pore nitrocellulose membrane in cold transfer buffer (Appendix A) at a constant voltage of 100V for one hour. Larger proteins (greater than

150kDa in weight) had 0.05% SDS added to the transfer buffer and were transferred at a rate of 30V constant current overnight at 4°C.

Confirmation of protein transfer was done using Ponceau S (Appendix A). Membranes were blocked in 5% Milk in 1X TBS-Tween for 60 minutes at room temperature. Membranes were subsequently incubated with the primary antibody (Appendix A for concentrations) overnight at 4°C. After the overnight incubation, membranes were washed x3 for 10 minutes with TBS-T and followed by 60 minutes incubation with the corresponding HRP (Horseradish Peroxidase) -conjugated secondary antibody in 5% milk. Membranes were washed x3 for 10 minutes each with TBS-T. Protein bands were detected by addition of a chemiluminescent substrate and the light emitted during the enzyme-catalysed decomposition reaction was captured by exposure to film for specific times. The sizes of protein bands detected were estimated relative to the molecular weight markers on the membrane.

2.5 Statistical analysis

Using Microsoft Excel, two tailed unpaired student's t-tests were performed on raw data from multiple repeat experiments to indicate if two sets of measurements were different. A p value of less than 0.05 was considered statistically significant.

Chapter III

Characterisation of breast cancer cell lines and primary breast cultures for the study of Junctional Adhesion Molecule-A in breast cancer

3.1 Introduction

As most human cancers are of epithelial origin, epithelial cell models form the basis for the majority of basic cancer biology studies. Malignant transformation in culture is a useful model for studying the early events in human carcinogenesis. However, human primary cells senesce and die after short periods in culture, and, in contrast to most rodent cells, do not undergo 'spontaneous' malignant transformation (Gregoire et al., 2001, Zhao et al., 2010).

The molecular mechanisms that underlie programmed cell death which occurs in most animal tissues during their development are still incompletely understood. Thus, the majority of breast cancer cell lines used for mechanistic studies of breast cancer have been chemically or virally immortalised to allow for controlled experimental studies exploring the mechanisms underlying cancer cell growth and proliferation, invasion and migration. By the same token, human primary cells have their use in research and are good models to study the behaviour and characteristics of cancer as will be discussed later in this chapter.

In the past, cancer research was based mainly on the identification of genetic alterations associated with cancer pathogenesis and less on the molecular determinants of carcinogenesis and the tumour microenvironment. Advances in cancer research often now focus on identifying the cellular and molecular determinants that support tumour growth (Amerasekera et al., 2004, Fidler, 2003). Most cancers (especially breast cancers) are heterogeneous in nature and structurally complex. Thus, an understanding of the molecular biology of the tumour microenvironment is important for our understanding of cancer progression.

A considerable part of our knowledge on breast cancers is based on *in vivo* and *in vitro* studies performed with breast cancer cell lines which provide an unlimited source of homogenous self-replicating material, free of contaminating stromal cells. *In vitro* cultures of established breast cancer cell lines are widely used for preclinical evaluation of disease progress. Most of these breast cancer cell lines originate from pleural effusions of metastatic breast cancer. Pleural effusions provide viable tumour cells with little contamination by fibroblasts or other tumour stromal cells (Lacroix and Leclercq, 2004), allowing the generation of permanent/immortalised breast cancer cell lines which have greatly advanced our understanding of the mechanisms underlying tumour initiation and evolution. Yet, despite the considerable role that cell lines continue to play in most aspects of cancer biology, due to the fact that they contain no stromal cells and lack three-dimensional structure, they are often viewed as non-representative models of the tumours from which they are derived (Lacroix and Leclercq, 2004). A study by Kim *et al.* suggested the possibility of merging human and animal models in the form of heterotransplanted tissues, implanted either heterotopically (subcutaneous) or orthotopically (mammary fad pad) into mice to help overcome these issues (Kim et al., 2004).

In the next section, *in vitro* and *ex vivo* models are defined and compared in terms of their value for studying the mechanisms of breast cancer initiation and progression.

3.1.1 Cell lines as *in vitro* models for studying breast cancer

Many experiments in cellular biology are conducted outside the organisms and such experimental results are annotated as *in vitro*. As test conditions do not always correspond to the conditions inside of the organism, false results may arise. Nonetheless, *in vitro*

studies are still important and valuable in research, as complex hormonal and neuronal influences which exist *in vivo* are eliminated. However, as a model, *in vitro* studies do not reproduce the complexity of the whole organism. Consequently, *in vitro* studies are usually followed by *in vivo* studies for validation.

In vitro research focuses on organs, tissues, cells, cellular components, proteins, and biomolecules. *In vitro* research is better suited than *in vivo* research for deducing biological mechanisms of action, and is highly productive and cheaper than its *in vivo* counterpart. Cell lines are common in laboratory research as they are easy to handle and exhibit a relatively high degree of homogeneity. They are also easy to source and are easily replaced from frozen stocks. However, cell lines are prone to genotypic and phenotypic drift during their continual culture (Burdall et al., 2003). This genotypic drift was best modelled by Tsuji *et al.,* using 35 primary breast tumours and 24 breast cancer cell lines showing gains and losses of several DNA copy number aberrations between the cell lines and their corresponding primary tumours; indicating that cell lines do not always represent the genotypes of parental tumour tissue specimens (Tsuji et al., 2010).

For our study, we employed two isogenic cell line series, the HMT-3522 and Hs578T cell line series. Isogenic cultures are derived from genetically identical organisms which have been environmentally differentiated into different phenotypes. They represent excellent model systems for studying breast tumour progression, recapitulating the stages in carcinogenesis in a more complex fashion than that permitted using single cell lines.

The isogenic HMT-3522 cell line series mimics what happens during carcinogenesis *in vivo* from cancer initiation to cancer progression, making them a very good model for

studying signalling pathways involved in breast cancer progression. This is further described in the next section.

3.1.1.1 HMT-3522 cell line series

The HMT-3522 cell line was derived from a fibrocystic lesion from the breast of a woman which propagated as a near-diploid, non-tumorigenic sub-line in serum free medium. These cells progressively changed after subculture in various conditions, acquiring p53 mutations, *MYC* amplification and EGF-independence accompanied by tumorigenicity in nude mice and the over-expression of EGFR, TGF alpha and c-erbB-2 (Lacroix and Leclercq, 2004). Thus from a non-malignant and non-tumorigenic origin, these cells were environmentally-driven to progressively become tumorigenic (Briand and Lykkesfeldt, 2001).

In more detail, the breast tissue used to generate the HMT-3522 series was explanted onto a collagen IV coating in a serum-free, chemically defined medium. After 34 subculture passages the cell line remained non-tumorigenic, and was designated as the S1 subline. In the early passages, the growth of this subline was dependent on the presence of EGF, transferrin, hydrocortisone, and insulin in the medium. In later passages (>70), only EGF was required in the medium to obtain optimal growth. Cells of passage 118 were exposed to EGF-free medium in an attempt to select/adapt them to grow independently of EGF. After 2–3 months, a subline, S2, was established, and after another 120 passages of the S2 subline, tumours appeared after subcutaneous inoculation of the cells in nude mice (Figure 3.1). After two *in vitro–in vivo* passages per subline, the subline designated T4-2 was cultured from an S2-derived tumour in a nude mouse. T4-2 cells were highly tumorigenic with progressive growth into tumours of 1cm in diameter within 3 weeks. Nonetheless,

after >400 passages the S1 cell line remained non-tumorigenic (Briand and Lykkesfeldt, 2001). Thus the HMT-3522 cell lines series encompasses phenotypically non-tumorigenic S1 cells and tumorigenic T4-2 cells from the same genetic background.

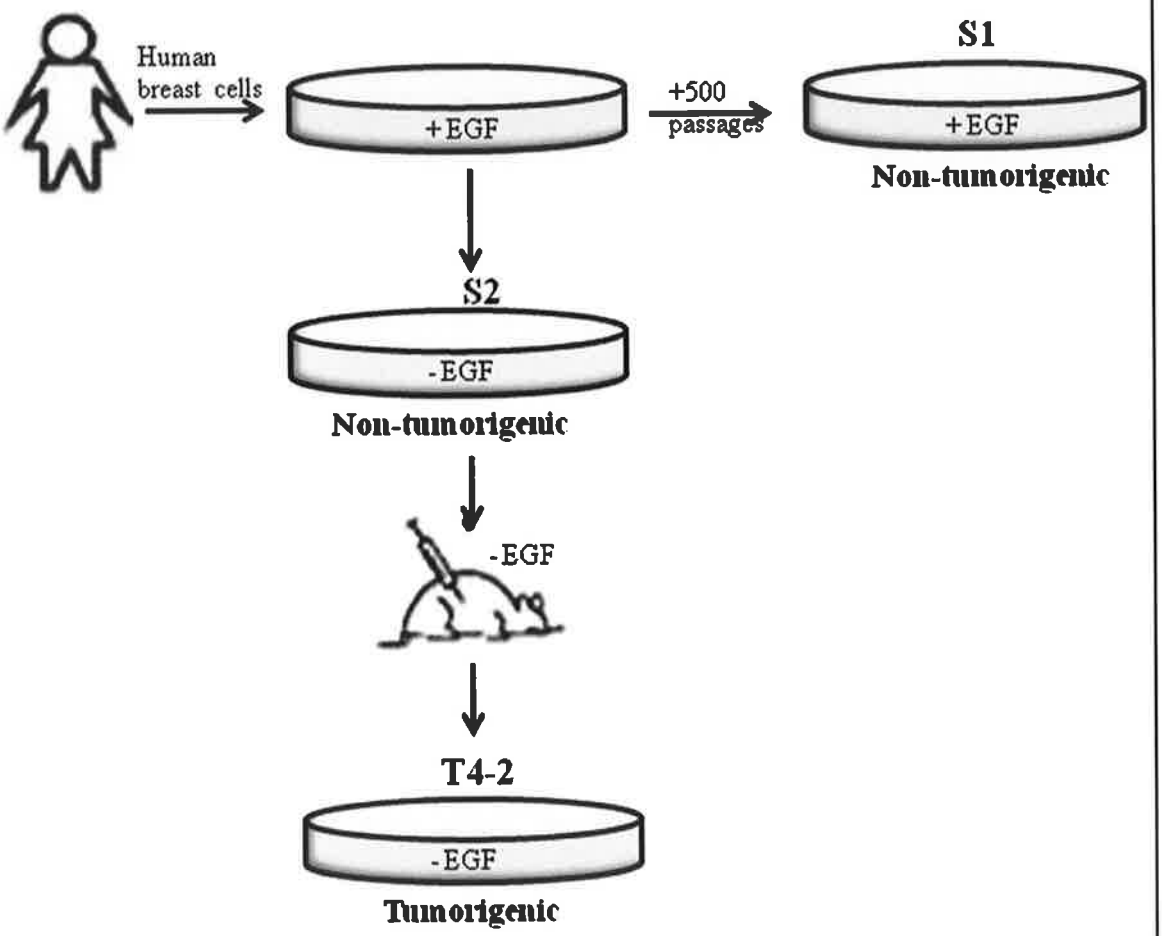


Figure 3.1: Schematic overview outlining the generation of the HMT-3522 cell line series (Briand et al., 1987).

The HMT-3522 cell line series comprises the phenotypically non-tumorigenic S1 cells and the phenotypically tumorigenic T4-2 cells; the latter growing independently of EGF.

It has been suggested that the phenotypic evolution of the HMT-3522 cell line series corresponds well with four well-described stages in breast cancer progression: benign disease, premalignant changes, *in situ* carcinoma, and carcinoma (Table 3.1). For our purposes, this spontaneously immortalised cell line series constituted a very good study model, representing both the early (stage 2) and late (stage 4) stages of tumour progression. We thus used this model to try and identify the role of junctional adhesion molecule-A (JAM-A) in each stage.

Stages *In vitro* in nude mice of breast cancer tumorigenicity
1. HMT-3522 Non tumorigenic Benign (Passage 1–40)
2. HMT-3522 S1 Non tumorigenic Benign (Passage >40) (Premalignant change)
3. HMT-3522 S2 tumorigenic *In situ* (Passage >238) (Stationary tumours)
4. HMT-3522 T4-2 tumorigenic Carcinoma (Invasive cancers)

Table 3.1: Comparison between stages in the *in vitro* model and human breast cancer (Briand and Lykkesfeldt, 2001). The HMT-3522 cells make for a very good model for study as both early stage and late stage cancer progression is represented within the model as represented on this table.

3.1.1.2 Hs578T cell line series

In the context of isogenic breast cancer models, the HMT-3522 cell line series offers many advantages for comparison studies. However, several other isogenic models have been described for breast cancer studies, and another isogenic cell line series of interest to this thesis is the Hs578T cell line series described below.

Hs578T cells provide other unique features which are useful in the study of breast disease. Hs578T cells were originally derived from a carcinosarcoma of epithelial origin (Hackett et al., 1977). The Hs578T line had a mixed polygonal morphology initially, but a stellate cell type was selected out during passaging by selective cloning. Isogenic subclones were isolated from the Hs578T cell lines using sequential passaging.

An invasive subclone annotated Hs578Ts(i)8 was isolated from the parental cell line Hs578T Parental (Hs578T-P) using a novel approach involving Matrigel™-coated invasion chambers (Hughes et al., 2008). After a 5 hour period, rapidly-invading cells were collected from the bottom of the invasion chambers by trypsinisation and propogated separately (Figure 3.3).

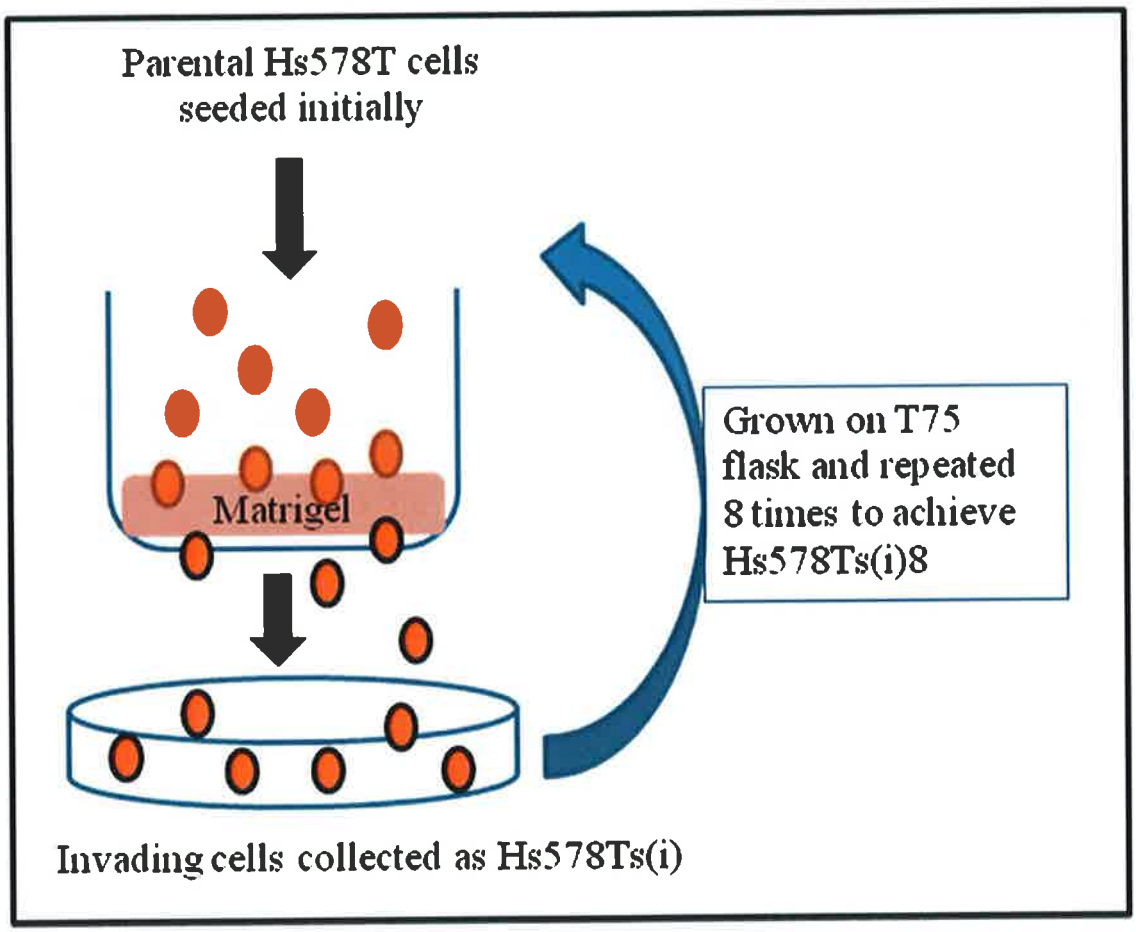


Figure 3.3: Schematic diagram outlining the generation of the invasive variant Hs578Ts(i)8 from Hs578T parental cells.

Parental Hs578T cells were seeded in 6-well invasion chambers coated with Matrigel™ and incubated for 5 hours. Following this, the invading cells (Hs578Ts(i)) were collected and seeded in a 24-well plate and progressively scaled up to a 75cm^2 flask. Once confluent, cells were again passed through invasion chambers, and the invasive cells selected out for subculture. This process was repeated 8 times to achieve the Hs578Ts(i)8 cells.

The selected invasive cells were grown to confluence and then subjected to a further 7 rounds of invasion assay. In each case the invading cells were collected and cultured to confluence (Hughes et al., 2008). After 8 rounds, the invasive-variant cells were noted to be 3-fold more invasive than the parental cell lines. Importantly, parental cells were also noted to be non-tumorigenic in nude mice, while those cultured after 8 rounds of invasion assays (Hs578Ts(i)8 cells) formed tumours in nude mice *in vivo*. Thus the HS578T cell line series model breast cancer progression from non-tumorigenic cells (Hs578T parental) to tumorigenic cells (Hs578Ts(i)8), thus illustrating its usefulness in studying and understanding breast cancer disease. In this thesis, we employed this breast cancer cell line model in studying the role of JAM in breast cancer progression

3.1.2 Primary breast cells as *in vitro* models for studying breast cancer

In addition to the cell lines discussed above, this thesis also employed the use of primary cell cultures in an effort to better recapitulate the complex biology of human breast cancer. Primary cultures are freshly isolated directly from tissues, and usually represent a heterogeneous population containing >1 constituent cell types from the parental tissue, and the expression of tissue-specific properties. Unlike established cell lines that have the ability to proliferate indefinitely, most primary cultures have a limited lifespan, and after several sub-cultures will senesce and stop dividing (Stampfer and Yaswen, 2000, Li et al., 2007). Only a minority of primary cultures, some derived from very aggressive tumours, spontaneously transform to become an immortal cell line. Established cell lines of this kind show many alterations from their initial primary cultures, including morphological changes and chromosomal variation (McCallum and Lowther, 1996). So although there is no ideal *in vitro* system which recapitulates all the complexities of cancer, primary breast cells provide a valuable accompaniment and/or alternative to using cell lines. A particular

advantage is that primary breast cells are accompanied by a detailed pathology linked to individual patients, thus allowing for better characterisation of the model. However although primary cultures are an excellent model with which to study the mechanisms involved in breast tumour progression, they do have some disadvantages. They mostly have a slow population doubling time and a limited lifespan, thus limiting their use in experiments requiring large number of cells. The possibility that primary tumour cultures may be infiltrated by normal epithelial cells and behave differently in culture compared to *in vivo* is also an issue that could compromise data interpretation. Nonetheless, primary cultures are still an excellent model which help recreate the *in vivo* complexities of cancer more closely than cell lines.

3.1.3 Tight junctions, JAM-A and cancer

Tight junctions play a very important role in the regulation of cell-cell adhesion and are important in maintenance of cellular polarity. Recent studies have suggested tight junctions as a potential barrier that must be overcome for successful tumour metastasis to occur (Martin and Jiang, 2009, Lee and Luk, 2010)

The major integral membrane constituents of tight junctions are claudin proteins, the junctional adhesion molecule (JAM) family and occludin. The claudin family are tight junction proteins expressed in both endothelial and epithelial cells which regulate the efflux of molecules through the paracellular space. Claudins are made up of over 20 different members, all with different expressions patterns depending on cell type. The exact function of claudin proteins within TJs is still unclear, but they appear to be important in TJ formation and function (Rangel et al., 2003). Occludin is a transmembrane protein with two extracellular loops and a long cytoplasmic tail containing several protein-

binding domains. Occludin is one of the major players in the formation of tight junctions and promotes cellular adhesion via a homophilic mechanism (Blaschuk et al., 2002).

Of particular interest in this thesis is the JAM family of TJ proteins, consisting of 5 members namely JAM-A, -B, -C, -4 and JAM-L. As mentioned in chapter 1, they contain an extracellular region, a single transmembrane domain and a short intracellular tail with a PDZ binding motif via which JAM-A interacts with the PDZ proteins AF6, Par-3, CASK, MUPP1, and ZO-1 (Ebnet et al., 2000). JAM-A was the first member of the JAM proteins to be identified (Martin-Padura et al., 1998) and is expressed on epithelial and endothelial cells. It has also been located in circulating platelets, monocytes, lymphocytes, neutrophils, dendritic cells and spermatozoa (Mandell and Parkos, 2005, Ueki et al., 2008).

Although much recent progress has been made in understanding its role in normal physiology, JAM-A is still a relatively new protein and as such not much is known about its role in pathophysiological states. However, several studies on inflammatory bowel disease have identified an important role for JAM-A in controlling mucosal homeostasis via regulation of cell integrity and permeability (Vetrano and Danese, 2009). JAM-A has also been implicated in cardiovascular disease via its control of proliferation and migration of smooth muscle cells and the initiation and growth of atherosclerotic plaques (Azari et al., 2010) and in the development of hypertension (Ong et al., 2009). A potential involvement for JAM-A in cancer is emerging and has been studied in prostate and pancreatic cancers (Shah et al., 2009).

Surprisingly however, the role of JAM-A in breast cancer is still a novel topic. Recently, a study by Naik *et al.* suggested that JAM-A was a negative regulator of cell migration and invasion in breast cancer (Naik et al., 2008). This was disputed by another recent study

showing that high JAM-A expression associated with poor prognosis in invasive breast cancer patients, possibly due to pro-migratory effects exerted by JAM-A (McSherry et al., 2009).

Given that loss of tissue architecture and disruption of cell polarity are prerequisites for breast cancer cell invasion, it is thus a strong possibility that disruption of tight junction proteins like JAM-A may play a role in breast cancer initiation and progression. We therefore aimed to investigate the differences between JAM-A expression and function in the non-tumorigenic versus tumorigenic variants of our isogenic cell line series HMT-3522 and Hs578T cells, in addition to carrying out selected investigations in primary breast cultures.

3.2 Specific aims

As mentioned in the section above, relatively little is known about JAM-A in breast cancer. Therefore the broad aim of this chapter was to examine the suitability of our cell line models to investigate the relationship between JAM-A and functional behaviours relevant to tumour progression.

