Identification of Proteins Associated with Cancerous and Invasive Phenotypes of Breast and Melanoma Cells using Proteomic approaches

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Identification of Proteins Associated with Cancerous and Invasive Phenotypes of Breast and Melanoma Cells using Proteomic approaches

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2009

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abstract

Breast cancer is the most commonly diagnosed cancer in women. To identify proteins that may be involved in transformation of normal cells into cancerous cells and invasion in breast cells, the proteomic profiles for cell lysates, conditioned media and membrane & membrane-associated proteins from normal mammary epithelial cells (HMEC), non-tumourigenic mammary immortalized cells (MCF-10A), non-invasive breast cancer cells (MCF7) and invasive breast cancer cells (BT20) were investigated using 2D-DIGE coupled with mass spectrometry. Thirty one interesting proteins (either not previously reported as associated with breast cancer or with limited information in the literature) were identified from this study. These include cytosolic proteins (EIF4A3, SERPINB1, UQCRC1, DCI and SOD1), secreted proteins (PDIA4, SERPINB1, SERPINB7, CTSA, NUCB1 and FHC) and membrane & membrane-associated proteins (IMMT, UQCRC1 S100A14 and FKBP5). A few cytosolic proteins (TXNRD1, 3-PGDH, TALDO1, CLP, ERp29 and QPRT), secreted proteins (TALDO1 and GSS) and membrane & membrane-associated proteins (GANAB, WDR1, RUVBL2 and C20ORF3) were identified that could be associated with invasion of both normal and cancer cells. We also identified cytosolic proteins (CLIC1, PSME1 and RAD23B), secreted proteins (DDAH1 and NUCB2) and membrane & membrane-associated proteins (ATP5B, RUVBL2, CES1, TALDO1 and AHCY) that could associated with breast cancer invasion.

The expression of two cellular proteins TXNRD1 and RAD23B which were observed differentially regulated from the cell lysates study, were down-regulated using siRNA. The knock down of TXNRD1 reduced invasion of the invasive cancer cell lines, SKBR-3 and MDA-MB 231 whereas down-regulation of RAD23B enhanced invasion of cancer and normal non-invasive cell lines (MCF-7 and MCF-10A). This suggests that TXNRD1 and RAD23B may play important roles in the regulation of invasion in the breast cancer cells.

New biomarkers could be valuable for the early detection of melanoma. The analysis of conditioned media from melanocytes, melanoma, normal mammary epithelial, breast and lung cancer cell lines using SELDI-ToF MS revealed that a small protein/peptide, identified as a possible fragment of bovine transferrin at 7.6kDa, was expressed only in

the melanoma cell lines. Gelatin zymography and specific inhibitor studies suggested that MMP 2 and MMP 9 secreted by melanoma cell lines are responsible for cleavage of the transferrin protein in the culture medium. We also investigated a number of other proteins/peptides (7.4kDa, 8.5kDa, 11.9kDa and 12kDa) that were up-regulated in conditioned media from the melanoma cell lines compared to melanocytes. The 8.5kDa protein was identified as ubiquitin. The transferrin fragment and ubiquitin could be valuable biomarkers for detection of melanoma. Thus proteomics approches are the power full tools for cancer biomarker discovery.

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Abbreviations

%	Percentage
2D-DIGE	Two-dimensional difference gel electrophoresis
2D-PAGE	Two-Dimensional Polyacrylamide Gel Electrophoresis
ATCC	All Type Cell Culture Media
BiP	Glucose-regulated protein, 78kDa
BSA	Bovine Serum Albumin
BVA	Biological Variance Analysis
CEA	Carcinoembryonic Antigen
CHPAS-	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Da	Daltons
DE	Differentially expressed
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylene diamine tetracetic acid
ER	Estrogen Receptor
FCS	Fetal Calf Serum
GAPDH	Glyceraldehyde-6-phosphate dehydrogenase
HER 2	Human Epidermal growth factor Receptor 2
HMEC	Human Mammary Epithelial Cells
Hrs	Hours
ICAT	Isotope Coded Affinity Tags
IEF	Isoelectric Focusing
IHC	Immunohistochemistry
IMS	Industrial Methylated Spirits
IPG	Immobiline DryStrip Gel
iTRAQ	Isobaric Tag for Relative and Absolute Quantitation

kDa	Kilo Daltons
L-15	Leibovitz's L-15 Medium
LC-MS/MS	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
M.W.	Molecular Weight Marker
m/z	Mass/charge ratio
MALDI-ToF MS	Matrix Assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrometry
MEM	Minimum Essential Medium
MMP	Matrix Metalloproteinase
MudPIT	Multidimensional Protein Identification Technology
N+C	Normal+Cancer
NHEM	Normal Human Epidermal Melanocytes
OD	Optical Density
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
pI	Isoelectric Point
PMF	Peptide Mass Fingerprints
PR	Progesterone Receptor
PTMs	Post-translational modifications
RNAi	RNA interference
rpm	Revolution(s) Per Minute
RPMI	Roswell Park Memorial InstituteMedia
SDS	Sodium Dodecyl Sulphate
SELDI-ToF MS	Surface Enhanced Laser Desorption/Ionization-Time of Flight Mass Spectrometry
SF	Serum-Free
SFM	Serum-Free Medium
SILAC	Stable isotope labelling with amino acids in cell culture
siRNA	Small interfering RNA
siRNAs	Small interfering RNAs
TE	Tris-EDTA
TEMED	N, N, N', N'-Tetramethyl-Ethylenediamine
TIMP	Tissue Inhibitor of Metalloproteinase
Tris	Tris(hydroxymethyl)aminomethane