Our first aim was to compare the protein expression and cellular localisation of JAM-A in the non-tumorigenic and tumorigenic variants of the HMT-3522 and Hs578T cell line series.

Our second aim was to compare tight junction integrity between the non-tumorigenic and tumorigenic variants of the HMT-3522 cell line.

Our final aim was to compare JAM-A protein levels between *ex vivo* human primary breast cells isolated from tumour and adjacent non-tumour tissue, and to correlate JAM-A expression with clinicopathological parameters. Based on this, we also evaluated the concentration of serum JAM-A in a cohort of breast cancer patients.

3.3 Results

3.3.1 Determination of JAM-A protein levels in HMT-3522 and Hs578T breast cancer cell lines

Western blot analysis was conducted to determine the protein level of JAM-A in the HMT-3522 cell line series over a time course of 10 days (Figure 3.4A). Both the S1 non-tumorigenic and T4-2 tumorigenic cells expressed similar levels of JAM-A, with an expression peak at day 7 in S1 cells and day 10 in T4-2 cells as shown in adjacent densitometry graphs. This was interesting considering that S1 cells generally grow more slowly than T42 cells. However, it is interesting to note that both the tumorigenic and the non-tumorigenic cells of this isogenic model exhibited a similar level of JAM-A up to day 7. Experiments past 10 days showed that the non-tumorigenic cells had a much lower expression of JAM-A tailoring off by day 16 compared to the tumorigenic cells whose JAM-A expression remained high till day 16 (Western blots not shown).

The Hs578T cell line series showed a lower level of JAM-A expression compared to the HMT-3522 series (Figure 3.4B). Hs578T parental cells had undetectable levels of JAM-A even at high loading protein concentrations, while Hs578Ts(i)8 cells showed a low expression of JAM-A. We conclude that only the invasive variant of the Hs578T cells expresses meaningful levels of JAM-A.

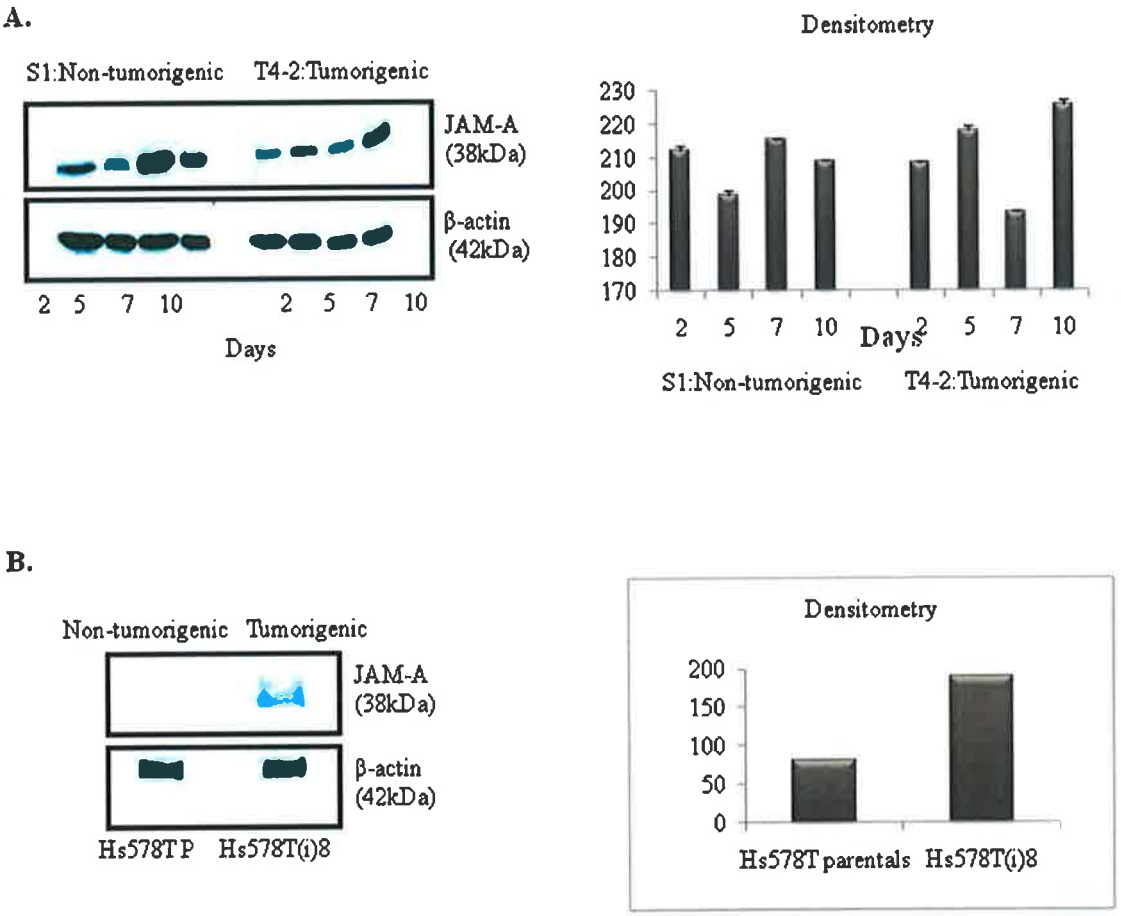


Figure 3.4: Protein levels of JAM-A in two series of isogenic breast cell lines.

(A) Western blot analysis of JAM-A expression in the HMT-3522 cell line series of S1 and T4-2 cells in a time course over 10 days. The densitometry graph represents 2 experiments, and error bars represent standard deviation. Both S1 and T4-2 cells express JAM-A protein. **(B)** Western blot analysis of JAM-A expression in Hs578T parental and Hs578Ts(i)8 cells at near confluence day 5. Densitometry graph shows one representative experiment. Hs578Ts(i)8 cells express a higher level of JAM-A protein than the normal Hs578T parental.

3.3.2 Determination of JAM-A localisation in HMT-3522 and Hs578T breast cancer cell lines

To determine whether the localization of JAM-A differed between the phenotypically non-tumorigenic and tumorigenic variants of each cell line, confocal immunofluorescence microscopy studies were performed (Figure 3.5). Confocal micrographs showed a similar membranous localisation of JAM-A in both the HMT-3522 S1 non-tumorigenic and T4-2 tumorigenic cells, consistent with its distribution in intracellular junctions. However, confocal micrographs revealed an undetectable JAM-A expression in the Hs578Ts(i)8 under the same staining conditions as in HMT-3522 cells, indicating the overall low level of JAM-A in the Hs578T cells relative to HMT-3522. Compared to other breast cancer cell lines like MCF 7, Hs578T cells also showed very low expression of JAM-A (data not shown).

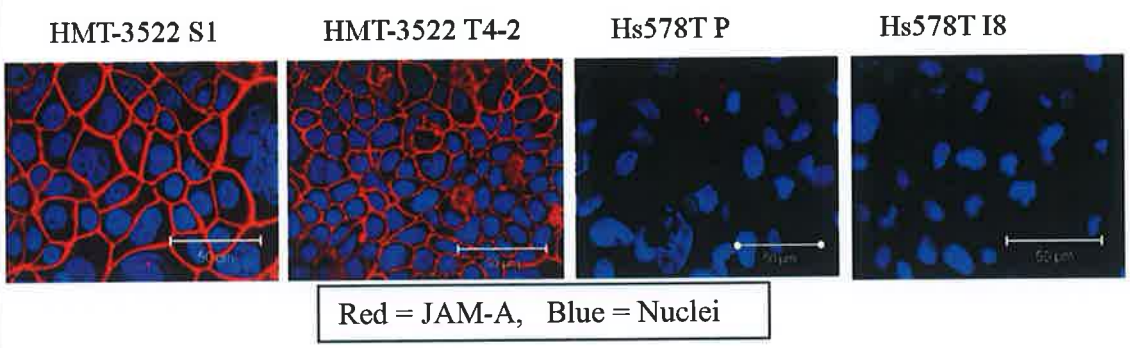


Figure 3.5: Localisation of JAM-A in two series of isogenic breast cell lines.

Confocal micrographs showing JAM-A localisation in confluent HMT-3522 S1 and T4-2 cells in addition to Hs578T parental and (i)8 cells. As can be seen from the images, the Hs578T series expressed a significantly lower level of JAM-A validating the western blot assays.

3.3.3 Tight junction function of the HMT-3522 breast cancer series

To evaluate tight junction gate function in the HMT-3522 isogenic cell line series, cells were grown on permeable support filters to establish full apico-basal polarity. Transepithelial electrical resistance (TER) was then measured over a number of days, using an epithelial voltohmmeter, as a well-established index of tight junction integrity (Figure 3.6A). HMT-3522 T4-2 cells displayed a higher TER than S1 cells, suggesting tighter adhesion between neighbouring cells or alternatively the presence of multi-layered cell sheets. The TER of HMT-3522 T4-2 cells peaked at 305$\Omega.cm^2$, which is much lower than the TER in many other epithelial cell lines such as MCF-7 and Caco-2 cell lines with TER values >600$\Omega.cm^2$ (Leonard et al., 2010, Somasiri et al., 2004).

Tight junction integrity (gate function) was also separately assessed by measuring how readily a small fluorosceinated dextran marker molecule was passively transported across the paracellular route (Figure 3.6B). Dextran flux increased steadily over time in both cell lines. However, in conjunction with the observation that S1 cells had a lower TER than T4-2 cells, dextran flux was higher across S1 cells. This supported evidence from the previous figure that S1 cells are leakier than T42 cells, despite expressing broadly similar levels of JAM-A.

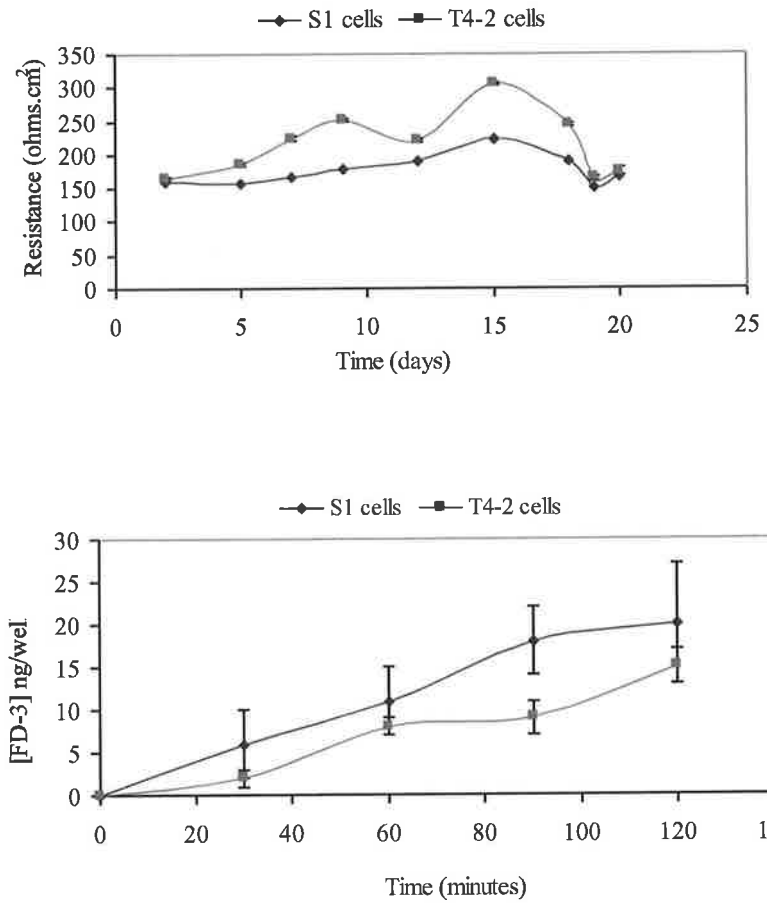


Figure 3.6: Measurement of tight junction integrity in HMT-3522 cell line series

(A) Transepithelial resistance in the HMT-3522 S1 and T4-2 cells over a 20 day period. T4-2 cells showed a higher transepithelial resistance than S1 cells Error bars represent standard deviation of replicates of three independent experiments. **(B)** Gate function measurement using fluorosceinated dextran to assess molecular flux from the apical to basal epithelial compartments over a 2 hour period. A higher flux rate was noted in the S1 cells than T4-2 cells. Error bars represent standard deviation of replicates of two independent experiments.

Having assessed tight junction integrity (gate function) using both the TER and dextran permeation methods, we next sought to assess tight junction fence function by estimating intra-membranous diffusion of a fluorescent lipid tracer molecule (sphingomyelin) between the apical and basal membranes. S1 and T42 cells were first grown to confluence on semi-permeable filter supports until peak TER had been achieved. Sphingolipid assays were then carried out, and all filters were stained and imaged in the reconstructed vertical (*xz*) plane using confocal microscopy (Figure 3.7). Sphingomyelin distribution (green) was observed to be diffusely spread across the apical and lateral membranes of both S1 and T4-2 cells; however a broader spread was noted in S1 epithelial sheets. In particular, vertical diffusion of sphingomyelin down the lateral membrane of T4-2 cells appeared to be more restricted than that in S1 cells. This suggested that the tight junction fence function of T4-2 cells was superior relative to that in S1 cells. This again could be explained by T4-2 cells being more tightly packed or forming multilayers, and thus making it more difficult for the sphingomyelin tracer to get through. Therefore both the gate and fence function assays validated the fact that HMT-3522 T4-2 cells had tighter junctions compared to S1 non-tumorigenic cells.

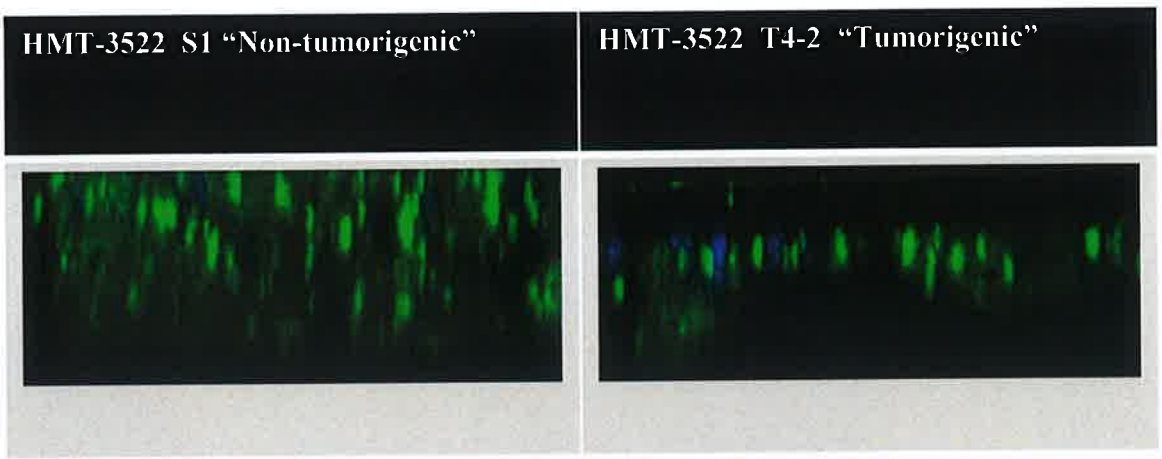


Figure 3.7: Intra-membranous diffusion of fluorescent lipid tracer sphingomyelin in isogenic HMT-3522 cell line series

Fence function assays in HMT-3522 S1 and T4-2 cells showed a diffuse spread of the fluorescently-labelled tracer molecule sphingomyelin (green).

3.3.4 Determination of JAM-A protein levels in primary breast cancer cultures

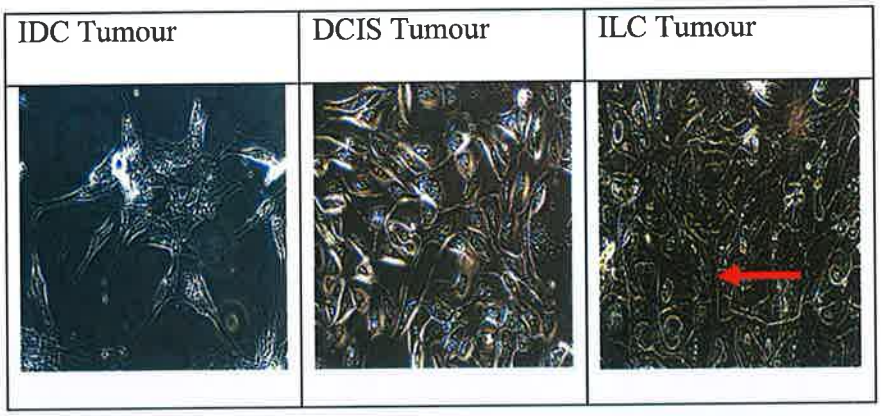
Primary epithelial cultures of tumour and non-tumour cells were generated from lumpectomy or mastectomy specimens obtained from breast cancer patients. Tumour tissue samples were from the centre of resected tumours, while non-tumour tissue samples were from beyond the tumour margins.

Primary breast cancer cells portrayed a variety of morphologies reflecting the heterogeneity of breast cancer disease, as observed in the representative phase contrast micrographs of Figure 3.8A. Flattening of cells from IDC tumours was noted, along with elongated spindle like edges. The DCIS micrograph shows a heterogeneous population of cells with cuboidal epithelial cells and flatter smaller cells which may be myoepithelial cells. The ILC micrograph portrays several populations of cells, with long flattened cells (possibly representing myoepithelial cells) surrounding more rounded cells (arrow). The possible presence of myoepithelial cells could be explained by the early passage numbers of these cultures, since myoepithelial cells have been reported to disappear after repeated sub-culturing and passaging of the cells (Bartsch et al., 2000).

We also noticed morphological differences between tumour and non-tumour cultures (Figure 3.8B), supporting the fact that our culture conditions supported the separate growth of both normal and abnormal cellular populations. Many tumour cultures grew rapidly into multi-cellular colonies regardless of the conditions provided. However, as evidenced by the non-tumour sample showing an isolated organoid attached to the culture flask with very few new cells emanating from it (arrow); non-tumour cells generally proliferated more slowly. The non-tumour samples on average proliferated to reach 50% confluence over 14 days while the tumour cells proliferated similarly within 3-5 days. An exception

was noted in the DCIS samples that showed similar proliferative rates in both tumour and non-tumour samples.

A.



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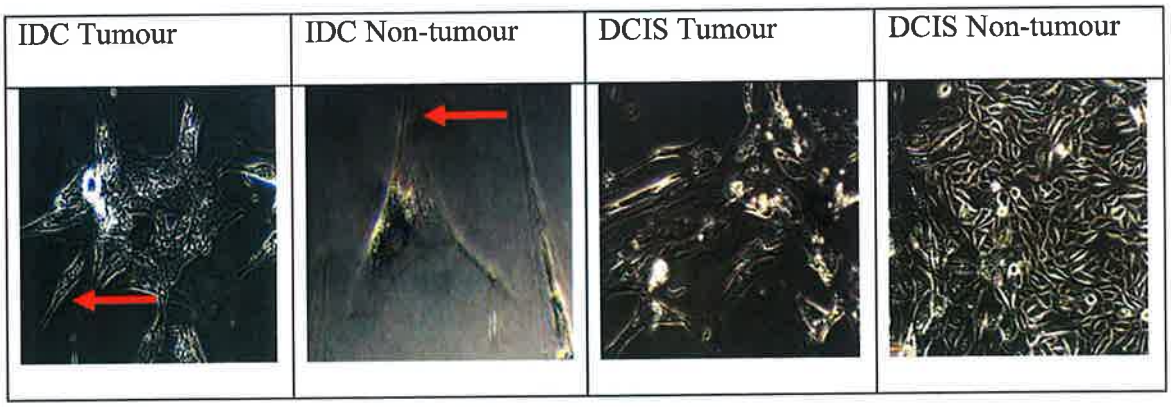


Figure 3.8: Morphological differences in primary breast culture cells

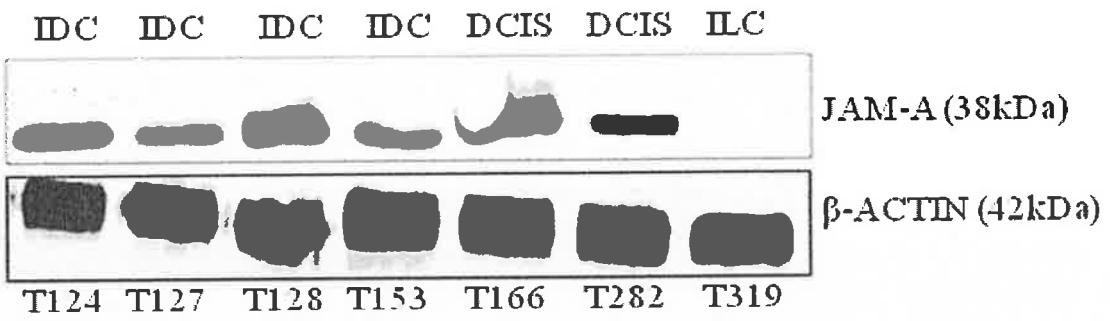
(A) Phase contrast images showing the heterogeneity of breast cancer primary cultures between invasive ductal carcinoma, ductal carcinoma *in situ* and invasive lobular carcinoma cells. Red arrow depicts long flattened cells surrounding more rounded cells, potentially myoepithelial and luminal epithelial cells respectively. **(B)** Phase contrast images showing differences in morphology between tumour and non-tumour primary cultures. Red arrows show single organoids with radial outgrowth of cells.

Western blot analysis was carried out to determine the protein levels of JAM-A in several independent primary breast cultures (Table 3.2). As seen by western blot analysis (Figure 3.9A), cultures from both invasive ductal carcinomas (IDC) and ductal carcinomas *in situ* (DCIS) expressed reasonable levels of JAM-A protein. Interestingly, decreased expression of JAM-A was noted in the invasive lobular carcinoma (ILC) culture shown. Sample size for the primary cultures were very small especially for the invasive lobular cancers as ILC cells are very hard to culture *in vitro*. Several of the ILC samples received grew fibroblasts and myoepithelial cells with very little epithelial cells to carry out the indicated experiments. On the contrary, IDC samples are very abundant as it makes up majority of breast cancer cases seen in our institution.

We proceeded to compare JAM-A protein expression between the primary breast cancer tumour and non-tumour cultures. As seen in the western blot in Figure 3.9B, tumour cultures (T) expressed much higher levels of JAM-A than cultures derived from non-tumour (NT) tissues. Table 3.2 shows the clinicopathological parameters of the primary culture samples in Figure 3.9A. Interestingly, we noted that two out of three of the Grade 2 IDC samples showed a moderate expression of JAM-A, while the one IDC sample of Grade 3 showed high levels of JAM-A. DCIS samples showed moderate to high expression of JAM-A in both Grade 2 and Grade 3 samples. The ILC sample displayed very little JAM-A. Overall, we concluded that higher grade tumour samples generally expressed higher levels of JAM-A. Nonetheless the sample sizes are very small and thus more samples would be needed to make a proper argument. However this novel result using primary cultures is in accordance with the recent publication by McSherry *et al.* (McSherry et al., 2009) correlating high levels of JAM-A gene and protein expression in breast cancer

tissues with poorer patient prognosis. We could thus suggest that JAM-A is a key contributor to breast cancer progression.

A.



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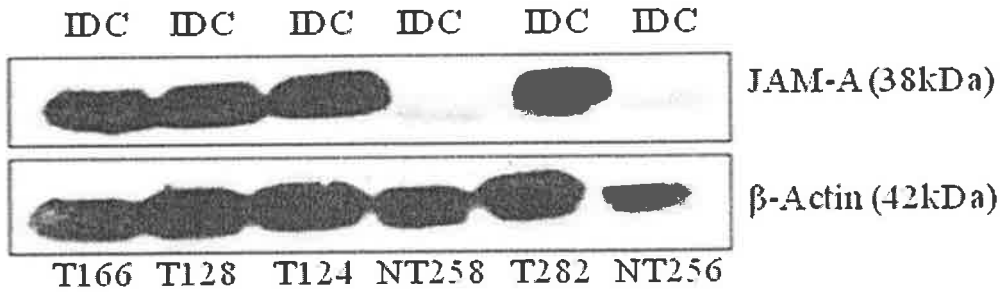


Figure 3.9: JAM-A protein expression between primary breast cancer tumour and non-tumour cultures. (T = tumour; NT = non-tumour)

(A) Western blot analysis for JAM-A protein level in a panel of independent primary breast cultures showing expression of JAM-A in all cells except the invasive lobular carcinoma. β-actin western blot controls for protein loading. **(B)** Western blot analysis of primary breast cultures comparing JAM-A protein level in four separate tumour samples versus two separate non-tumour samples. Non-tumour samples showed a lower expression of JAM-A protein than the tumour samples.

	T124	T127	T128	T153	T166	T282	T319
Tumour type	**IDC**	**IDC**	**IDC**	**IDC**	**DCIS**	**DCIS**	**ILC**
Grade	**3**	**2**	**2**	**2**	**3**	**3**	**2**
ER status	+	+	+	+	*	+	+
PR status	+	-	-	+	*	+	+
HER2 status	-	-	-	-	*	+	-
JAM-A Expression	**3**	**2**	**3**	**2**	**3**	**2**	**1**

Table 3.2: Clinicopathological parameters of the primary culture samples.

The relative expression levels of JAM-A were scored 1-3 depending on the protein level in each sample in the western blot in Figure 3.9A. A score of 1 was given for low expression, 2 for medium expression and 3 for high expression of JAM-A. Tissues with higher grades showed higher JAM-A expression. The total number of samples was 7, representing tissue samples of tumours from 7 breast cancer patients post resection of tumour.

3.3.5 Detection of JAM-A levels in serum samples of breast cancer patients

As mentioned in the last section, we noted an increase in JAM-A expression among high grade tumours leading us to believe that high JAM-A expression correlates with breast cancer aggressiveness. We thus proceeded to analyse a panel of blood serum samples from patients who had undergone breast surgery for breast cancer, in order to determine if serum JAM-A could act as a biomarker for breast cancer aggressiveness.

To carry out this experiment, we identified 16 random samples of IDC, DCIS and benign (non-tumour) of varying grades, and measured serum JAM-A levels by ELISA. In Figure 3.10, our results show a higher concentration of serum JAM-A per unit of protein in the benign samples compared to the invasive ductal cancers. We pooled IDC grade 1&2 for calculations due to small sample number. There was a statistically significant difference between serum JAM-A levels in benign versus the invasive ductal cancers of grade 1&2.

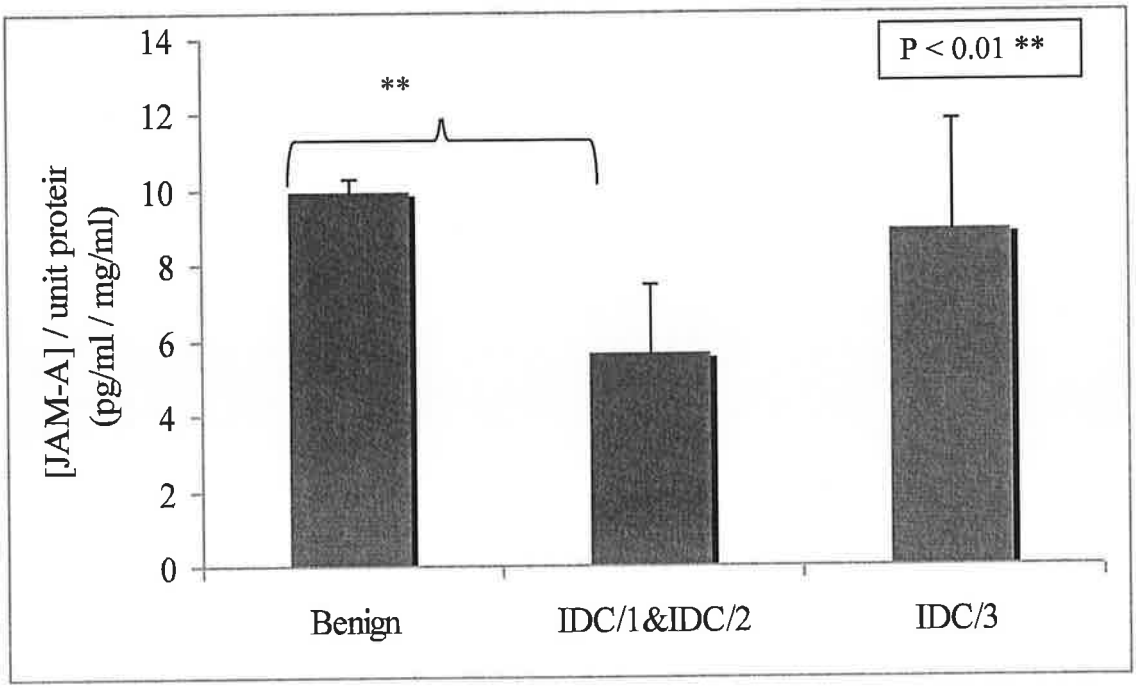


Figure 3.10: Measurement of serum JAM-A levels in breast cancer patients

Bar graph depicting the level of soluble JAM-A in breast cancer patient serum. An ELISA assay was done on 16 randomly selected pre-operative serum samples including benign and invasive ductal disease from breast cancer patients. The graph shows the concentration of serum JAM-A per unit of protein and depicts a higher concentration in the benign cells. Error bars refer to standard deviation of 3-8 individual patient samples, with three replicates per patient sample.

3.4 Discussion

As outlined in chapter 1, correct adhesion at epithelial cell-cell and cell-matrix sites plays an important role in normal breast tissue architecture. It has been suggested that dysregulation of adhesion is involved in cancer invasion and metastasis (Potter et al., 1999) One way in which this could be manifested is via the phenomenon of epithelial-to-mesenchymal transition (EMT). EMT has been associated with breast cancer metastasis by many studies in recent years (Savagner, 2010, Whipple et al.). During metastasis, tumour cells respond to the changing microenvironment with internal adaptations that are reflected in the cytoskeleton (Whipple et al.). EMT consists of a rapid and often reversible change of cellular phenotype, in which. epithelial cells loosen their cell–cell adhesion structures (including tight junctions), modulate their polarity and rearrange their cytoskeleton (Thiery et al., 2009). Cells become isolated, motile and may be resistant to apoptosis (Savagner, 2010). Based on the heterogeneity amongst cells, it is likely that the contribution of a process like EMT to cancer progression depends on the tumour type.

Given the contribution of altered adhesion to EMT and cancer progression in general, we were interested to model the role of alterations in tight junctions (TJs) to cancer progression. TJs are composed of several different transmembrane and intracellular proteins including occludin, claudins, JAMs, zonula occludens (Vetrano and Danese, 2009). Of particular interest is the JAM family which was explored in this chapter. JAM-A is involved in the regulation of junctional assembly and maintenance of cell integrity (Mandell and Parkos, 2005, Bradfield et al., 2007). Controversy has recently been generated over the role JAM-A in breast cancer progression (McSherry et al., 2009, Naik et al., 2008) In this chapter, we aimed to search for differences in JAM-A expression and

localisation in breast cancer cell lines that mimicked non-tumorigenic and tumorigenic phenotypes.

We identified that the HMT-3522 S1 (non-tumorigenic) and T4-2 (tumorigenic) cells showed slightly different levels of JAM-A expression at different time points, with that in S1 cells peaking earlier than that in T42 cells. In the Hs578T cell line series, the invasive variant showed a higher level of JAM-A protein expression. This was also validated in the primary breast cultures which showed, interestingly, that primary breast cultures of higher grade had higher levels of JAM-A protein expression. In parallel, non-tumour samples were found to express low levels of JAM-A. These findings are in keeping with recently published data by McSherry *et al.* (McSherry et al., 2009) stating that aggressive tumours over-express JAM-A protein. Furthermore it suggests that the Hs578T series may be more valuable than the HMT-3522 series for investigating the contribution of JAM-A to breast cancer progression.

To return to what has been published regarding JAM-A in breast cancer, over-expression of JAM-A on a tissue microarray of breast cancer patients corresponded to poorer prognosis (McSherry et al., 2009). In the same study, JAM-A knockdown at both gene and protein level was noted to decrease migration and invasion. It is an interesting possibility that disruption of JAM proteins plays a key role in disease progression, as loss of tissue architecture and cell polarity is a prerequisite for breast cancer invasion and metastasis (Bordin et al., 2004). In fact, in colonic carcinoma cell lines there has been direct evidence for JAM-A-mediated regulation of intestinal epithelial barrier function by influencing claudin expression and proliferation (Laukoetter et al., 2007). To explain the conflicts with another recently-published study suggesting the opposite, that JAM-A has a negative correlation with breast cancer progression (Naik et al., 2008), Mc Sherry *et al.* suggested

that low expression / under expression of JAM-A may impair cellular adhesion and polarity potentially contributing to cancer initiation, while over-expression of JAM-A could promote migratory events favouring tumour progression (McSherry et al., 2009). Therefore future work dissecting the contributions of altered JAM-A expression to breast cancer must carefully focus on time-dependent elements.

We proceed to assess the tight junction gate function of HMT-3522 cells by measuring their transepithelial resistances (TER). This refers to the role of the tight junction as a physiological gate restricting the paracellular transport of water, ions, and non-ionic small molecules. Surprisingly, TER was found to be increased in HMT-3522 T4-2 tumorigenic cells relative to the S1 non-tumorigenic cells. The fact that T4-2 cells are more invasive and proliferative means that they pack together more tightly, potentially accounting for the higher TER and the lower flux of FITC-Dextran tracer across the cells. This may also coincide with the fact that the invasive / tumorigenic cells showed higher JAM-A expression than S1 cells at later time points, hence tighter junctions or enhanced JAM-dependent signalling.

In this chapter we also employed the use of primary cultures to validate our cell line studies. We noted that on evaluation of JAM-A protein expression that invasive ductal carcinomas and ductal carcinomas *in situ* expressed similar levels of JAM-A, while invasive lobular cancers showed very little JAM-A expression. Comparing tumour and non-tumour samples for JAM-A expression, we observed that non-tumour samples showed much lower levels of JAM-A thus validating the work done early on cell lines. Following this, we proceeded to investigate the concentration of JAM-A in serum of breast cancer patients. We observed a significantly higher JAM-A concentration in the benign samples compared to the invasive ductal cancers. It is tempting to speculate that loss of serum

JAM-A may be a biomarker in breast cancer disease, but, due to the significant variation in JAM-A levels among our small patient cohort, future studies will need to expand and refine these observations.

In conclusion, the HMT-3522 S1 and T4-2 cell line series exhibited similar levels of JAM-A protein expression (despite peak expression at different stages), but featured differences in their tight junction integrity. The Hs578T cells showed a variable expression of JAM-A protein with the parental "normal" cells having significantly lower protein levels of JAM-A than their invasive counterparts. Primary breast cultures also validated the higher level of JAM-A expression in the invasive samples compared to the non-tumour samples. In the ELISA assay, the blood serum level of JAM-A in primary cultures showed a higher concentration of soluble JAM-A in benign versus invasive cancer. Our results show both isogenic breast cancer cell line series are useful models with which to study the role of JAM-A in breast cancer progression, while the Hs578T cells have added value due to parallels between with their JAM-A expression and that in primary breast cells.

Chapter IV

Functional relevance of Junctional Adhesion Molecule-A in breast cancer progression

4.1 Introduction

4.1.1 Proliferation and cancer

Cancer is frequently considered as a disease of abnormal proliferation. Tight junctions are best known for their roles in regulating permeability and barrier function, but recently they have been implicated in other roles like morphogenesis, cell polarity and cell proliferation and differentiation (Schneeberger and Lynch, 2004).

We and others hypothesize that alterations in tight junction protein signalling may contribute to cancer. The tight junction adhesion proteins JAM-A is the main interest of this thesis. As previously emphasised, JAM proteins have important functions in numerous cellular adhesive processes including intercellular junction assembly (Liang et al., 2000), cell morphology (Mandell et al., 2005) and leukocyte migration (Martin-Padura et al., 1998, Ostermann et al., 2002). Recently, a role for JAM-A dysregulation in breast cancer has been proposed (McSherry et al., 2009, Naik et al., 2008). In breast cancer and indeed most cancers, loss of cell polarity is a prerequisite for cancer invasion and thus metastasis (Man and Sang, 2004). In fact, JAM-A disruption in murine carcinoma cells has been shown to induce conversion of cells from a static, polarized state to a pro-migratory phenotype (Mandicourt et al., 2007).

We thus speculate that functional disruption of JAM proteins may play a key role in models of breast cancer progression. In the next section of this chapter, we discuss the role of 3-dimensional culturing in breast cancer cell lines and how the model was used to study the functional role of JAM-A in cancer progression.

4.1.2 3-Dimensional culturing to recapitulate breast acinar morphology

In research most *in vitro* studies on cancer and proliferation are done using 2-dimensional monolayer cultures, where cells are forced to adjust to unnatural substrates different from the natural 3-dimensional environment. In this section, we discuss the benefits of growing cells in 3-dimensional cultures. Organotypic culture is the growth of cells in a three-dimensional environment rather than in traditional two-dimensional culture dishes. 3-dimensional culturing is biochemically and physiologically similar to *in vivo* tissue, although some *in vivo* complexities (for example, diffusion of nutrients) can never be fully recapitulated in *in vitro* (Stefanova et al., 2009). Nonetheless *in vivo* conditions can be somewhat reconstructed in 3-dimensional culture systems, for example those that incorporate combinations of epithelial and stromal cell growth in semisolid matrices such as Matrigel™; a basement membrane matrix extracted from the Engelbreth–Holm–Swarm (EHS) mouse tumour (Falkner et al., 2003). Matrigel™ is well-known to be rich in extracellular matrix proteins, with laminin, collagen IV, heparan sulphate proteoglycans, entactin and nidogenas as major components. Various growth factors are also present, including TGF-beta, FGF and plasminogen activators. Matrigel™ polymerises under normal physiological conditions to produce a reconstituted, biologically active matrix that is effective for the attachment and differentiation of cellular material (Lacroix and Leclercq, 2004).

In the context of breast physiology, 3-dimensional Matrigel™ cultures have been described to closely approximate the *in vivo* mammary functional unit or acinus. The acinus is typically a polarised structure with a central lumen, which in these culture models, can be achieved depending on the integration of cues from the surrounding microenvironment (Mi et al., 2009, Weigelt and Bissell, 2008).

4.2 Specific Aims

The previous chapter dealt with characterising the isogenic breast cancer models of HMT-3522 S1 "non-tumorigenic" and T4-2 "tumorigenic" cells based on their tight junction integrity. We explored the differences between the Hs578T parental "normal" and Hs578T (i) 8 "invasive" cells showing a higher JAM-A expression in the invasive cells, in common with that in high grade tumour primary cultures. In this chapter these cell lines were utilised to assess functional parameters relevant to breast cancer initiation and progression in relation to JAM-A modulation.

Our first aim was to identify any differences in the proliferative patterns between non-tumorigenic and tumorigenic variants of the isogenic cells using MTT proliferation assay and to characterise differences in proliferation and JAM-A expression between non-tumorigenic and tumorigenic cells upon treatment with J10.4, an inhibitory antibody to JAM-A.

Our second aim was to identify if inhibition of JAM-A has a role in cell migration using scratch wound assays in cells treated with J10.4 antibody.

Our third aim was to grow cells in 3-dimensional culture conditions to mimic the *in vivo* conditions in order to determine if JAM-A antagonism has an effect on polarisation or phenotype of the isogenic cells.

4.3 Results

4.3.1 Proliferation characteristics of HMT-3522 and Hs578T cell line series.

MTT proliferation assays were carried out in both cell line series over a four day period. From the graph shown in Figure 4.1, it was observed that invasive HMT-3522 T4-2 and Hs578T (i) 8 cells (dotted lines) proliferated faster and at a higher rate than their normal counterparts (solid lines). This phenomenon could be accounted for by the fact that the HMT-3522 T4-2 and the Hs578T (i) 8 cells are invasive cells and may have the propensity to grow as multilayers thus recapitulating their tumorigenic phenotype.

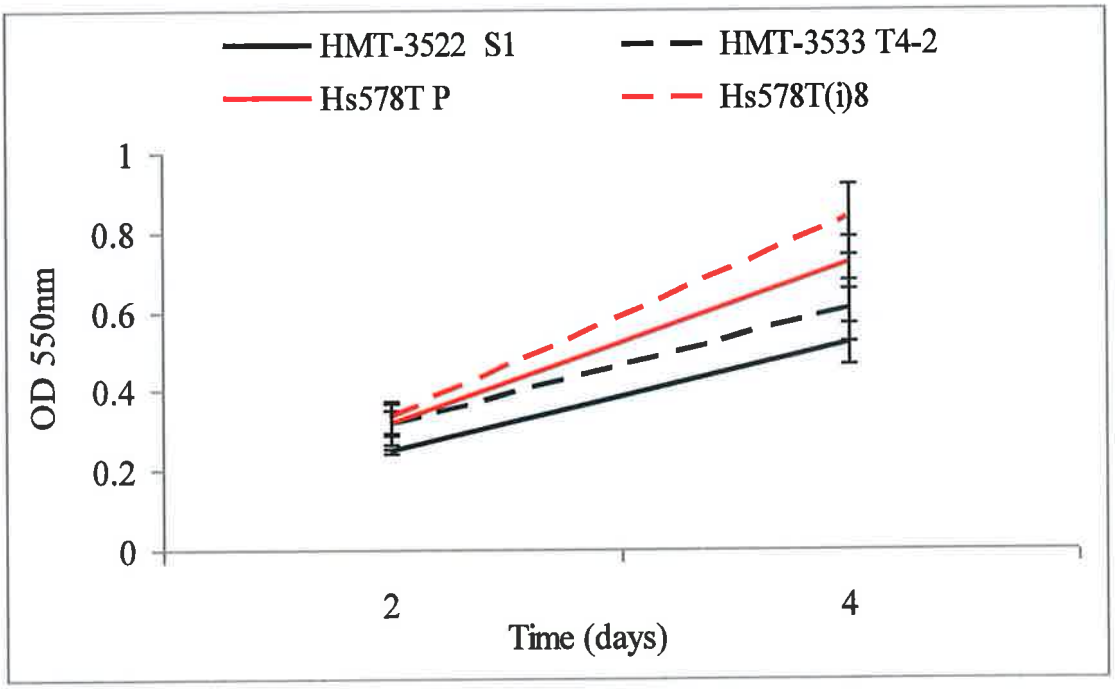


Figure 4.1: Proliferation characteristics of HMT-3522 and Hs578T cell lines.

Cells ($2x10^4$) were plated in triplicate wells of two 96-well plates on day 0. MTT (5mg/ml) solution was added to one plate at days 2 and 4 and incubated for 4 hours. MTT was removed, DMSO added and absorbance measured at 540nm. Error bars refer to standard deviation of four independent experiments. The tumorigenic variants of both cell lines showed a higher proliferation compared to the non-tumorigenic.

4.3.2 Investigation of the effects of JAM-A antagonism on cellular proliferation

In the above section (Figure 4.1), we showed the proliferative patterns of the HMT-3522 and Hs578T cell lines. Next, MTT proliferation assays were also carried out in the presence of J10.4 antibody. Surprisingly however in Figure 4.2, HMT-3522 S1 cells showed no proliferative differences between control and J10.4-treated cells, while the HMT-3522 T4-2 cells showed a slight (but non-statistically significant) increase in proliferation upon JAM-A inhibition. Cyquant proliferation assays were also carried out with similar results (data not shown).