UHP	Ultra high pure water
UHP	Ultra High Pressure Water
uPA	Urokinase Plasminogen Activator
v/v	volume/volume
w/v	weight per vol

Section 1

Introduction

1.1 General Introduction

More than 11 million people are diagnosed with cancer every year. It is estimated that there will be 16 million new cases every year by 2020. From a total of 58 million deaths worldwide in 2005, cancer accounts for 7.6 million (or 13%) of the global mortality (Cho 2007). Deaths from cancer in the world are projected to continue rising, with an estimated 9 million people dying from cancer in 2015 and 11.4 million dying in 2030. Cancer is a disease of uncontrolled cell growth caused by exposure to carcinogens (cancer-causing substances), genetic defects and/or viruses. These cells can multiply and form a large mass of tissue called a tumor. Some tumours are limited to one location and can be surgically removed. These tumors may cause little harm and are therefore called benign. Cancer cells of other tumors may invade to surrounding tissue and spread to the other organs of the body. Such aggressive tumors are called malignant or cancer.

Cancer is today recognized as a highly heterogeneous disease. More than 100 distinct types of human cancer have been described, and various tumour subtypes can be found within specific organs (Grizzi and Chiriva-Internati 2006, Brafford and Herlyn 2005). In addition, tumours have somatic mutations and epigenetic changes, many of which are specific to the individual neoplasm (Ramaswamy 2004). It is now recognized that this genetic and phenotypical variability primarily determines the growth and invasiveness of cells (Grizzi and Chiriva-Internati 2006). The metastatic potential of neoplastic disease and its response or resistance to therapy could also be dependent on the genetic and phenotypical variability of cells. Therefore it seems that the multi-level complexity of cancer explains the clinical diversity of histologically similar neoplasia.

During the development of most types of human cancer, primary tumours produces cells that move out, invade adjacent tissues, and travel to distant sites where they may succeed in founding new colonies. These distant settlements of tumour cells, metastases, are the cause of 90% of human cancer deaths (Sporn 1996). The capability for invasion and metastasis enables cancer cells to escape the primary tumour mass and colonize a new terrain in the body where, at least initially, nutrients and space are not limiting.

Invasion and metastasis are exceedingly complex processes, and their genetic and biochemical determinants remain incompletely understood. At the mechanistic level, they are closely allied processes, which justify their association with one another as one

general capability of cancer cells. Both utilize similar operational strategies, involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases. A cascade of complex interactions between the cancer cell and its surroundings results in the metastatic cascade. For successful metastasis, cells must penetrate the vessels (intravasation), travel through the blood stream or lymph system and then exit the vessels at the new site (extravasation), and proliferate. The series of steps of this metastatic cascade are depicted in Figure 1.1.