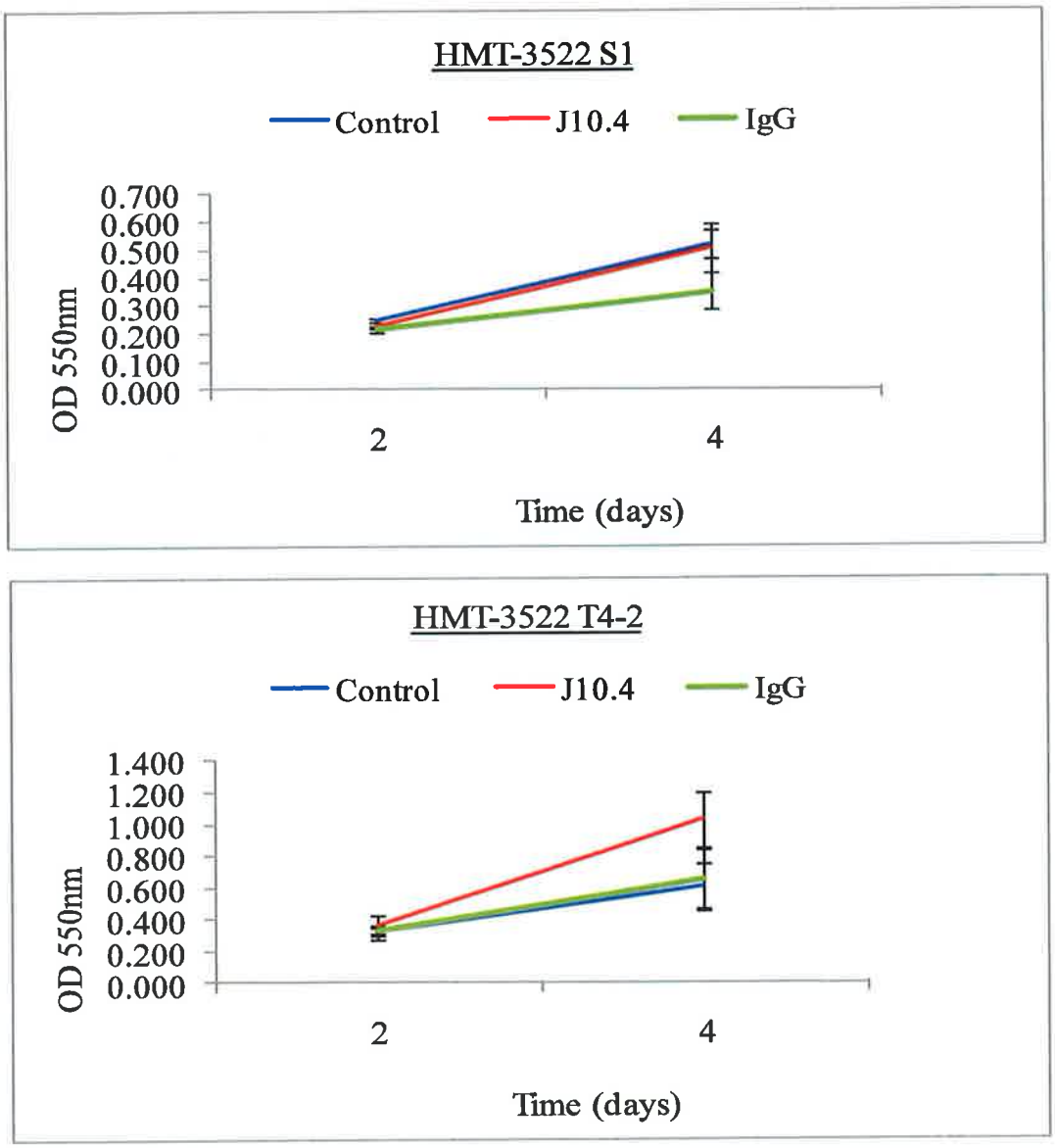


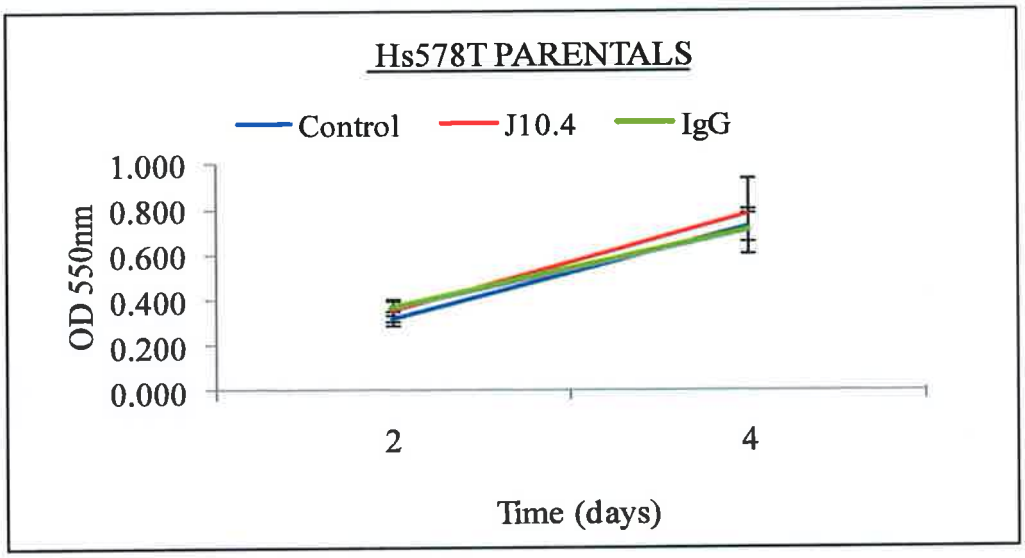
Figure 4.2: Proliferation of HMT-3522 S1 and T4-2 cell lines in the presence of JAM-A inhibitory antibody (J10.4) or IgG as a negative control.

Cells ($2x10^4$) were plated in triplicate wells of two 96-well plates on day 0. MTT (5mg/ml) solution was added to one plate at days 2 and 4 and incubated for 4 hours. MTT was removed, DMSO added and absorbance measured at 540nm. Error bars are indicative of standard deviation of four independent experiments.

Further to the discussion above, we proceeded to test whether JAM-A antagonism had anti-proliferative effects using a different cell line series. However as shown in Figure 4.3, the Hs578T cells similarly showed no significant differences in proliferative patterns between controls and J10.4-treated cells in either of the parental or invasive cell line variants. Interestingly, we noted in Hs578T parental cells that the control, IgG and J10.4 graphs completely overlapped; possibly reflecting the fact that these cells express minimal JAM-A as seen in chapter 3. The Hs578T (i) 8 invasive cells showed a marginal decrease in proliferation upon J10.4 treatment which was not statistically significant.

Both the HMT-3522 and Hs578T cell lines used in the proliferation assays showed a very low expression level of JAM-A in comparison to other breast cancer cell lines, for example MCF7 cells. Other work done in our lab showed a decrease in proliferation upon JAM-A inhibition in MCF7 cells. This suggests that since there is less JAM-A on HMT-3522 and Hs578T cells for the inhibitory antibody to bind, there may be thus very little overall effect. On the other hand, MTT and Cyquant proliferation assays may not be sensitive enough to portray subtle anti-proliferative properties of the JAM-A inhibitory antibody.

Thus, further analysis of the anti-proliferative effects of JAM-A needs to be evaluated with a breast cancer cell line expressing high levels of JAM-A using an alternate method for example a transient knockdown of JAM-A in breast cancer cell lines using siRNA.



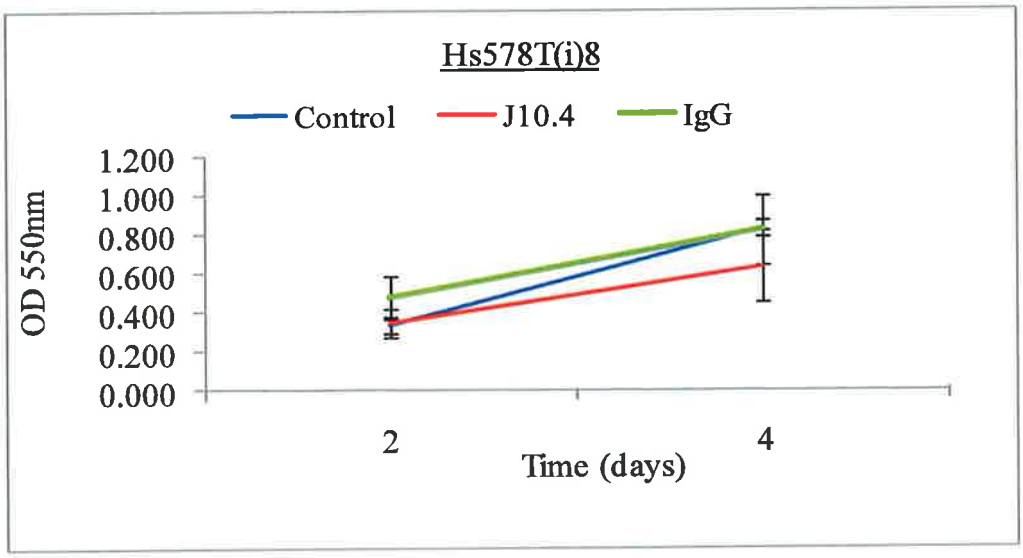


Figure 4.3: Proliferation of Hs578T cell lines in the presence of JAM-A inhibitory antibody (J10.4) or IgG as a negative control.

Cells ($2x10^4$) were plated in triplicate wells of two 96-well plates on day 0. MTT (5mg/ml) solution was added to one plate at days 2 and 4 and incubated for 4 hours. MTT was removed, DMSO added and absorbance measured at 540nm. Error bars are indicative of standard deviation of four independent experiments.

4.3.3 Assessment of cellular morphology and JAM-A protein levels following JAM-A inhibition

Having observed no proliferative differences between the non-tumorigenic and tumorigenic variants of two isogenic cell line series, Hs578T and HMT-3522, upon JAM-A inhibition, we also looked qualitatively at cell growth after antagonising JAM-A function. JAM-A was pharmacologically antagonised using the inhibitory antibody, J10.4, which prevents the dimerisation (and thus signalling functions) of JAM-A (Mandell et al., 2004).

Cells were grown on 6-well tissue culture plates and treated with either media only, JAM-A inhibitory antibody or an IgG negative control antibody (added at time 0). Phase contrast images were acquired at 48 and 72 hours. Figure 4.4A shows that JAM-A-inhibited cells grew in more scattered colonies, particularly in HMT-3522 cells and primary breast cultures. There was an obvious decrease in the number of cells present in all J10.4-treated cells, suggesting (contrary to proliferation assays) that JAM-A antagonism has an anti-proliferative effect.

To determine whether JAM-A antagonism altered JAM-A protein expression, cells were grown to confluence on tissue culture dishes and treated with 5μg/ml of J10.4 for 24 hours. JAM-A expression was subsequently determined by western blot analysis. As shown in Figure 4.4B, a decrease in the protein expression of JAM-A was observed in both S1 and T4-2 cells upon JAM-A inhibition. Interestingly, a greater decrease was noted in S1 compared to T4-2 cells. This may reflect the higher proliferation rate of T4-2 cells (as noted in figure 4.1), allowing reductions in JAM-A expression to be quickly compensated for by new cell growth.

A.

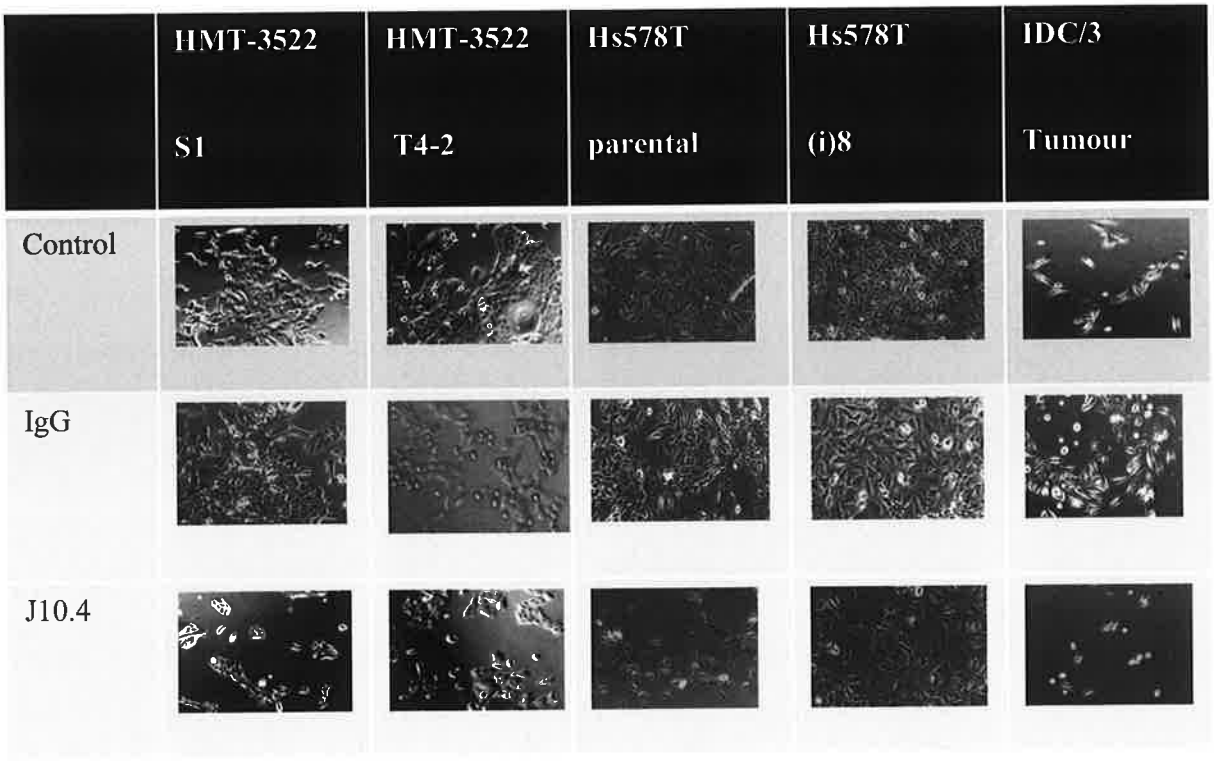


Figure 4.4: Cellular morphology following treatment with JAM-A inhibitory antibody.

(A) Phase contrast photographs of HMT-3522, Hs578T and primary culture breast cells grown for 48h in the presence or absence of the JAM-A inhibitory antibody J10.4. JAM-A antagonism induced a reduction in cell numbers compared to control conditions (no treatment and IgG negative control).

B.

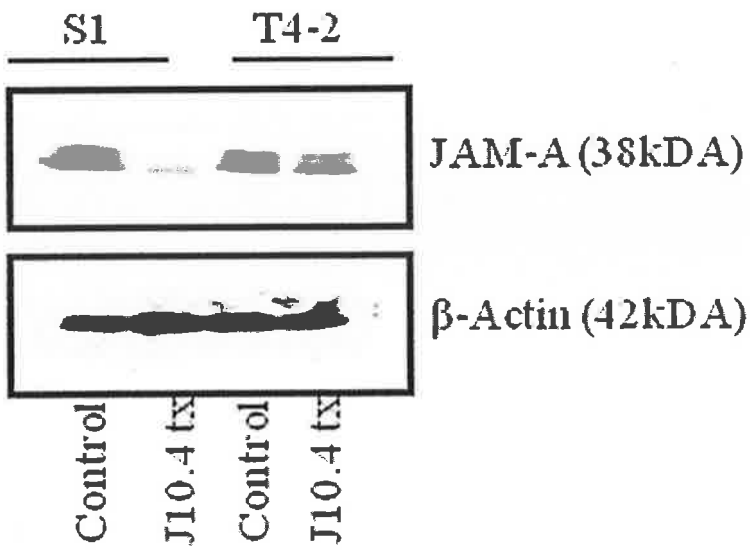


Figure 4.4: JAM-A protein level following treatment with JAM-A inhibitory antibody.

(B) Western blot analysis showing JAM-A expression on HMT-3522 S1 and T4-2 cells following 24h treatment with J10.4 (JAM-A inhibitory antibody), with a reduction in protein levels in J10.4 treated cells.

4.3.4 Migration and invasion of J10.4 treated cells

Having shown J10.4 to have a possible anti-proliferative effect (from phase contrast images) and to induce a decrease in protein levels of JAM-A, we next sought to investigate the role of JAM-A inhibition on cell migration Migration assays were chosen because although many different factors contribute to aggressive behaviour in breast cancer, cell motility is a crucial step in the invasion and metastasis of cancer.

The migratory capacities of HMT-3522 cells in response to media only versus J10.4 treatment were first compared using scratch wounding migration assays (Figure 4.5). Migration was quantitated as % wound closure at 0, 2, 6, and 24 hours post injury. Results showed a significant difference in wound closure between control (untreated) and J10.4-treated cells in both HMT-3522 S1 and also T4-2 cells. This indicates a decrease in the migratory properties of both non-tumorigenic and tumorigenic cells upon JAM-A inhibition.

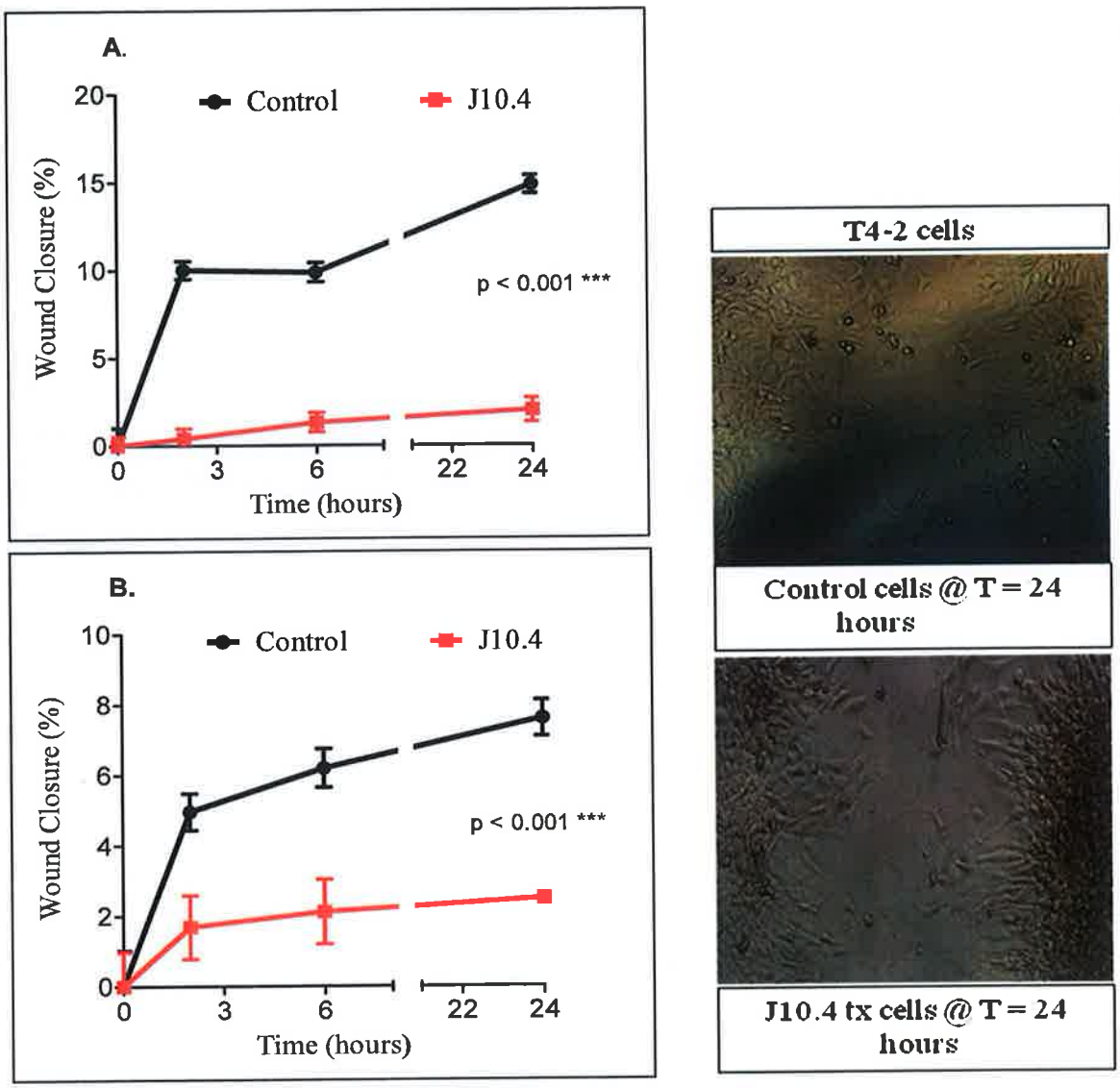


Figure 4.5: Assessment of migration in HMT-3522 cells in the presence of J10.4. (A) Migration of HMT-3522 S1 cells and (B) HMT-3522 T4-2 cells was assessed by scratch wound migration assay. Cells were grown to confluence in 24-well plates and wounded by scratching across the monolayer 2 hours after treatment with J10.4 inhibitory antibody or media alone. Phase contrast images of the wounded region were taken and used to analyse percentage closure over time. Errors bars refer to standard deviation of two independent experiments, with 3 replicate wells in each experiment. Inset shows phase contrast images representative of wound closure.

4.3.5 3-Dimensional morphological characteristics of HMT-3522 cell lines, Hs578T cell lines and primary breast cultures.

Having assessed the proliferative and migratory properties of HMT-3522 cell lines in response to JAM-A inhibition in 2-dimensional environments, we proceeded to grow cells in 3-dimensional cultures to better mimic the *in vivo* environment. We first needed to identify if our particular breast cancer cell lines and primary cultures formed 3-dimensional spheroids (resembling breast acini), in order to subsequently investigate the effects of JAM-A antagonism on 3-dimensional polarization.

As shown in Figure 4.6, it was observed that the HMT-3522 cell line series formed spheroids in 3-dimensional Matrigel™ cultures. Specifically, HMT-3522 S1 cells recapitulated the phenotypic characteristics of normal breast tissue *in vivo*. Approximately 30% of S1 cultures formed acinus-like structures after 14 days with polarised cells surrounding a central lumen; very similar in structure to the breast acinus *in vivo* . The other 70% of S1 cells formed organised spheroids which had not yet developed lumens. In contrast, T4-2 cells mostly formed large disorganised, undifferentiated clumps of cells, which were reminiscent of unpolarized tumour masses. Interestingly, a primary breast culture from invasive ductal carcinoma (IDC) tissue was also noted to form a spheroid-like structure with somewhat polarised cells. The primary culture modelled here was only of intermediate grade (grade 2), which could explain the partial formation of a polarised structure. In contrast, the Hs578T cell line of parental and (i) 8 cells grew in a flat 2-dimensional manner in Matrigel™. They showed no spheroid or acinus formation. This was also observed in a paper showing the morphology of 24 breast cancer cell lines in 3-dimensional cultures (Kenny et al., 2007).

A.

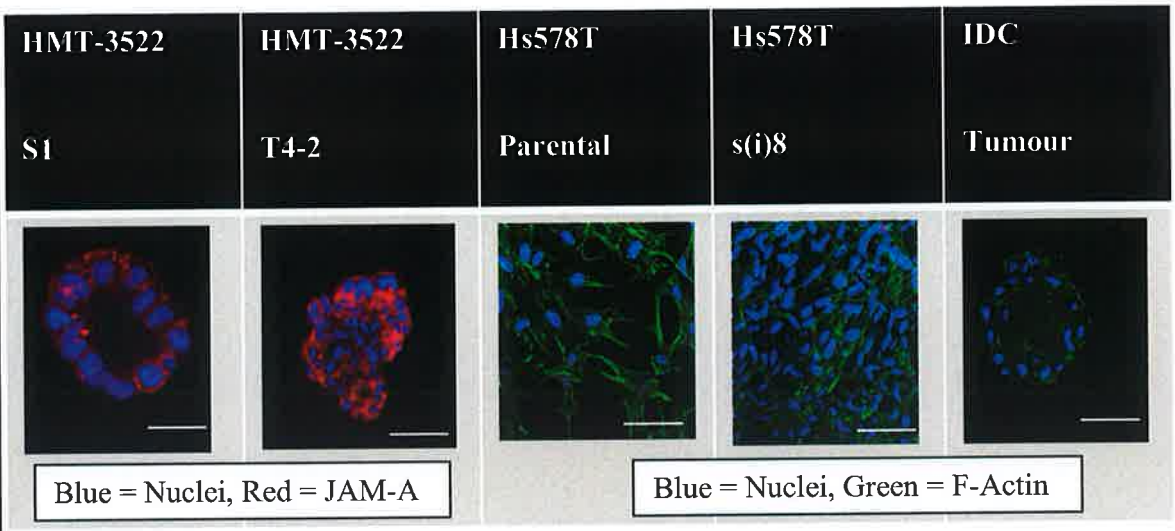


Figure 4.6 Morphology of breast cancer cell lines and a representative primary breast culture in 3-dimensional culture conditions.

Cells were grown in chamber slides in the extracellular matrix Matrigel™ for 14 days. Regular feeding was carried out every third day with media containing 5% Matrigel™. At day 14, cells were fixed, stained for JAM-A in the HMT-3522 cells and for F-actin in the Hs578T and primary culture cells as Hs578T cells do not express high levels of JAM-A as shown in Figure 3.5. The cells were analysed using confocal microscopy. Images shown represent a single *xy (en face)* image through the widest part of any spheroids formed. HMT-3522 cell lines and primary breast cells formed spheroids while Hs578T cell lines formed no spheroids in 3-dimensional cultures. Scale bar =50μm.

4.3.6 Determination of the functional effects of JAM-A inhibition on 3-dimensional cultures of HMT-3522 cell lines and primary breast cultures.

Weaver *et al.* showed that inhibition of β1-integrin function resulted in a reduction of tumorigenicity and reversed the malignant phenotype of T4-2 invasive breast cancer cells (Weaver et al., 1997). Based on these findings and some evidence that JAM-A signals via β1-integrin (Severson et al., 2009a, McSherry et al., 2009), we sought to investigate whether JAM-A antagonism too could normalise the tumorigenic appearance of HMT-3522 T4-2 cells grown in 3-dimensional cultures.

Having shown in Figure 4.6 that both HMT-3522 S1 and T4-2 cells formed spheroids or clumps of cells (respectively) when embedded in a 3-dimensional Matrigel™ microenvironment, we proceeded to test the influence of JAM-A antagonism on these morphologies. Cells were grown as 3-dimensional cultures in the presence or absence of inhibitory JAM-A antibody (J10.4; present from seeding), fed regularly with media alone or media containing J10.4 or IgG (used as a negative control – micrographs not shown), and harvested at day 14 for staining and analysis by confocal microscopy.

Figure 4.7 shows the morphology of HMT-3522 S1 and T42 breast cancer cells and a representative breast primary culture following treatment with J10.4. A significant decrease in size of 3-dimensional structures was observed both in HMT-3522 and primary cultures upon JAM-A inhibition. The S1 non-tumorigenic cells, even with J10.4 treatment, grew as polarised structures although smaller in size. The T4-2 tumorigenic cells (that had initially been noted to grow as unpolarized clumps of cells resembling tumours) displayed an array of morphologies upon J10.4 treatment. A decrease in structure size was firstly observed, but more importantly while some of these spheroids retained their initial

tumorigenic morphology, others formed more organised structures (discussed in detail in the next section). Interestingly, the HMT-3522 T4-2 cells also showed a change in nuclear morphology after J10.4 treatment, with an increase in size and several nuclei noted to be joined together. The human breast primary culture exhibited here also showed a reduction in spheroid size following J10.4 treatment.

In an effort to quantitate the observed effects of J10.4 on diminishing 3-dimensional spheroid size, the circumference of approximately 20 spheroids per condition were measured using image analysis software and plotted on the graph shown in Figure 4.8. Statistically-significant reductions in spheroid circumferences were noted between control (media alone or control IgG treatment) and J10.4-treated conditions, corroborating the qualitative observations made in Figure 4.7.

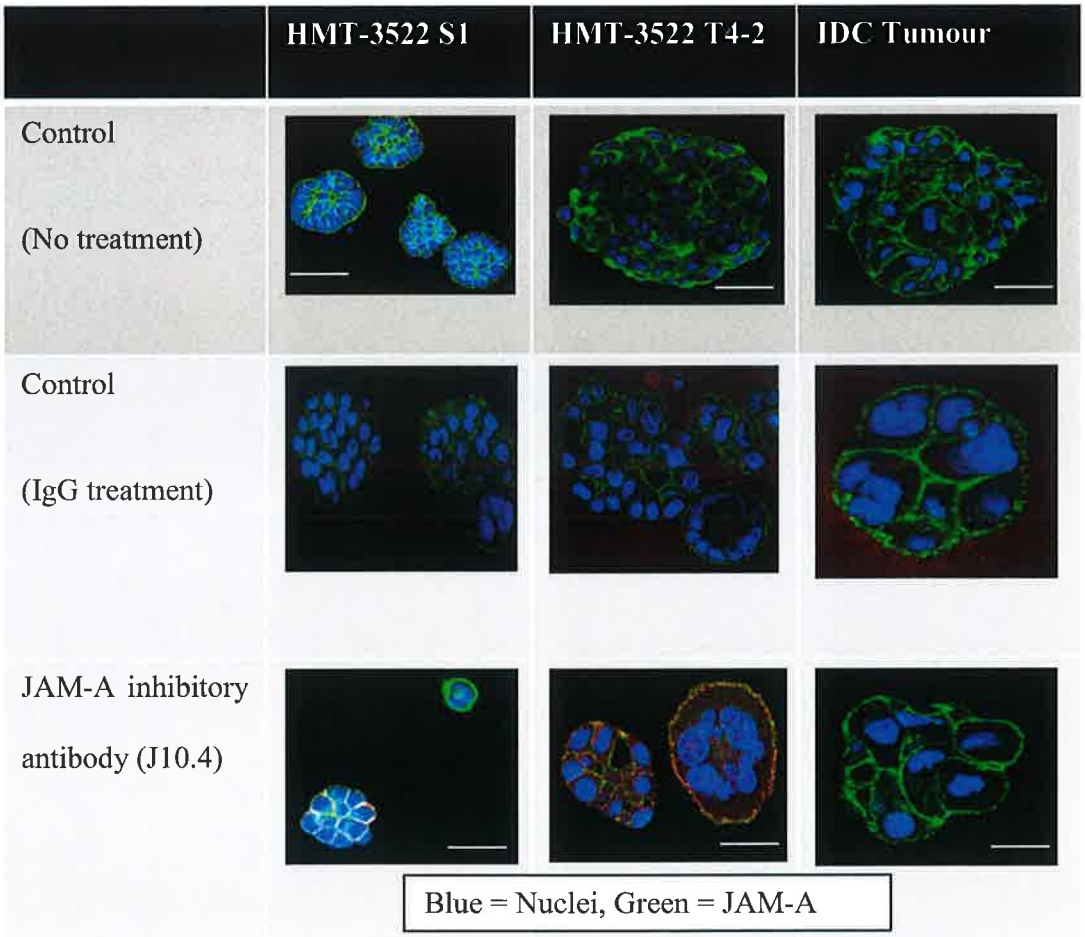


Figure 4.7: Morphology of 3-dimensional cultures following treatment with JAM-A inhibitory antibody.

Cells were grown in Matrigel™ for 14 days prior to harvesting, fixing and staining for analysis by confocal microscopy. HMT-3522 S1 "non-tumorigenic" and T4-2 "tumorigenic" cell lines and primary breast invasive ductal carcinoma cell all showed a reduction in the number of cells per spheroid upon J10.4 treatment compared with the controls. Scale bar =50μm.

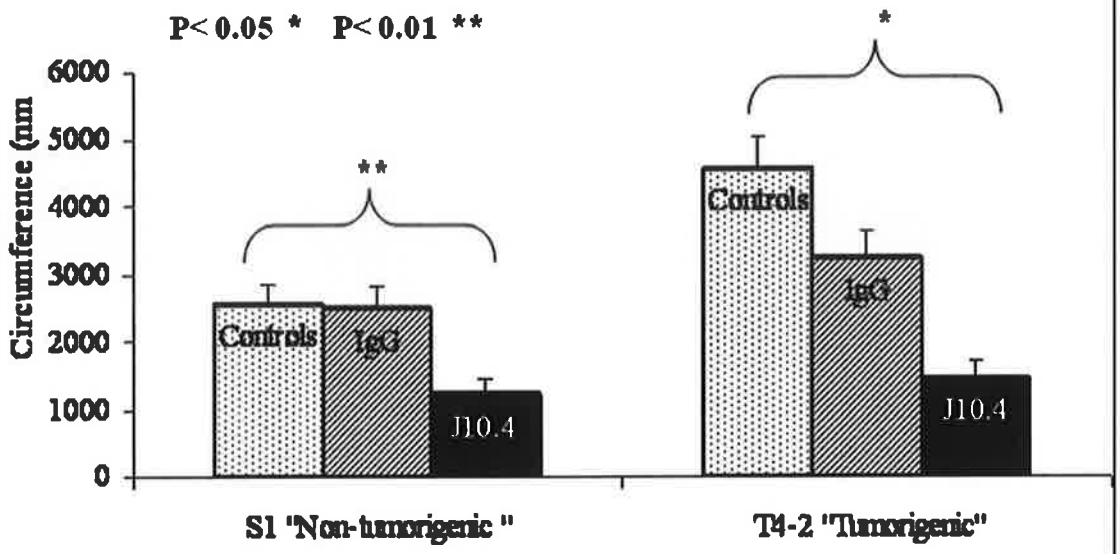


Figure 4.8: Spheroid circumferences of 3-dimensional cultures following treatment with JAM-A inhibitory antibody.

Cells were grown in Matrigel™ for 14 days prior to harvesting, fixing and staining for analysis using confocal microscope. The graph shows the mean spheroid circumference from five independent experiments. Error bars are indicative of standard deviation. Spheroid circumference was reduced in cells of HMT-3522 series following treatment with J10.4

Since JAM-A inhibition induced a significant reduction in spheroid size; we asked whether treatment with J10.4 also influenced the morphologically-abnormal phenotype of T42 cells. Remarkably, not only was there a marked cellular clearout of cells leading to a decrease in spheroid circumference, but we also noted that T4-2 cells showed a partial reversion towards a more normal phenotype (Figure 4.9). Specifically, approximately 40% of T42 cultures treated with J10.4 developed polarised structures with a single layer of cells encircling defined luminal spaces, in contrast to the unpolarised cellular clumps of untreated T4-2 cells. Partial re-polarization and the induction of lumen formation was only evident in invasive T4-2 cells, as J10.4 treatment did not alter the polarity or enhance the formation of lumens in normal S1 cells.

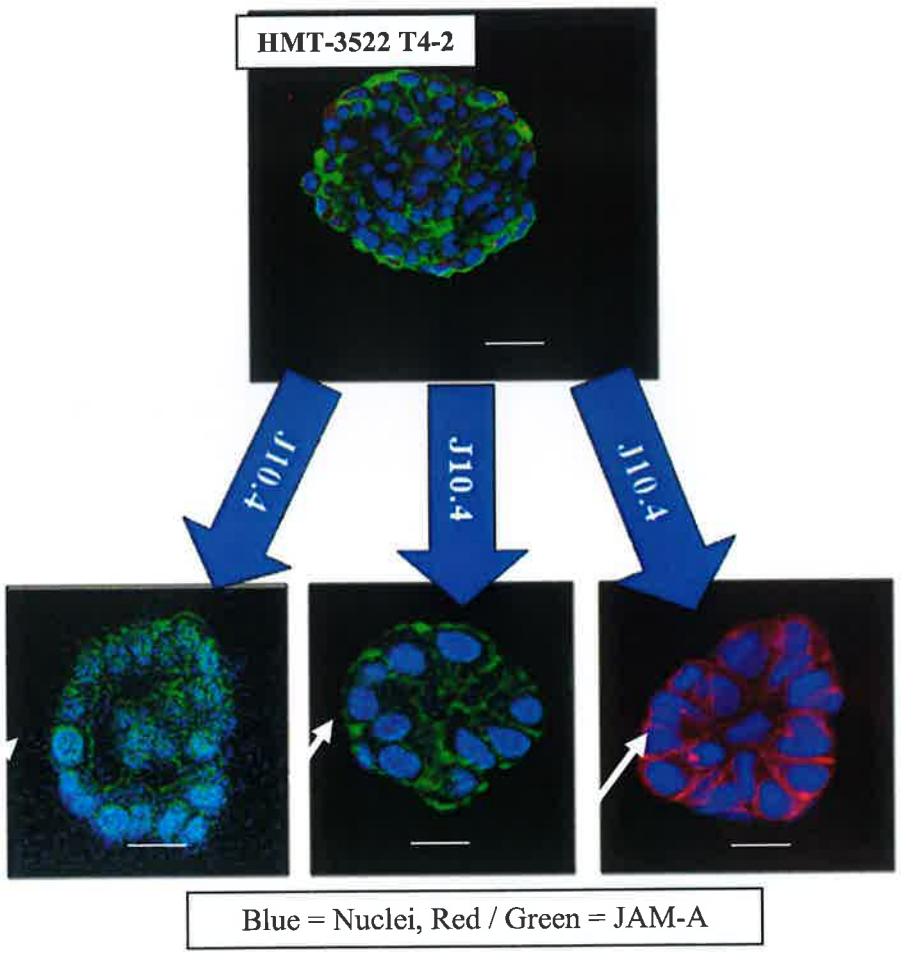


Figure 4.9: Morphology of 3-dimensional cultures following treatment with JAM-A inhibitory antibody.

Spheroids represented here are HMT-3522 T4-2 tumorigenic cells. J10.4 treatment induced the formation of acinus-like polarised structures with partial lumen clearance (arrow). Scale bars =50μm. Images representative of 3 different experiments indicating partial lumen clearance.

4.4 Discussion

The transmembrane tight junction protein JAM-A has been implicated in several cellular functions including the control of mucosal homeostasis and tight junction integrity (Vetrano et al., 2008, Yeung et al., 2008, Laukoetter et al., 2007), regulation of cell migration (Severson et al., 2008) and leukocyte migration (Woodfin et al., 2007). However, as previously alluded to, very little is known about the expression of JAM-A in breast epithelium and its derived cancers. Two interesting studies on JAM-A and breast cancer gave conflicting evidence of a potential role for JAM-A in breast cancer progression. Naik *et al.* initially published a paper in 2008 stating that knockdown of JAM-A enhanced invasiveness in two breast cell lines - MDA-MB-231 and T47D, thus suggesting that JAM-A is a negative regulator of breast cancer invasion (Naik et al., 2008). This was disputed by McSherry *et al.* who showed that on an invasive breast cancer tissue microarray of 270 patients, high JAM-A expression correlated with reduced breast cancer disease–specific survival, reduced 5-year recurrence-free survival and higher tumour grade (McSherry et al., 2009).

Since neither of those publications was in print at the time our study was started, the role of JAM-A alterations in breast cancer progression was still completely unknown. Having established cellular models (in chapter 3) with which to investigate this area, we showed that high protein expression of JAM-A correlated with aggressive tumour phenotypes in primary cell cultures and in an isogenic cell line series consisting of normal and invasive cells. Based on this finding, we sought to determine if antagonism of JAM-A would alter tumorigenic phenotypes in breast cancer cell lines, estimated by measuring their proliferative and migratory characteristics.

Our results in this chapter demonstrated that JAM-A antagonism decreased proliferation in both 2-dimensional and 3-dimensional cultures treated with the inhibitory antibody J10.4. By light and confocal microscopy we noted a decrease in the number of cells in all JAM-inhibited cell lines compared to controls. Curiously however, anti-proliferative changes were not observed in MTT proliferation assays in either the Hs578T or HMT-3522 cell lines. No published studies to date have investigated the role of JAM-A in proliferation and breast cancer. However, Azari *et al.* recently showed that JAM-A is required for proliferation of vascular smooth muscle cells in coronary artery disease (Azari et al., 2010). Therefore in the context of JAM-dependent regulation of breast cell proliferation, much remains to be investigated in future studies.

However proliferation is only one of several functional behaviours that are relevant to cancer progression. For example, cell migration is a key contributor to early invasive behaviour during metastasis. Thus we proceeded to investigate the impact of JAM-A antagonism on cell migration in non-tumorigenic and tumorigenic HMT-3522 cells. We observed a significant decrease in cell migration in both cell types upon JAM-A inhibition with J10.4. This contrasted with the work stating that over-expression of JAM-A inhibited cell migration and invasion (Naik et al., 2008). However our results are supported by a study showing a reduction in migration of colonic epithelial cells upon JAM-A antagonism using the same antibody (J10.4) (Severson et al., 2008); in addition to work by McSherry *et al.* showing that knockdown of JAM-A led to decreased cell migration (McSherry et al., 2009). A very recent paper also validates this theory; having shown that over-expression of the micro RNA miR145 in breast cancer cells led to a downregulation of JAM-A which in turn led to decreased migration and invasiveness (Gotte et al., 2010).

One study has suggested that JAM-A affects migration through a pathway involving β1-integrin, as disruptions in JAM-A dimerisation led to internalisation and degradation of β1-integrin (Severson et al., 2008). Integrins are involved in the directionality of cell movement and the subsequent breakdown of cell-cell adhesive structure (Hynes, 2002). They also play an important role in migration of cells in breast cancer, not only for physically tethering cells to the matrix, but for sending and receiving molecular signals that regulate these processes (Hood and Cheresh, 2002). Studies have shown that over expression of AKT2 (a serine/threonine protein kinase involved in multiple cellular processes such as cell proliferation, apoptosis and cell migration) in breast cancer cells leads to an upregulation of β1-integrin and promotes breast cancer cell invasion (Arboleda et al., 2003). Results from our laboratory have also suggested that JAM-A over-expression enhances β1-integrin expression in breast cancer tissues (McSherry et al., 2009). To further elucidate the pathway of JAM-A signalling in breast cancer, we sought to investigate whether JAM-A was signalling downstream to β1-integrin in breast cancer cell lines, by determining whether a stimulatory β1-integrin antibody could reverse the anti-migratory effects of JAM-A antagonism in invasive Hs578Ts(i)8 cells. Migration assays did not yield definitive results because of technical issues surrounding the best method of quantification that still need to be overcome, however it will be important to design alternative future studies to determine whether β1-integrin is a direct downstream target of JAM-A in invasive cells.

In addition to its regulatory control of cell migration, β1-integrin also plays important roles in regulating growth and apoptosis (Guo and Giancotti, 2004). It has been reported that an inhibitory antibody to β1-integrin induced a striking morphological and functional reversion of tumorigenic cells to a normal phenotype (Weaver et al., 1997). Based on this

knowledge, we sought to investigate if JAM-A antagonism in 3-dimensional cultures decreased proliferation (as noted in the 2-dimensional cultures earlier) and influenced the abnormal morphology of T4-2 cells. We noted a significant reduction in the number and size of 3-dimensional spheroids upon treatment with inhibitory JAM-A antibody. Interestingly, we also noted a partial reversion of the abnormal phenotype in invasive T42 cell clusters; supporting our hypothesis that JAM-A signalling may contribute to breast cancer progression and that JAM-A is a likely upstream regulator of β1-integrin in this context.

Taken together, our results have shown that dimerisation of JAM-A is involved in downstream signalling events that promote cell migration. JAM-A is also an important regulator of cell polarity, and inhibition of JAM-A in invasive breast cancer cells induces partial reversion to a more normal phenotype. We thus suggest that pharmacological antagonism of JAM-A in breast cancer may offer hope as a potential therapy and so further research into JAM-A and breast cancer is both necessary and essential.

Chapter V

Discussion

Overall discussion

Breast cancer is the leading cause of female cancer, with approximately 2300 new cases in Ireland yearly (National Cancer Registry - www.ncri.ie) Breast cancer is a heterogeneous disease with many aetiological factors contributing to development of the disease, including environmental, genetic and hormonal factors. Most tumours originate in the milk ducts, which are composed of a layer of polarized epithelial cells surrounded by myoepithelial cells. Loss of cell polarity is an early hallmark of breast cancer.

One of the epithelial tight junction proteins, JAM-A, has been implicated in controlling cell polarity. JAM-A regulates many cellular adhesive processes including intercellular junction assembly, cell morphology, and leukocyte migration (Mandell et al., 2005, Martin-Padura et al., 1998). Dysregulation of various tight junction proteins has been reported in several cancers (Isabel J. Latorre et al., 2000) but little is known about whether JAM-A has a role to play in breast cancer. Thus this thesis focussed on the functional relevance of JAM-A in breast cancer progression.

To initiate our studies, two isogenic breast cancer cell line series (HMT-3522 and Hs578T) were employed. Both isogenic models portrayed non-tumorigenic and tumorigenic variants of the same genotypic backgrounds. The isogenic cell line HMT-3522 was identified to mimic breast cancer progression from premalignant to invasive carcinoma (Briand and Lykkesfeldt, 2001); while the isogenic Hs578T cell line was noted to model cancer invasiveness from non-tumorigenic parental cells to tumorigenic cells (Hughes et al., 2008). We first characterised differences between the normal and invasive cells of the isogenic models in order to examine their suitability as a model in investigating the role of JAM-A in breast cancer progression.

One of the main functions of tight junctions is the regulation of epithelial barrier integrity. In this thesis we showed differences in the tight junction function between HMT-3522 S1 and T4-2 cells, namely that T4-2 (tumorigenic) cells had a higher transepithelial resistance and a lower transepithelial solute flux, indicating tighter junctions than in S1 (non-tumorigenic) cells. We also observed higher expression of JAM-A in the invasive variant of Hs578T cells compared to their normal counterparts, and found that JAM-A levels were higher in human breast tumour primary cultures relative to non-tumour cultures. Interestingly, JAM-A expression in primary cultures from invasive ductal carcinomas of grade 3 was higher than that of the lower grades.

Having demonstrated high levels of JAM-A expression in aggressive tumour samples, we further investigated whether JAM-A might be shed from tumour cells and act as a serum biomarker of disease. Interestingly, measurement of soluble JAM-A in the serum of patients with breast cancer (collected prior to surgery) revealed the opposite correlation to that noted in tissue samples. Specifically, benign samples showed higher concentrations of JAM-A in serum compared to that in invasive carcinoma samples. From this result, we speculate that JAM-A expression is being retained on breast tissues during disease progression, possibly contributing to tumorigenic signalling events, whereas that on the surface of normal cells is being shed into the bloodstream so those signalling pathways remain silent. Interesting correlations have been reported between levels of serum JAM-A and various cardiovascular diseases (Azari et al., 2010). Azari *et al.* showed that JAM-A is needed for the growth of atherosclerotic plaques and subsequent migration of inflamed smooth muscle cells. Thus, silencing of JAM-A in vascular smooth muscle cells has an anti-proliferative effect. In relation to our study, it is intriguing to speculate that high serum JAM-A levels noted in benign samples produce anti-proliferative and anti-migratory effects. However further studies must be performed to confirm or deny these effects.

Taken together, we observed higher JAM-A expression in more invasive cells in primary cultures and in Hs578T cells, while serum levels of JAM-A showed higher concentrations in benign samples compared to invasive samples. To date, only one report has been published on the effects of JAM-A over-expression and cancer aggressiveness (McSherry et al., 2009); and this supports our findings. In that study, an inhibitory antibody to JAM-A inhibited migration in MCF7 cells (high JAM-A expressing cells). In contrast, Naik *et al.*. (Naik et al., 2008) reported a decrease in the migratory properties of MDA-MB-231 cells (low JAM-A expressing cells) upon over-expression of JAM-A. They also reported that cell lines with high endogenous JAM-A portrayed non-invasive properties while those with low levels of JAM-A were highly invasive cells.

Due to these recent conflicting findings on whether JAM-A positively or negatively regulates tumorigenic behaviour, we investigated further the functional effects of JAM-A inhibition in the isogenic cell lines using an inhibitory antibody to JAM-A. Proliferation is one of the cellular mechanisms involved in cancer progression. We carried out proliferation assays to evaluate differences between normal and invasive cells following treatment with a JAM-A function-blocking antibody, J10.4. While there was no significant decrease in the proliferation of J10.4 treated cells in either of the cell lines using these assays, we however observed qualitative reductions in cell number upon J10.4 treatment (evidenced by phase contrast micrographs and confocal micrographs of 3-dimensional cultures). This discrepancy between qualitative and quantitative data highlights that future studies will have to address more specifically the possible mechanisms whereby JAM-A might influence proliferation. It also highlights the limitations of *in vitro* assays if considered in isolation. Further evidence of these limitations was seen in immunoprecipitation assays carried out to compare the protein binding partners of JAM-A in HMT-3522 S1 and T4-2 cells (data not shown). Interestingly, though much is known

about the binding partners of JAM-A in other cellular systems (Brennan et al., 2010), we did not detect binding between JAM-A and its traditional binding partners in the HMT-3522 cell line model, further indicating that *in vitro* studies must be interpreted with caution.

To further evaluate the functional effects of JAM-A inhibition via another mechanism, migration assays were carried out with the isogenic cells. Scratch wound assays demonstrated significant reductions in migration of HMT-3522 cell lines upon treatment with the inhibitory antibody J10.4, which acts by blocking the dimerisation of JAM-A. This finding is in keeping with recent papers stating that knockdown of JAM-A leads to decreased cell migration in breast cells (McSherry et al., 2009) and that over expression of the micro RNA miR145 down regulates JAM-A leading to decreased migration and invasion (Gotte et al., 2010).

Some studies have suggested that JAM-A dimerisation is required for the downstream promotion of cancer cell migration, and that it may function through β1-integrin as a downstream effector (McSherry *et al.*., 2009, Severson *et al.*., 2009). Additionally, expression levels of β4-integrin and E-cadherin (cell-cell adhesion proteins) were unchanged upon JAM-A knockdown while β1-integrin levels were decreased (McSherry et al., 2009). Cell-surface integrin receptors bind to components of the extracellular matrix and are important for cell movement; therefore we next investigated the relationship between JAM-A and β1-integrins in our cell line series. We hypothesized that if JAM-A inhibition decreased migration and β1-integrin is involved in migration, it is possible that both JAM-A and β1-integrin could be in the same pathway. Using Hs578T invasive cells (which express high levels of JAM-A) we sought to investigate if JAM-A and β1-integrin are in a linear pathway. For this approach, a JAM-A inhibitory antibody (J10.4) and a stimulatory β1-integrin antibody (TS2/16) were used. Although our results were

inconclusive (data not shown), we speculate that future investigations will validate JAM-A to signal downstream through β1-integrin to control migration.

β1-integrin also has a role to play in polarization, as evidenced by an important study showing that inhibition of β1-integrin function reduced tumorigenicity and the malignant phenotype of T4-2 tumorigenic breast cancer cells (Weaver et al., 1997). A similar study using β1-integrin blocking antibodies in prostate cancer cells was also observed to revert tumorigenic F6 cells to polarised acinar-like structures in 3-dimensional cultures (Zhang et al., 2009). Thus we speculated that if JAM-A and β1-integrin were in the same pathway, JAM-A inhibition in 3-dimensional breast cultures could potentially revert the tumorigenic phenotype in the same way that b1-integrin antagonism has been shown to do. Our study showed formation of 3-dimensional spheroids in the HMT-3522 cell line series and not in the Hs578T cells. Specifically, the non-tumorigenic (S1) variant of HMT-3522 cells formed acinus-like structures while the tumorigenic variant cells (T4-2) formed clumps of cells reminiscent of tumours. A highlight and very novel finding of this thesis was that the tumorigenic phenotype of T4-2 cells was partially normalised upon JAM-A inhibition. This is an exciting result which, in light of the β1-integrin blocking studies mentioned above, supports the possibility that β1-integrin functions downstream of JAM-A in controlling cellular behaviours relevant to the progression of breast cancer.

These findings, although promising, must be contextualized with the fact that the role of JAM-A in a clinical setting of breast cancer is still yet to be discovered. Tight juctions as a whole are primary regulators of paracellular transport across epithelial cells (Gonzalez-Mariscal et al., 2005), and successful drug delivery may require modulation of tight junction proteins to allow drug molecules to pass (Matsuhisa et al., 2009). To date, there are no cancer therapies on the market which specifically target tight junctions. However

several tight junction proteins have been described as receptors for specific molecules or organisms, and as such, these might provide valid and novel targets for drug delivery. For instance, Claudin-3 and -4 have been suggested as drug delivery targets because they act as the receptor for *Clostridium perfringens* enterotoxin (CPE). The ability of CPE to rapidly and specifically lyse cells expressing claudin-3 or -4 could potentially be exploited in the treatment of breast cancers over expressing these proteins.(Santin et al., 2007, Morin, 2005, Katahira et al., 1997) As so little is known in the field of JAM-A and drug delivery, future investigations of tight junction proteins as candidates for drug targeting to prevent or limit breast cancer progression will be valuable. Our study in this thesis has suggested much potential for future consideration of JAM-A as a potential therapeutic target, a biomarker or even as a drug delivery system in breast cancer.

In this thesis, the use of two isogenic cell line series of the same genotype but different phenotypes - one being phenotypically "normal" and other phenotypically "invasive" - has allowed us a unique opportunity to begin studying the fundamental role of JAM-A in the regulation of growth and tissue morphogenesis. The HMT-3522 cell line series provided an excellent model to study the role JAM-A plays in functional reversion of an invasive phenotype using 3-dimensional cultures. The Hs578T cells showed marked differences between their invasiveness and expression of JAM-A in support of published data on the higher expression of JAM-A in more aggressive cells. However, it must be noted that neither of the isogenic cells proved to be good models for assessing JAM-A function in proliferation.

Overall, we have identified that JAM-A influences morphology, proliferation and migration of breast cancer cells. Subsequently we showed that JAM-A antagonism normalises a tumorigenic phenotype in 3-dimensional breast cancer cultures, leading us to

hypothesise that these influences are via β1-integrin acting downstream of JAM-A. This wealth of evidence indicates the potential involvement of JAM-A in breast cancer initiation and progression and thus, further studies on JAM-A will allow a more comprehensive understanding of the behaviour and contributions of JAM-A to breast cancer tumour progression.

Future work

The work on JAM-A and breast cancer is very novel with so many avenues worthy of future study. Several studies have implicated JAM-A in the control of proliferation, though not yet in breast cancer disease. Further experiments in breast cancer cell lines and possibly *in vivo* studies to explore the functional role of JAM-A in proliferation would be valuable. Our work also showed high levels of JAM-A in primary breast tissues with a corresponding low JAM-A levels in serum of breast cancer patients. We propose further evaluation of this theory in a larger cohort of patients to establish if JAM-A could be a potential biomarker in breast cancer disease. Mandell *et al.* and Severson *et al.* observed that JAM-A regulates epithelial morphology and migration via β1-integrin in colonic cells. Preliminary results done in this thesis to evaluate JAM-A signalling pathway were ambiguous (data not shown). We thus propose that further work to elucidate the functional role of β1-integrin in JAM-A signalling will help in understanding the cellular mechanisms whereby JAM-A might regulate cellular behaviours relevant to breast cancer progression.

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Appendices

Appendix A

Tissue Culture Reagents and Media

- **Hs578T**

DMEM	500ml
Insulin	0.01mg/ml
L-Glutamine	5% Final concentration
Penicillin / Streptomycin(100X)	5mls (1X)
Foetal Bovine Serum	10% Final concentration

- **HMT-3522 cells medium composition**

S1 / T4-2 cells

Add to DMEM/F12 medium the following additives

Ingredients	Stock reagents	Vol. in 10 ml	Final conc.	Expiration	
				4° C	-20° C
Insulin	100 µg/ml	25 µl	250 ng/ml	1 mo	5 mo
Transferrin	20 mg/ml	25 µl	10 µg/ml	1 mo	3 mo
Sodium Selenite	2.6 µg/ml	50 µl	2.6 ng/ml	1 wk	1 mo
Estradiol	10^{-7} M	50 µl	10^{10} M	3 mo	6 mo
Hydrocortisone	$1.4x10^{-3}$ M	50 µl	$1.4x10^{-6}$ M	1 mo	6 mo
Prolactin	1 mg/ml (30u/ml)	250 µl	5 µg/ml	1 mo	6 mo
EGF (S1 only)	20 µg/ml	25 µl	10 ng/ml	2 wk	3 mo

Insulin: Dissolve 20 mg in 10 ml of 5 mM HCl. Sterile filter and aliquot in 1ml portions. Dilute 1 ml of the former stock into 19 ml of distilled water to give 100 micrograms/ml. This was sterile filtered and aliquoted into 1ml portions as the working stock. Store in the -80°C freezer.

Transferrin: Dissolve 200mg in 10mls of purified water. This was sterile filtered and aliquoted into 200µl volumes as the working stock. Store in the -80°C freezer.

Sodium Selenite: Dissolve 100mg in 5ml purified water. This stock was further diluted by taking 52µl of previous stock in 10mls purified water. This was aliquoted into 250µl portions and stored in the -80°C freezer.

Estradiol: This chemical is light sensitive so perform in the dark. Dissolve 25mg in 3.125ml of 95% ethanol or pure ethanol. Serial dilutions are made in ethanol in 1mM, 10µM, 0.1µM concentrations. 0.1 µM stock is the working concentration. This was aliquoted into 200µl portions, wrapped in foil and stored in the -80°C freezer.

Hydrocortisone: Dissolve 50mg in 10ml of 95% ethanol or pure ethanol. This is aliquoted into 1ml portions and stored in the -80°C freezer. The working stock was made by diluting 1ml of the former stock into 9ml ethanol.

Prolactin: $NaHCO_3$ solution was prepared by dissolving 110.83mg in 50 mls of H_20.

1000IU prolactin was dissolved into 33.3ml $NaHCO_3$. This was sterile filtered and aliquoted into 1ml portions as the working stock.

EGF: 100µg was dissolved in 5mls purified water. This was aliquoted into 100µl portions and stored at -80°C.

- **Primary Breast culture cells**

Digestion mix = Digestion media + Digestion Enzymes

Digestion Media

DMEM-F12 Ham	500ml
10% fetal bovine serum	50ml
10µg/ml insulin	5ml
5µg/ml Fungizone	5ml
10X penicillin/Streptomycin/neomycin	5ml

Digestion Enzymes

100U/ml Hyaluronidases / 200U/ml Collagenases 1X mix

Feeding media

MEBM	500mls
Pen / Strep (100X)	5mls (1X)
Bullet Kit®	

Appendix B

Protein Extraction / Western Blotting Buffers and Solutions

Relax Buffer

100mM KCl	1.49g
3mM NaCl	0.035g
3.5mM MgCl	0.067g
10mM HEPES	2mls

Made up in dH_2O

pH 7.4 with HCl or NaCl

Add 1% TX-100 and 1:100 protease and phosphatase inhibitor cocktails before use

10% w/v Sodium dodecyl sulphate (SDS)

SDS	2g
H_20	20mls

4X Tris-HCl/SDS Buffer pH8.8

Trizma base	90.83g
20% SDS	10ml
dH_2O	200ml

pH to 8.8 with concentrated HCl (~8ml)

Make up to 500ml with dH_2O

4X Tris-HCl/SDS Buffer pH6.8

Trizma base	15.14g
20% SDS	5ml
dH_2O	200ml

pH to 6.8 with concentrated HCl (~6ml)

Make up to 250ml with dH_2O

10% Ammonium Persulphate

Ammonium Persulphate	1g
dH_20	10ml

store at 4°C

SDS-PAGE Gels

Separating Gel

Chemicals	*6% Gel*	*7.5% Gel*	10% Gel
30% Acrylamide/ 0.8% Bisacrylamide	4ml	3.75ml	5ml
Tris-HCl/SDS pH 8.8	3.75ml	3.75ml	3.75ml
dH_2O	8.25ml	7.5ml	6.25ml
10% Ammonium Persulphate	50µl	50µl	50µl
TEMED	10µl	10µl	10µl

Stacking Gel

Chemicals	**Volume**
30% Acrylamide/0.8% Bisacrylamide	0.65ml
Tris-HCl/SDS pH 6.8	1.25ml
dH_2O	3.05ml
10% Ammonium Persulphate	25µl
TEMED	5µl

2X Sample Buffer

10% SDS	300µl
Glycerol	200µl
Tris Blue (1.5M Tris)	500µl

pH 8.8 plus Bromophenol blue)

+40µl DTT (40mM in 2X)

4X Sample Buffer

SDS	0.5g
Tris HCl pH 6.8	5ml
Glycerol	4ml

Bromophenol blue (to achieve the desired colour)

1ml dH_2O

+800µl DTT (80mM in 4X)

10X Tris Glycine

Tris base	30.3g
Glycine	144g

Make up to 1litre with dH_2O

Running Buffer

Tris-Glycine 10X	100ml
20% SDS	5ml
dH_2O	890ml

Transfer Buffer

Glycine	11.5g
Trizma Base	2.5g
dH_2O	640ml
Methanol	160ml

(+ 4ml 10% SDS for proteins >150kda)

Ponceau Red

Ponceau S	50mg
dH_2O	50ml
Glacial acetic acid	150µl

10X TBS

NaCl	80g
KCl	2g

Trisma base	30g
dH_20	800mls

pH 7.4 with conc HCl.

Add dH_20 to 1L final vol.

1X TBS

Dilute 100mls 10x TBS in 1L dH_20

1X TBS Tween

10X TBS	100mls
100% Tween-20	1ml

dH_20 to 1L final vol.

Stripping Buffer

50mM Tris, pH 6.8	25mls
20% SDS	40mls
dH_20	335mls

Before use, add 100mM β-mercaptoethanol

RIPA Buffer

Tris HCl 20 μM	6.057g
NaCl 150 μM	0.877g
EDTA 5 μM	0.146g

1% Triton X-100

Immunofloresecence Staining for Coverslips and 3D cultures

3.7% Paraformaldehyde

Paraformaldehyde	0.74g

Dissolved in 10mls hot PBS

pH to 7.4 with 1M NaOH and 1M HCL

(Use litmus paper as too corrosive for pH electrodes)

Make to a final volume of 20mls with PBS

10X PHOSPAHATE BUFFERED SALINE (PBS)

NaCl	80g
KCl	2g
Na_2HPO_4	14.4g
KH_2PO_4	2.4g

dH_20	800mls

Adjust to PH 7.4 with conc HCl.

Add dH_20 to 1litre final vol.

10X IF WASH

NaCl	7.6g
Na_2HPO_4	1.876g
NaH_2PO_4	0.414g
NaN_3	0.5g
BSA	1g
Triton X-100	2mls
Tween-20	0.41mls

10X PBS/ GLYCINE

NaCl	30.0g
Na_2HPO_4	9.38g
NaH_2PO_4	2.07g
Glycine	37.5g

Bring up to a total volume of 500mls. pH 7.4

Functional Assays

Hanks Balanced Salt Solution

$HBSS^+$ and $HBSS^-$ were prepared according to the manufacturer's instructions. Briefly, 1 bottle of reagent was dissolved in 800ml dH_2O. 0.35g sodium bicarbonate was added and NaCl and HCl were used to pH the solution (pH 7.4). 10ml (10mM) HEPES was added and the solution made up to 1l with dH_2O.

Isolation Buffer

30mM EDTA

52mM NaCl

5mM KCl

10mM HEPES

2mM DTT

60mM HCl

Made up in Ca^{2+}-free HBSS.

pH 7.1 with 2.5M Tris

P Buffer

10mM HEPES ph 7.4	200 µl
1mM sodium pyruvate	200 µl
10mM glucose	3.6g
3mM $CaCl_2$	2.94g
145Mm NaCl	1.17g
Total Volume	20mls

Appendix C

Chemical Suppliers

Reagents	Product code	Supplier
Acrylamide/bis-acrylamide	A3699	Sigma
Ammonium persulfate	A3678	Sigma
Bromophenol blue	B8026	Sigma
BCA Protein assay kit	23227	Pierce
Collagenases	C0130	Sigma
Cyquant Assay Kit	7026	Invitrogen
DAPI	D9163	Sigma
DMEM/Ham's F12	D8437	Sigma
DMSO	D5879	Sigma
DTT	D9163	Sigma
ECL Western blotting substrate	32106	Pierce
EDTA	E9884	Sigma
Estradiol	E-2758	Sigma
Foetal Bovine Serum	DE14-801F	Lonza
Glycerol	G5516	Sigma
Glycine	G7126	Sigma
Goat anti-mouse HRP	A9917	Sigma
Goat anti-rabbit HRP	A0545	Sigma
HBSS without phenol red	H1387	Sigma

HBSS without phenol red, $Ca2^+$, $Mg2^+$	H4891	Sigma
HEPES	H0887	Sigma
Hydrochloric acid (HCl)	84426	Sigma
Hydrocortisone	H-0888	Sigma
Hyaluronidases	H3506	Sigma
Insulin	I-6634	Sigma
Kodak developer/replenisher	P7042	Sigma
Kodak fixer/replenisher	P7167	Sigma
Matrigel	354230	BD Bioscience
MEBM+BulletKit®	cc3151	Lonza
β-Mercaptoethanol	M7154	Sigma
Methanol	24229	Sigma
MTT	M2128	Sigma
Paraformaldehyde	15812-7	Sigma
p-Phenylenediamine	P6001	Sigma
Ponceau S	78376	Sigma
Potassium chloride (KCl)	P4504	Sigma
Prolactin	L-6520	Sigma
Protein G-sepharose beads	17-0618-01	GE Healthcare
Rabbit anti-Actin	A2066-0.2ml	Sigma
Sodium bicarbonate	S8875	Sigma
SDS	71730	Sigma
Sodium chloride (NaCl)	S9625	Sigma
SuperSignal West Femto Maximum Sensitivity Substrate	(34095)	Pierce
TEMED	T9281	Sigma

Transferrin (Human)	T-2252	Sigma
Triton-X 100	93426	Fluka
Trizma base	T6066	Sigma

Antibodies	Product Code	Supplier
Mouse anti-ZO-1	610966	BD Bioscicences
Mouse anti-Occludin	33-1500	Zymed
Rabbit anti-Occludin	71-1500	Zymed
Rabbit anti-ZO-1	61-7300	Zymed
Rabbit anti-ZO-1	38-9000	Zymed
Mouse monoclonal anti-JAM-A (J10.4)	SC-53623	Santacruz
Mouse monoclonal anti-JAM-A (J10.4) (without azide or gelatin)	SC-53623L	Santacruz
Rat anti β1 integrin	552828	BD Biosciences
Goat anti Rabbit HRP	7074	Cell Signalling
Goat anti Mouse HRP	A 9917	Sigma
Goat anti Rat HRP	A 9037	Sigma
IgG Mouse	I 5381	Sigma
Alexa Fluor® 488 phalloidin	A 12379	Molecular Probes
IgG Rat	I 4131	Sigma

Concentrations of antibodies used in Western Blotting

Antibodies	Concentrations	Supplier
Mouse anti-ZO-1	1:500	BD Biosciences
Mouse anti-Occludin	1:500	Zymed
Rabbit anti-Occludin	1:500	Zymed
Rabbit anti-ZO-1	1:500	Zymed
Rat anti β1 integrin	1:500	BD Biosciences
Goat anti Rabbit HRP	1:3000	Cell Signalling
Goat anti Mouse HRP	1:5000	Sigma
Goat anti Rat HRP	1:5000	Sigma
Rabbit anti-JAM-A	1:500	Zymed

Publications

(Attached at rear of thesis)

Manuscripts Published-

Brennan K, **Offiah G**, McSherry EA, Hopkins A.M. "Tight junctions – a barrier to the initiation and progression of breast cancer?" (Review) *J. Biomed. Biotechnol.* 2010. PMID: 19920867

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Manuscript in preparation: **Offiah G**, Hill ADK, Hopkins AM. "HMT3522 cell lines series as a good model in elucidating mechanistic pathways in breast cancer progression".

Presentations

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Offiah G, McSherry EA, Hill ADK, Hopkins AM. The role of JAM-A in breast cancer invasion. Cancer conference, St. James' Hospital, Dublin May 2009; Poster presentation

Offiah G, Hill ADK, Hopkins AM. JAM – a sticky situation in breast cancer? Waterford Surgical Research Meeting 2009; Poster presentation. (**First prize in category**).

Offiah G, Hill ADK, Hopkins AM. The role of JAM-A in breast cancer invasion. Research Day, Beaumont Hospital, April 2009; Poster presentation

Offiah G, Hill ADK, Hopkins AM. Junction Adhesion Molecule – A: A new target in Breast Cancer Invasion? International Mater breast conference, May 2009; Poster presentation

Offiah G, McSherry EA, Hill ADK, Hopkins AM. Potential of JAM-A as an anti-proliferative target in breast cancer. RAMI conference, Registrar's prize Jan 2010; Oral presentation

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Offiah G, McSherry EA, Hill ADK, Hopkins AM. Junctional Adhesion Molecule-A (JAM-A): A Potential Target for Breast Cancer Anti-proliferative Therapy? Sylvester O'Halloran Surgical Meeting, Limerick March 2010; Oral presentation

Offiah G, McSherry EA, Hill ADK, Hopkins AM. Junctional Adhesion Molecule-A (JAM-A): A Potential Target for Breast Cancer Anti-proliferative Therapy? RCSI research day (April 2010); Poster presentation

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Review Article

Tight Junctions: A Barrier to the Initiation and Progression of Breast Cancer?

Kieran Brennan, Gozie Offiah, Elaine A. McSherry, and Ann M. Hopkins

Department of Surgery, Royal College of Surgeons in Ireland, Dublin, Ireland

Correspondence should be addressed to Ann M. Hopkins, annhopkins@rcsi.ie

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Breast cancer is a complex and heterogeneous disease that arises from epithelial cells lining the breast ducts and lobules. Correct adhesion between adjacent epithelial cells is important in determining the normal structure and function of epithelial tissues, and there is accumulating evidence that dysregulated cell-cell adhesion is associated with many cancers. This review will focus on one cell-cell adhesion complex, the tight junction (TJ), and summarize recent evidence that TJs may participate in breast cancer development or progression. We will first outline the protein composition of TJs and discuss the functions of the TJ complex. Secondly we will examine how alterations in these functions might facilitate breast cancer initiation or progression; by focussing on the regulatory influence of TJs on cell polarity, cell fate and cell migration. Finally we will outline how pharmacological targeting of TJ proteins may be useful in limiting breast cancer progression. Overall we hope to illustrate that the relationship between TJ alterations and breast cancer is a complex one; but that this area offers promise in uncovering fundamental mechanisms linked to breast cancer progression.

1. Introduction

Breast cancer is the most common form of cancer among women in North America and the majority of European nations. Each year, it is diagnosed in an estimated 1 million women worldwide, and is the cause of death of over 400 000 [1]. The incidence of breast cancer increases with age and doubles every 10 years until the menopause, supporting a link with hormonal status [2]. Specific life events associated with an enhanced breast cancer risk include reproductive factors, nulliparity, radiation exposure, hormonal status, obesity, family history, and many others [3, 4].

Breast cancer is a heterogeneous disease in which genetic and environmental factors interact to initiate carcinogenesis. However, 10% of all breast cancer cases have a strong hereditary component in which half carry a deleterious mutation in the high penetrance genes BRCA1 or BRCA2. These account for over 50% of familial breast cancer cases and confer a lifetime risk of 60–80% [5]. In its simplest forms, breast cancer can be subclassified into preinvasive and invasive disease categories. Neoplastic conversion to invasive cancer likely occurs sometime during the preinvasive histological phases of usual hyperplasia, atypical hyperplasia, and ductal carcinoma in situ (DCIS) [6–11]. One hypothesis suggests the existence of genetically distinct subgroups of DCIS, only some of which subsequently progress to invasive ductal carcinoma (IDC) [12–14]. An alternate theory proposes that DCIS progresses from low to high grades and then to invasive cancer with progressive accumulation of genomic changes. However, the large extent to which the genome is altered in DCIS indicates that genomic instability most likely precedes phenotypic evidence of invasion, and highlights the importance of environmental components on the development of invasive cancer [6].

Recent data have shown significant reductions in the mortality rates of breast cancer, which have been mainly attributed to improved screening techniques, improved surgical and radiotherapy interventions and also the utilization of traditional chemotherapies in a more efficacious manner. Large-scale translational research studies have also identified many important new biomarkers predictive of poor prognosis in breast cancer patients [15–17]. However, much remains

to be understood about the development and progression of breast cancer. Our review will address the contribution of altered epithelial cell-cell adhesion to the development and progression of breast cancer, with particular emphasis on the role of the tight junction (TJ) adhesion complex in these processes.

2. TJs and Physiological Cell-Cell Adhesion

Cell-cell adhesion is necessary for the assembly of coherent sheets of barrier-forming epithelial cells that line the breast ducts and lobules. However cell-cell contacts are far from being static structures which maintain barriers by simply holding cells together. In fact cell-cell contacts undergo constant remodelling to allow the extrusion of apoptotic cells as well as the incorporation of newly differentiated epithelial cells, derived from progenitor cells, without loss of barrier function [18]. Cell-cell contacts must also be remodelled depending on the developmental stage of the breast, whether in response to increased proliferative demands of puberty and pregnancy, increased differentiation during lactation, or increased apoptosis in conjunction with gland remodelling during involution [19]. Finally, during wound healing, epithelial cells can undergo coordinated movement and proliferation to bridge the wound, and establish new cell-cell contacts with epithelial cells from the opposing side of the wound [20].

Epithelial cell-cell contacts consist of three main adhesive structures: tight junctions (TJs), adherens junctions and desmosomes, as well as gap junctions for cell-cell communication (Figure 1). In polarized epithelial cells the tight junction and adherens junction are asymmetrically distributed at the apical region of the lateral membrane forming the apical junctional complex, which encircles the apex of the cells and marks the border between the apical and basolateral membrane domains [21]. These adhesive structures are composed of integral membrane proteins that link the neighbouring cells through homophilic and heterophilic interactions, and the presence of cytoplasmic scaffolding proteins that organise signalling complexes and anchor cell-cell contacts to the actin cytoskeleton (or intermediate filaments in the case of desmosomes) [22].

In this review we will first outline the protein components of the TJ and discuss the biological roles of the TJ complex, review how alterations in these roles could facilitate breast cancer initiation or progression, and finally mention pharmacological approaches towards targeting TJ proteins that could have value in limiting breast cancer progression.

2.1. Protein Components of the TJ. Proteins within TJ can be grouped into integral membrane proteins, scaffolding proteins, or signalling proteins as outlined in what follows.

2.1.1. Integral Membrane Proteins. The first protein to be discovered at the tight junction, occludin [23], is a 68 kDa transmembrane protein with two extracellular loops and a long cytoplasmic tail containing several protein-binding domains. It exists in unphosphorylated and serine/threonine

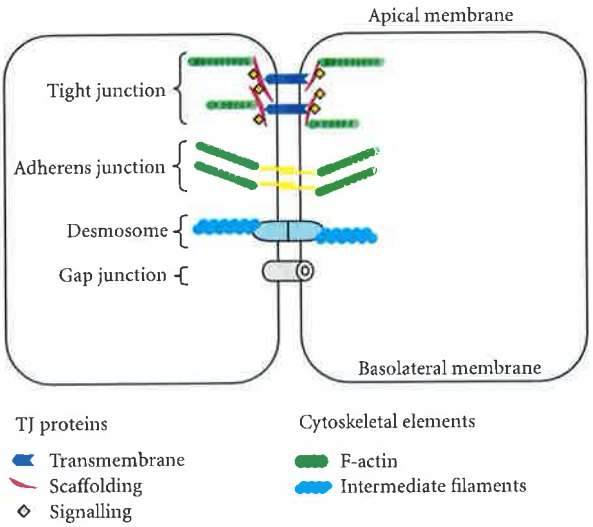

FIGURE 1: Epithelial cell-cell adhesion complexes.

and tyrosine phosphorylated forms, with the degree of phosphorylation affecting tight junction assembly, transepithelial resistance, and localisation of occludin to the tight junction [24, 25]. Several enzymes are involved in regulating these phosphorylation events, including PKC [26], CK2 [27], and the nonreceptor tyrosine kinase c-Yes [24].

The claudin family of TJ transmembrane proteins consists of 24 members between 20 and 27 kDa in size, mostly with short cytoplasmic tails which bind to the PDZ (PSD-95, Dlg, and ZO-1) domains of other TJ proteins including ZO-1, -2, and -3 [28]. Various claudins are expressed in a tissue-specific manner, with the subtle differences in their extracellular loops determining ion selectivity of the paracellular pathway [29, 30].

The junctional adhesion molecule (JAM) family consists of "JAM-A, -B, -C, -L and JAM-4, ..." which are found at TJs of epithelial/endothelial cells and on various hematopoietic cells [31–33]. They contain an extracellular region with two Ig-like domains, a single transmembrane domain, and a short intracellular tail with a PDZ binding motif through which JAM-A interacts with the PDZ proteins AF6, Par-3, CASK, MUPP1, and ZO-1 [34–38].

The coxackie and adenovirus receptor (CAR) is a 46 kDa integral membrane protein with one transmembrane region, a long cytoplasmic tail, and an extracellular region composed of two Ig-like domains. The carboxyl terminal domain of CAR contains a PDZ binding motif which interacts with ZO-1, MUPP-1, MAGI, and PICK1 [39–41]. CAR is required for MUPP1 localization at the tight junction [39].

Crumbs3 (CRB3) is a single-pass membrane protein located at the apical cell membrane with a small fraction in the upper part of tight junctions of epithelial cells. It is involved in the establishment of cell polarity in mammalian epithelial cells and regulates the morphogenesis of tight junctions. CRB3 interacts with PAR6, PALS1, and PATJ [42–45].

Tricellulin is a 66 kDa membrane protein which has multiple phosphorylated states but exists predominantly in its unphosphorylated form. It is enriched only at tricellular tight junctions, where it enforces the barrier function of epithelial cell sheets [46].

2.1.2. Scaffolding and Signalling Proteins. *Zona occludens* (ZO) family members ZO-1, ZO-2, and ZO-3 belong to the MAGUK family of TJ-associated scaffolding proteins. ZO proteins interact with each other [47, 48] as well as with cingulin [49], claudin-1–8 [28], actin [47], α-catenin [50], and occludin [47]. Interactions vary between family members, as illustrated by the fact that ZO-3 can interact with PATJ [51] while only ZO-1 can interact with JAMs A-C [34, 37], EGFR [52], and AF-6 [53]. In addition, ZO-1 binds the Y-box transcription factor ZONAB and the heat shock protein Apg-2 [54, 55].

Afadin (AF-6) is a Ras-binding, PDZ domain-containing scaffolding protein which interacts with nectin, JAM-A, ZO-1, profilin, ponsin, Rap1, and signal-induced proliferation-associated protein 1 (SPA-1, a Rap1 GTPase activating protein) [35, 56–58]. The nectin-AF-6 complex is involved in the formation of adherens and tight junctions.

The membrane associated guanylate kinase inverted (MAGI) family consists of MAGI-1, MAGI-2, and MAGI-3. MAGI-1 was first identified in mouse as a protein interacting with k-RasB [59]. MAGI-2 was initially identified in rat as a protein interacting with N-methyl-D-aspartate receptors (NMDA-Rs) and neuronal cell adhesion proteins [60]. MAGI-3 was identified in a two-hybrid screening as a protein interacting with the tumour suppressor PTEN [61], and Receptor Tyrosine Phosphatase beta [62].

Cingulin is a 140–160 kDa protein consisting of a globular "head" domain, a coiled-coil "rod" domain, and a globular "tail" domain. *In vitro* binding studies have revealed that cingulin interacts with various components of tight junctions including JAM, ZO-1, ZO-2, ZO-3, myosin and F-actin, suggesting a role for cingulin as a linker between the TJ membrane and F-actin [49, 63–65]. Cingulin also functions to sequester and inactivate the RhoA activator GEF-H1 at TJs, resulting in inhibited RhoA signalling and G1/S phase transition [66, 67]. Loss or mutation of cingulin does not perturb the formation of tight junctions, but results in increased claudin-2 expression and cellular proliferation, which are dependent on increased RhoA activity [68].

Amot is a scaffolding protein with a coiled-coil region and a PDZ binding motif [69], which forms a complex with the Rho GTPase-activating protein Rich1 and is targeted to the tight junction interaction with PATJ or MUPP1 [70, 71]. Two Amot-like proteins, JEAP and MASCOT, have also been identified as TJ proteins [72, 73] which interact with MUPP1 [70].

Atypical protein kinase C is located at the TJ and plays a crucial role in maintaining tight junction structure and cell polarity through phosphorylation and stabilization of junction-associated proteins [74, 75]. Activation of classical protein kinase C and novel protein kinase C has been shown to disassemble TJs [76].

2.2. Biological Functions of the TJ Complex

2.2.1. Gate and Fence Function. In polarized epithelial cells the TJ forms a belt-like structure at the apical-most region of the lateral membrane, and represents a boundary between apical and basolateral membranes [77]. The main functions attributed to TJs are the regulation of paracellular permeability (gate or barrier function), and the formation of a physical barrier preventing intramembranous movement of lipids and proteins (fence function). Gate function regulates the passage of ions and solutes across epithelial sheets in an organ-specific manner, and can be modified depending on the specific requirements of the organ [30, 78]. Fence function is required to maintain asymmetric distribution of membrane components and to develop membrane polarity [77]. Epithelial barrier function relies heavily on the claudin family of TJ proteins, which form strands controlling selective permeability by forming size- and charge-selective aqueous pores [30, 78, 79]. Epithelial fence function on the other hand is not solely reliant on one subset of integral membrane proteins but instead requires cooperation between integral membrane proteins and several TJ scaffolding proteins and signalling molecules [80].

2.2.2. Regulation of Adhesion and Migration. Epithelial cells control adhesion to the basement membrane and extracellular matrix to maintain an intact barrier that can reseal quickly in response to injury. Breaching of the epithelial barrier stimulates cells to extend protrusions into the wound space, which can result in TJ disruption and release of proteins such as PATJ, Par3, aPKC [81], Cdc42, and Par6 [82] from their scaffolds. Retargeting these (and other) proteins to the migrating edge helps polarize migrating cells in the direction of movement via reorientation of the Golgi, centrosome and the microtubule cytoskeleton along the axis of migration [82, 83].

2.2.3. Regulation of Polarity and Differentiation. TJs regulate epithelial polarity by controlling the assembly of three main polarity complexes; the CRB3 complex, the Par complex, and the Scrib complex, which will be discussed in the following section. The apical junctional complex serves to restrict the movement of these complexes in order to form distinct apical and basolateral domains. Apico-basolateral polarity allows for terminal differentiation of epithelial barriers by apical orientation of the trans-Golgi network, which can sort membrane proteins toward either apical or basolateral membranes. Specialized membrane trafficking leads to the accumulation of receptors and channels in either apical or basolateral membranes, allowing electrochemical gradients to develop across epithelial sheets [84, 85].

From the afore-mentioned information, it can be observed that TJ proteins exert fundamental influences over cellular processes that regulate polarity, differentiation and migration; all of which are processes central to cancer progression. Therefore, TJ and other cell-cell adhesion proteins are gaining increasing attention in breast cancer research [86–89]. Most work to date has focused on adherens junction

proteins (such as cadherins) in breast cancer progression; and in fact loss of E-cadherin is a defining feature of lobular breast carcinoma [90, 91]. However, TJ proteins have also been found to be dysregulated in several human cancers including breast, and have been suggested as promising targets for cancer detection, diagnosis, and therapy [88]. In this review, we will attempt to summarize current knowledge on the impact of TJ proteins on breast cancer progression; based on the ability of TJs to control polarity, differentiation, and migration.

3. The Contribution of TJ Alterations to Breast Cancer

Our review will focus on three aspects whereby functional alterations in TJs may impact breast cancer progression by altering cell polarity, cell fate, and cell migration. For a broad overview of TJ alterations in cancer metastasis of other tumours, the reader is directed to a recent review [92].

3.1. TJ-Mediated Alterations in Polarity—Role in Breast Cancer Progression. Formation of the TJ adhesion belt allows the targeting of scaffolding proteins which regulate the cellular polarity machinery. This machinery is composed of three polarity complexes which identify separate regions of the cell. These polarity complexes were originally identified in *C. elegans* and *Drosophila*, but have been found to be highly conserved in mice and higher mammals. The CRB complex identifies the apical region due to apical concentration of CRB3 [93], which is targeted to the tight junction by PATJ and ZO-3 [51, 94]. The Par complex localizes at TJs through interactions between Par-3 and JAM-A [38]. Finally, the Scribble (Scrib) complex identifies the basolateral region of the cell, and is targeted to adherens junctions through interaction of Scrib and Dlg with E-cadherin [95–98].

The CRB complex is the most apically located polarity complex in epithelial cells, and acts as an apical anchor for the targeting of cytoplasmic proteins during polarisation [93]. It is composed of the transmembrane protein CRB3 and the scaffolding proteins PALS1 and PATJ. Several components of the CRB complex are reportedly dysregulated in breast cancer. For example, CRB3 and PATJ expressions were shown to be repressed by the transcription factor ZEB1, which is upregulated in invasive ductal and lobular breast cancers [99]. ZEB1 has been implicated in epithelial to mesenchymal transition (EMT), a dedifferentiation programme associated with cancer metastasis in which epithelial junctions and cell polarity are disrupted, contributing to increased cell motility [100]. PATJ also binds a negative regulator of mTOR called TSC2, which regulates survival, apoptosis, and cell cycle progression [101–103]. The mTOR pathway has been shown to be frequently deregulated in various cancers including breast [16, 18]. Massey-Harroche et al. reported that PATJ knockdown in intestinal epithelial cells resulted in the upregulation of the mTOR pathway [104]; and it is possible that loss of PATJ in cancers such as breast could facilitate tumour progression by allowing the prosurvival effects of mTOR activation to go unchecked. PATJ is also important for the proper localisation of claudin-1, ZO-3, CRB3, occludin, aPKC, and ZO-1 at TJs [51, 94, 105]. Loss of PATJ could, therefore, also promote "leaky" junctions, resulting in increased access of luminal growth factors to the basolateral epithelial surface. It is intriguing to speculate that this leakiness could promote tumour progression by feeding the developing tumour, as proposed in the nutritional model of carcinogenesis [106]. This along with the dysregulation of aPKC has the potential to induce substantial increases in proliferation as well as a loss of polarity, all of which are hallmarks of cancer progression.

Other members of the CRB polarity complex could also play a role in breast cancer progression. Knockdown of the PALS1 binding partner lin-7 in renal epithelial cells was shown to reduce expression levels of PALS1, PALS2, Dlg2, Dlg3, and PATJ [107]. A resulting failure to recruit aPKC to TJs resulted in reduced epithelial barrier function, delayed polarization, and impaired lumen clearance in three-dimensional morphogenesis models [107, 108]. Loss of PALS1 also resulted in defects in E-cadherin trafficking [109], which, taken together, suggests that analagous disruption of the CRB3 complex during breast cancer could impair barrier function and polarity, and potentially facilitate occlusion of breast duct lumens with tumour cells.

The PAR complex is made up of Par3, Par6, aPKC, and Cdc42/Rac1. Recently, Par6B was reported to be transcriptionally upregulated in breast cancer tissues by quantitative PCR [110]. Interestingly, MCF-10A breast epithelial cells overexpressing Par6 polarized normally in three-dimensional culture models, but showed higher proliferation rates which were dependent upon Par6 interactions with aPKC and Cdc42 [110]. Increased Par6 signalling has also been reported in MCF-10A cells overexpressing activated ErbB2 [111, 112], the growth factor receptor which is amplified in 25–30% of breast cancers and which identifies a subtype of highly aggressive tumour [113]. Activation of ErbB2 in these cells induces the formation of multiacinar structures with abnormal filled lumens, in a manner dependent on interactions of ErbB2 with the Par6-aPKC complex [112]. Mutation of Par6 in cells overexpressing activated ErbB2 was observed to restore lumen formation, suggesting an inhibitory tone of Par6-ErbB2 interactions on apoptotic clearance of developing lumens [112]. The role of Par6 in apoptosis is suggested to be due to the activation of aPKC by Par6 [114]. Par6-aPKC interactions have also been shown to activate Rac1 in non-small cell lung cancer cells, resulting in anchorage-independent growth and invasion through activation of matrix metalloproteinase-10 (MMP-10) expression [115, 116]. Thus dysregulation of Par6 in cancer cells has the potential to impact tumour progression via direct effects on polarity, migration, and even apoptosis.

In contrast to Par6, Par3 expression has been found to be reduced in oesophageal squamous cell carcinomas in association with lymph node metastasis and poor differentiation [117]. Certain forms of EMT have also been shown to downregulate Par3 expression, with Par3 overexpression capable of rescuing the loss of E-cadherin during EMT in a rat kidney epithelial model [100]. Given the putative link between EMT induction and breast cancer progression, it

will be interesting to uncover whether Par3 expression might also be lost in breast cancer. Since Par3 regulates Par6, loss of Par3, in turn, is likely to exert an influence over the control of proliferation, polarity, and apoptosis resistance by Par6 signalling in cancer cells.

The scribble complex is an evolutionarily conserved complex consisting of three members, Scrib, lethal giant larvae homolog (LGL), and discs large homolog (DLG). Loss of function mutations of scrib, DLG, and LGL in *Drosophila* have demonstrated abnormal cell polarity with increased proliferation without tumour cell overgrowth, possibly due to increased apoptosis [118–120]. Scrib has been shown to colocalise with DLG and E-cadherin at adherens junctions [98], and is required for formation of this junction and proper localisation of DLG and LGL as well as apical targeting of CRB3 [97, 121]. Scrib staining was shown by immunohistochemistry to be reduced and mislocalized in human breast cancer tissues [98, 122]. Zhan et al. have suggested a role for Scrib in breast cancer development by reducing apoptosis in c-myc over-expressing breast epithelial cells [122]. Activation of c-myc enhanced the formation of a Scrib complex which activated the small GTPases Rac and Cdc42 and increased the expression of a proapoptotic protein Bim. Conversely, loss of Scrib suppressed the ability of c-myc to induce Bim expression [122]; suggesting a mechanism for reduced apoptosis and increased resistance of breast cancer cells to cytotoxic stresses in the event of Scrib loss.

LGL is a cytoplasmic protein which is targeted to the lateral epithelial membrane during polarisation [123]. Like CRB3 and PATJ, LGL1 is repressed by the transcription factor ZEB1, whose expression is upregulated in several forms of breast cancer [99]. Therefore, dysregulation of LGL may play a role in EMT events associated with breast cancer progression. Alterations in the final member of the Scrib complex, DLG, may also play a role in cancer progression. DLG3 has been shown to be reduced in gastric carcinoma [124]; whilst overexpression of DLG1 and DLG3 inhibits cellular proliferation via a block in G1/S phase transition of the cell cycle [125, 126]. DLG4 interacts with Frizzled proteins to regulate the WNT signalling pathway [127], inappropriate activation of which has been implicated in oncogenesis due to myriad influences on cell adhesion, migration, proliferation, and cell death [128]. DLG1 and DLG3 also regulate WNT signalling through DLG3-mediated β-catenin degradation and the binding of DLG1 to APC and by modulating the antiproliferative effects of APC [125, 126, 129]. In unpolarized cells, DLG1 is ubiquitinated and degraded, and only upon junctional formation is it hyperphosphorylated and stabilised [130]. This indicates that the integrity of cell-cell contacts regulates the Scrib polarity complex and that disruption of this complex promotes dysregulated growth and resistance to apoptosis.

As illustrated above, alterations in CRB3, Scrib, and Par polarity complexes can promote proliferation, cell cycle progression and evasion of apoptosis as a result of disrupted apical-basolateral polarity in a variety of models. Although several of the seminal observations were originally made in *Drosophila* and *C. elegans*, many findings have since been confirmed in higher mammals and during *in vitro* studies on human breast and other carcinoma cell lines. Thus insights from simple organismal models of polarity are highly relevant not only to the control of normal human physiology by the polarity machinery but also to the development of many cancers including breast. However, abnormalities in the polarity machinery are only one of several ways in which TJ dysfunction can impact upon breast cancer progression. The role of TJ-mediated alterations in cell fate will be discussed in the following section.

3.2. TJ-Mediated Alterations in Cell Fate—Role in Breast Cancer Progression. Although cancer is frequently considered as a disease of abnormal proliferation, cancer progression is not determined solely by proliferative advantage within tumour cells. Other factors such as apoptosis resistance and the ability to bypass senescence pathways contribute to an environment supporting breast cancer progression. The role of individual TJ proteins in modulating these aspects of breast cancer progression will be addressed in what follows.

Occludin expression is known to be downregulated in several cancers including breast [131]; its loss correlating with glandular dedifferentiation and progression of human endometrial, colorectal, and lung carcinomas [132–134]. In recent studies, occludin overexpression was found to promote detachment-induced apoptosis (anoikis) in AC2M2 murine breast carcinoma cells, while endogenous occludin re-expression correlated with downregulation of apoptosis-inhibitory genes (bcl-2, survivin) and upregulation of apoptosis-inducing genes (apaf-1, bax) [135]. TUNEL assays also revealed that HeLa cells constitutively overexpressing wild-type occludin exhibited increased sensitivity to oxidant-induced apoptosis. Occludin overexpression was also shown to induce premature senescence in AC2M2 cells, as assessed by increased senescence-associated β-galactosidase enzymatic activity and the upregulation of negative cell cycle regulators such as p16INK4A, p21Waf1/Cip1, and p27Kip1 but not p53 [131]. The ability of cells to autoinduce growth arrest based upon the expression of TJ proteins such as occludin could has a profound inhibitory effect on tumour growth, and illustrates how significant a loss of such proteins could be for tumour progression.

Similarly to occludin, claudin-1, -4, -6 have also been reported as downregulated in breast cancer [131, 136–139]. Suppression of endogenous claudin-6 expression by siRNA in MCF7 breast cancer cells increases resistance to oxidant-induced apoptosis and anoikis, thereby promoting colony formation in two- and three-dimensional cultures [140]. In a complementary approach, forced induction of claudin-1 expression in MDA-MB-361 breast cancer cells resulted in elevated apoptosis in three-dimensional cultures. Enhanced apoptosis correlated with increased spheroid size, suggesting a positive effect of nutrient and growth factor diffusion into spheroids [136]. This supports the hypothesis that cancer formation may be promoted in premalignant epithelial tissues that have become chronically leaky to growth factors [141]. Claudin-1 may also play a role in the control of cell fate, with observations of increased expression in senescent

breast epithelial cells [139] and reduced expression in invasive breast cancers.

In an interesting contrast, expression of claudin-3, -4 and -7 have actually been observed to increase in both breast and ovarian cancers [88, 142–145]. Overexpression of claudin-3 and -4 in HOSE-B ovarian cells enhanced cell survival in clonogenic assays [88], further supporting a role for either upregulation or downregulation of key claudins in the controlling cancer cell fate.

Occludin-interacting proteins ZO-1 and ZO-2 have also been shown to be repressed with cancer progression [52, 146, 147], where decreased ZO-1 staining correlates with decreased glandular differentiation of breast tumour specimens [146]. ZO-1 and ZO-2 regulate cell cycle progression and proliferation in a cell density-dependent manner [148–151] through transcription factors such as ZONAB. ZONAB localizes to the nucleus and the TJs in proliferating MDCK cells [151], but is not detectable in the nucleus of nonproliferating high-density cells [148]. Evidence indicates that cytoplasmic ZONAB immunoprecipitates with both CDK4 and cyclin D1 and assists in the nuclear accumulation of cdk4, promoting G1/S phase transition and cell cycle progression [148]. ZONAB also upregulates ErbB2 expression [151], which (as discussed earlier) could profoundly impact progression of a subset of breast cancers. ZO-2 blocks cell cycle progression by downregulating cyclin D transcription and inhibiting cdk2 and cdk4 [150, 152]. ZO-2 also controls cyclin D expression and interacts with the transcription factors jun, fos, and C/EBP to regulate proliferation [150]. Thus, it can be observed that ZO proteins control cellular proliferation in a density-dependent manner by sequestering transcription factors at the tight junction. Loss of ZO proteins during breast cancer may, therefore, promote proliferation via a loss of control over cell cycle progression.

Finally, in addition to the many TJ structural proteins which exert regulatory control over cell fate, signalling proteins affiliated with the TJ complex could also play a part in breast cancer progression. For example, many small GTPases have been described to affiliate with the TJ complex [153]. As key signalling molecules which regulate actin dynamics, GTPases profoundly impact processes that are central to cancer initiation and progression [154]. For example, RhoA promotes cell cycle progression through the regulation of p21 and p27 levels [155]. TJs regulate RhoA activity by cingulin-mediated sequestration of the RhoA activator GEF-H1 and inhibition of G1/S phase transition [67]. Increased GEF-H1 levels can arise by mutations in p53 [156], a frequent genetic alteration observed in breast cancer.

These alterations suggest a relationship between TJ alterations and the malignant potential of several carcinomas, via deficits in controlled proliferation, regulated cell cycle progression and apoptosis. This suggests that the dysregulation of cell-cell contact machinery may be a prerequisite for cancer progression in order to turn off specific epithelial regulatory pathways. The loss of membrane polarity via tight junction abnormalities may also alter cell-cell and cell-extracellular matrix interactions, and might, therefore, facilitate migration, invasion, and the development of metastasis, which will be reviewed in the following section.

3.3. TJ-Mediated Alterations in Cell Migration—Role in Breast Cancer Progression. As discussed earlier, altered cell-cell adhesion contributes to a loss in polarity and contact inhibition, culminating in uncontrolled proliferation during breast cancer initiation. There is also evidence that altered cell adhesion plays a fundamental role in breast cancer progression by freeing tumour cells from both neighbouring cells and the underlying matrix; and in parallel by conferring a motile or migratory advantage to cells during invasion and metastasis [19, 157, 158]. In this section, we will attempt to summarize current knowledge on the impact of TJ proteins on breast cancer progression.

3.3.1. TJ Integral Membrane Proteins. Occludin has been linked with cancer progression in endometrial carcinoma; where decreasing expression was correlated with increasing grade, myometrial invasion, and lymph node metastasis [132]. Forced expression of occludin in breast cancer cells has been shown to decrease cancer cell migration and invasion both *in vitro* and *in vivo* [135]. Interestingly, the occludin gene can be silenced by hypermethylation, and it may be that the methylator phenotype promotes tumourigenic, invasive, and metastatic properties of cancer cells [135].

A wealth of evidence has also implicated the claudin family in breast cancer cell migration. Tumours with low expression of claudin-3, -4 and -7 and high expression of stem cell and epithelial-mesenchymal markers were recently shown to associate with poorer overall survival [159], as mirrored in other studies [160]. Like occludin, claudin-1 protein levels are reportedly reduced in breast tumours and breast cancer cell lines [161]. Importantly, claudin-1 has been detected in the membranes of normal breast ductal epithelial cells and in some DCIS tumour cells, but is frequently absent from invasive tumours [142]. Furthermore, claudin-1 expression has been demonstrated as a good predictor of disease recurrence and malignant potential in breast cancer. Morohashi et al. demonstrated that recurrent breast tumours displayed significant reductions in claudin-1 expression compared to primary tumours; while reduced claudin-1 expression has also been associated with lymph node involvement and decreased disease-free survival [162]. Taken together, these data suggest a role for claudin-1 in invasion and metastasis. Furthermore, MDA-MB-361 breast cancer cells deficient in claudin-1 grow as multicellular filled spheroids in three-dimensional cultures and re-expression of claudin-1 induces central lumen formation [136]; perhaps by nutritional deprivation of innermost cells inducing apoptosis as already discussed [141].

Other than claudin-1, the loss of several other family members has been reported in breast cancer [142, 162–166]. Osanai et al. demonstrated that decreased expression of claudin-6 in breast cancer cells (by siRNA or epigenetic silencing) increases MMP activity, likely facilitating increased cancer cell migration and invasion [140]. Subsequent reintroduction of claudin-6 increased cellular adhesion and

abrogated enhanced invasion and migration. Expression of claudin-7 has been shown to be reduced in IDC cells compared to those of the normal breast [164], and reduced in fine needle aspirates from IDC patients [166]. In both studies, loss of claudin-7 expression also correlated with increasing tumour grade and metastatic disease. In an intriguing study, treatment of MCF7 breast cancer cells expressing high levels of claudin-7 with HGF/scatter factor (which decreases cell-cell adhesion) led to a dramatic decrease in claudin-7 expression, further linking the loss of claudin-7 and cell cohesion in breast cancer [164]. Most recently, forced expression of claudin-16 in MDA-MB-231 breast cancer cells has been reported to induce junctional formation and concurrently reduce aggressive and motile behaviour *in vitro* and *in vivo* [165].

In contrast to the reduced expression of several claudins, claudin-3 and -4 have in fact been found to be elevated in breast cancer at both mRNA and protein level [143–145, 167]. Interestingly, overexpression of either claudin-3 or claudin-4 in human ovarian epithelial cells has been reported to increase migratory and invasive capabilities [88]. As discussed later in this review, the ability of both claudins-3 and -4 to function as receptors for *Clostridium perfringens* enterotoxin (CPE) [168] may provide a unique targeting mechanism to eliminate cancer cells overexpressing these proteins.

JAM proteins regulate numerous cellular adhesive processes including intercellular junction assembly [169], cell morphology [170], and leukocyte migration [171, 172]; while JAM-A dysregulation has recently been implicated in breast cancer [173, 174]. JAM-A has been shown to regulate epithelial cell morphology and enhance β1-integrin expression through modulation of Rap1 GTPase activity [170]. Since loss of tissue architecture and cell polarity is a prerequisite for breast cancer invasion and metastasis [175], disruption of JAM proteins may, therefore, play key roles in disease progression. Indeed, disruption of JAM-A in a colonic carcinoma cell line was shown to convert cells from a stationery, polarized state to a migratory phenotype [176]. Recently, Naik et al. reported that JAM-A overexpression decreased migration and invasion in breast cancer cell lines, while knockdown of JAM-A expression enhanced invasiveness [174]. It was hypothesized, therefore, that the loss of JAM-A may correlate with poor clinical prognosis. However, a subsequent study by McSherry et al. revealed a significant association between *high* JAM-A gene and protein expression and poor survival in 2 large cohorts of human invasive breast cancer tissue specimens [173]. Furthermore, knockdown or antagonism of JAM-A significantly decreased migration in MCF7 breast cancer cells expressing high endogenous levels of JAM-A. The apparent conflict between these two studies may be resolved by the fact that *under*expression of JAM-A is likely to impair cellular adhesion and polarity (favouring tumour initiation), whereas *over*expression of JAM-A could promote integrin-mediated migratory events that favour tumour progression. These data clearly implicate an imbalance of JAM-A expression patterns in breast cancer, and, as discussed later, may also form an interesting therapeutic target.

3.3.2. TJ Adaptor and Signalling Proteins. Few studies have specifically focussed upon the involvement of TJ adaptor and signalling proteins in breast cancer progression. However, ZO-1 loss has been linked to poor prognosis in breast cancer, with significantly reduced levels of TJ-associated ZO-1 in patients with metastatic disease compared to those remaining disease-free [177]. Polette et al. also showed that expression of the matrix metalloproteinase MT1-MMP in invasive breast tumour cell lines is correlated with cytoplasmic localization of ZO-1 and with occludin loss [178]. ZO-1 has also been shown to be reduced or lost in ductal breast cancer tissues, in parallel with increased dedifferentiation [146]. Reduced ZO-1 expression has been significantly associated with reduced E-cadherin expression; whose loss is inextricably linked with lobular breast cancer [86]. Furthermore, ZO-1 has been reported to play an important role in controlling expression of the ErbB2 gene [179]. Downregulation of another family member, ZO-2, has also been reported in breast carcinoma [180]. In addition, ZO-2 has been reported as crucial for the tumour-inducing capabilities of the Adenovirus type 9 E4 protein. Expression of mutant ZO-2 protein lacking the E4 binding site inhibits E4-mediated tumour initiation in mammary glands [180, 181]. Taken together, it can, therefore, be considered that ZO proteins play important roles in the migratory events associated with breast cancer progression.

The Par complex (Par3-Par6-aPKC) promotes normal junction assembly by regulation of actin dynamics and is known to be altered in many cancers including breast (reviewed in [111]). Indeed, as previously addressed in this review, Par6, through association with aPKC and ErbB2, has been shown to disrupt apical-basal polarity and protect cells from apoptosis [112]. Other potential links between Par proteins and breast cancer involve EMT and the regulation of Rho family small G proteins including Rac [182], Rho [183], and Cdc42 [184]. For example, Par6 reportedly interacts with TNFα in inducing EMT and TJ loss via degradation of RhoA [185]. *In vitro* experiments have revealed that the Par complex along with Rac signalling stabilizes front-rear polarization of noncontacting keratinocytes, thereby stimulating chemotactic migration [182]. Indeed, important biological processes such as migration and invasion are highly regulated by the Rho family. The Rho guanine nucleotide exchange factor (GEF) Tiam has been shown to increase with increasing breast tumour grade or cell line invasiveness [183]; and Tiam-mediated Rac1 activation has been correlated with tumour cell migration and invasion *in vitro* [186]. Integrin-mediated adhesion through Rho family GTPase activity has been reported as essential in regulating cell polarity and membrane protrusiveness [41]. Specifically, Rac1 and Cdc42 have been linked with integrin-mediated motility and invasion through PI3K signalling in breast cancer cell lines [187]. Furthermore, the Rap GTPase Rap1 has been identified as a crucial signalling element downstream of β1 integrin [170], responsible for regulating breast acinar structure and inducing mammary gland lumen formation [188]. Yet another signalling protein downstream of TJs, PKC [189], has also been linked to cancer initiation and progression. PKC overexpression and altered localization

has been demonstrated in breast cancer [190], and PKC signalling is required for EGF-induced chemotaxis of human breast cancer cells [191].

This wealth of evidence indicates the potential involvement of several TJ adhesion cascades in the migratory events associated with breast cancer progression. Further studies on these proteins will allow a more comprehensive understanding of their behaviour and contributions to tumour progression, ultimately defining candidate breast cancer prognostic markers. The study of compounds designed to specifically target and block the action of adhesion proteins involved in cancer invasion could be of substantial therapeutic benefit in preventing breast cancer invasion.

4. TJs and Breast Cancer Drug Therapies

Targeted therapeutic agents for breast cancer represent a growing proportion of new drugs entering clinical testing. Since carcinogenesis is a multistep process characterized by alterations in many key growth and development pathways, there are numerous opportunities for pharmacologic targeting. Selection of appropriate drug targets and the ability to effectively deliver drugs to those targets are pivotal issues in drug development. This section will review current knowledge on TJs as breast cancer drug targets, and as targets for therapeutic modulation of cancer drug delivery.

In spite of the regulatory influences exerted by TJs on diverse processes relevant to cancer progression (as discussed in previous sections), there are currently no cancer therapies on the market which specifically target TJs. However, clues to potential TJ targets of value have come from many sources, including translational research studies involving patient databases. For example, overexpression of claudin-3 and -4 proteins has been demonstrated in over 90% of primary breast carcinomas in a patient group of 188 [192], and in 60–80% of breast tumours in a tissue microarray of 314 patients [193]. Claudin-3 and -4 overexpression has also been noted in other neoplasias including ovarian, prostate, pancreatic, and endometrial [193–196]. These proteins form an intriguing potential target for cancer therapies, since both claudin-3 and -4 have been identified as the receptor for *Clostridium perfringens* enterotoxin (CPE) and the only claudin family members capable of mediating CPE binding and cytolysis [195–198].

CPE is a well-known virulence factor responsible for the gastrointestinal symptoms associated with *C. perfringens* type A food poisoning. By inducing permeability alterations in host intestinal epithelial cells, CPE induces cell death and epithelial desquamation. CPE is thus a multifunctional toxin with cytotoxic, TJ-damaging, and proinflammatory activities [199, 200]. This ability of CPE to rapidly and specifically lyse cells expressing claudin-3 or -4 could potentially be exploited in the treatment of breast cancers overexpressing these proteins. Accordingly, it has been shown that claudin-3 and -4 expressing breast cancer cell lines grown in cell culture and as xenograft tumours underwent rapid and dose-dependent cytolysis in response to CPE treatment [143]. Even more promisingly, administration of CPE has been shown to reduce the growth of claudin-4 overexpressing human ovarian and pancreatic tumours [201]. Thus, local delivery of native CPE may be useful in the treatment of preneoplastic lesions such as DCIS and in neoadjuvant settings such as the locoregional control of locally advanced breast carcinoma, as well as in tumour downstaging to allow breast conservation therapy [143]. In addition to this, the documented ability of CPE to downregulate the epithelial barrier through interference with claudin-3 and -4 may enhance local drug delivery for other treatment modalities. However, at least two caveats must be noted. Firstly (as discussed in prior sections), loss of TJ-based adhesion may imbalance cellular polarity which by itself is likely to be protumourogenic. Secondly, claudin-3 and -4 are expressed in several normal human tissues including gut, lung, and kidney; therefore the potential high toxicity of CPE at doses used for systemic cancer therapy in animal models might limit its use in humans to local treatments [194, 202].

Claudin-3 and -4 overexpression in breast cancer could alternatively be targeted by toxin- or radionuclide-conjugated antibodies, which would either destroy the cancer cells directly or target them for attack by the host immune system. The high sequence identity between claudin-3 and -4 may allow generation of antibodies recognizing both proteins. Potential indications for anticlaudin antibody-based therapeutics include carcinomas of colorectal, breast, ovarian, and prostate origin [203].

In common with the discovery of claudin-3 and -4 as the CPE receptor, other TJ proteins are known to be hijacked as pathogen receptors. The TJ protein CAR acts as the primary site for adenovirus attachment during infection, a feature which has been exploited as a delivery mechanism for gene therapies [204, 205]. CAR expression has been shown to significantly increase in breast tumour tissue along with increasing tumour grade [206]. Breast tissue samples showing elevated CAR expression have been associated with poor patient prognosis [206]. While the biological roles of CAR are incompletely defined, emerging evidence suggests that it may function in regulating cell proliferation [207]. Whether CAR overexpression in breast cancer could be successfully targeted by nonpathogenic components of the virus in order to diminish cancer cell proliferation remains an intriguing question.

Another TJ protein of interest for breast cancer drug discovery is occludin. Gumbiner et al. showed that occludin homotypic interactions and turnover, but not synthesis, could be affected by treating cells with peptides to the extracellular loop of occludin [208, 209]. Nusrat et al. also identified occludin peptides capable of binding TJ structural and signalling proteins [210], and demonstrated that the second extracellular loop of occludin regulates cellular transformation by oncogenes such as Raf-1 [211]. Given that occludin has been reported to be dysregulated in some breast cancers, it is intriguing to speculate that occludin could be a target for peptide-based cancer drugs.

Another TJ protein implicated in breast cancer progression is ZO-1, which as noted, has sequence homology with the *Drosophila* tumour suppressor Dlg, implying that ZO-1 could possess similar functions as a tumour suppressor

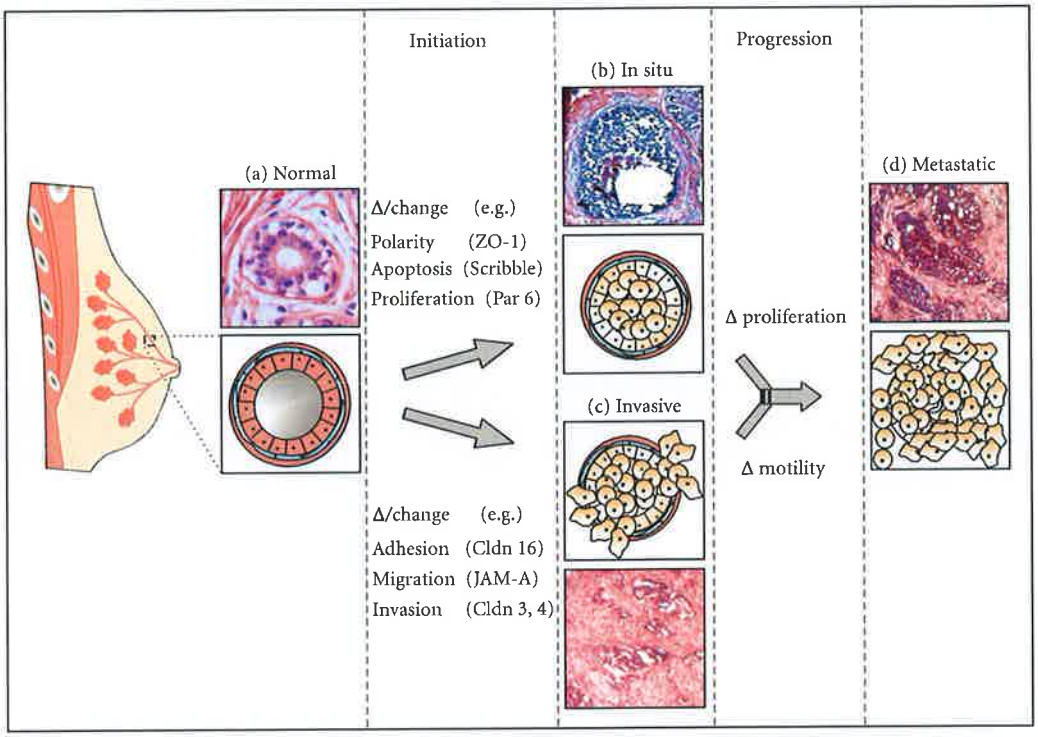

FIGURE 2: The potential influence of TJ proteins on breast cancer development. Breast cancer predominantly begins in luminal epithelial cells lining the normal breast ducts (a). Alterations in TJ proteins may lead to the initiation of breast tumourigenesis in at least two ways. Firstly, TJ alterations may favour decreased cellular apoptosis and increased proliferation leading to uncontrolled growth, such as is seen in ductal carcinoma *in situ* lesions (b). Secondly, TJ alterations may decrease cell adhesion and increase motility, facilitating cancer cell migration as seen during invasion and basement membrane breakdown in early primary invasive breast carcinoma (c). Breast carcinoma likely requires coordinated efforts of both increased proliferation and increased motility to progress to metastatic stages (d).

gene in mammalian epithelia [19]. Martin et al. observed decreased ZO-1 staining in several invasive breast cancer cell lines supporting the tumour-suppressive characteristics of ZO-1 [177].

Even more recently, it has been observed that increased JAM-A expression in human breast cancer tissues correlates with poor patient prognosis [173]. Since this mechanism is thought to involve promotion of integrin-mediated migratory events at the cell-matrix interface, it is, therefore, interesting to speculate that targeting JAM-A dimerization to reduce signalling could be a promising and novel target to reduce breast cancer cell motility during the early stages of invasion or metastasis.

A final point regarding TJs as breast cancer therapeutic targets relates to drug delivery. In order for therapeutic agents to reach their target *in vivo*, they must cross epithelial and/or endothelial barriers. Since the TJ is the primary regulator of paracellular transport across such cells [212], successful drug delivery may require modulation of TJ proteins to allow drug molecules to pass [213]. However, as before, it must be noted that disruption of TJ proteins purely for drug delivery purposes may itself promote cancer progression by upsetting homeostatic mechanisms of polarity, differentiation, cell fate, and migration which are tightly regulated by TJs in normal tissues.

To conclude this section, we note that therapeutic modulation of breast cancer via selective targeting of tight junction structural proteins is in its infancy. At present, CPE offers the best-developed strategy via targeting of claudin-3 and -4. While many signalling proteins and enzymes loosely affiliated with the TJ plaque may prove easier pharmacological targets, full discussion of this topic is beyond the scope of this review. Interested readers are directed to a comprehensive review by Schneeberger and Lynch [214]. It is clear however that further investigations into the cell biology of tight junctions are necessary to provide insights into putative future applications of TJ components as candidates for drug discovery to prevent or limit breast cancer progression.

5. Conclusions

Finally, we summarize the role of TJs in breast cancer initiation and progression as follows (see Figure 2). During the initiation phase of cancer, fundamental alterations in the TJ complex may impair its functional control over important cellular processes such as polarity and cell fate determination, or cell motility characteristics. Dysregulation of either of these aspects likely contributes to the pathologies which we recognise as ductal breast carcinoma *in situ* or invasive ductal

carcinoma. Further dysregulation of a combination of these (and other) events is likely to be required for the most serious step of breast cancer progression, the transition to a metastatic phenotype.

It must be noted that there is not a simple relationship between TJ protein loss or gain and breast cancer. As we have described in this review, both loss and gain of TJ proteins can impart a growth advantage to breast cancer cells, as well as increased resistance to apoptosis, loss of polarity, and increased migratory or invasive characteristics. Through these important regulatory influences on polarity, cell fate and cell movement, we suggest that an intact and functional TJ complex acts as a barrier to the initiation and progression of breast cancer. However, any imbalance in the protein components of this complex (whether increased or decreased) will, in turn, imbalance the strict homeostatic control required to maintain breast tissue in its differentiated state, increasing the risk of inducing a pathologically dedifferentiated state such as breast cancer.

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