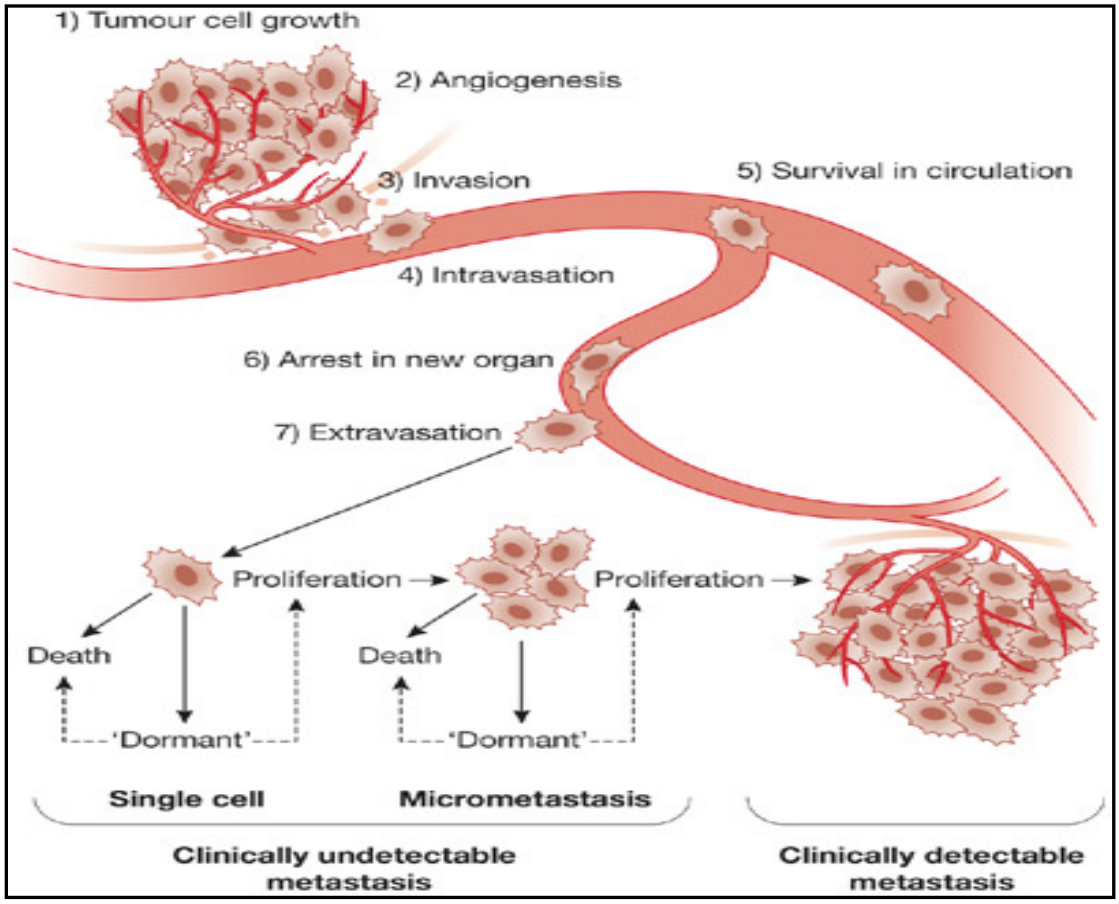


Figure 1.1. **Invasion and metastasis**. During the metastatic process, cancer cells proceed through a series of distinct steps to form a secondary tumour. Cells detach from the primary tumour, invade adjacent tissue and then enter the lymphatic or circulatory system. Size constraints cause the cells to be arrested in distant organs, where they extravasate and enter the surrounding microenvironment. At this point, the cell might proliferate to form a clinically detectable metastasis or it might remain dormant as a single cell or micrometastasis. The specific factors governing the fate of disseminated cells are as yet unknown; however, it is thought to be largely dependent on favourable interactions between the tumour cell and its microenvironment (McGee *et al.* 2006).

1.2 Invasion

It has been established that the expression of the invasive phenotype is not caused by one single gene or protein. The invasive process involves genetic deregulation of the tumour cells leading to an imbalance of stimulatory and inhibitory physiological events. This deregulation occurs in a small subset of tumour cells; less than 0.05% of circulating tumour cells establish invasion (Nicolson 1991). Generally the invasion process requires local attachment, proteolysis of the extracellular matrix, and cell migration (Albelda 1993).

1.2.1 Attachment of cells

The extracellular matrix (ECM) and adhesion molecules play an important role in the regulation of cell attachment during invasion.

1.2.1.1 The extracellular matrix (ECM)

Invasive cancer cells must degrade natural tissue barriers. The most important natural barriers is ECM which is composed of basement membrane and interstitial connective tissue and plays an important role in cell and tissue support, anatomic compartmentalisation and cellular differentiation (Duffy 1987, Liotta 1986, Kalluri 2003). ECM consists of 4 main components; structural proteins (collagens), specialised proteins (fibronectin and laminin), proteoglycans and glycosaminoglycans.

1.2.1.1.1 Collagen

The collagens are the major protein comprising the ECM. There are at least 12 types of collagen. Type I, II and III are the most abundant and form fibrils of similar structure. These collagens are characterised by their long and helical structures. Three alpha chains coil together to form a triple superhelix. Collagens are also rich in proline and hydroxyproline. Collagens organise the extracellular matrix and give it resilience (Tryggvason *et al.* 1987).

1.2.1.1.2 Fibronectin

Fibronectin is a large glycoprotein that is found in all vertebrates and the first well-characterised adhesive protein. Fibronectin contains at least six folded domains, each with a high affinity for collagen, heparin, fibrin, specific integrins and receptors on the surfaces of various types of cells. Expression of fibronectin has been shown to directly

influence the establishment and maintenance of the transformed phenotype (Brown *et al.* 2006).

1.2.1.1.3 Laminin

Laminins are flexible four-armed, glycoproteins and anchor cell surfaces to the basal lamina. Laminin monomers polymerise into 3D structures in a time- and concentration-dependent manner. Laminins interact with cells through various receptors that mostly belong to the family of integrins (Ruoslahti 1999). Many epithelial tumours exhibit altered localisation or expression of laminin-binding integrins (e.g. α6β4/α6β1). This promotes invasion through the basement membrane and increases motility in the stroma, where tumour cells can remodel the matrix by depositing laminin (Colognato and Yurchenco 2000, Holler 2005).

1.2.1.2 Adhesion molecules in cell attachment

For invading, tumours cells must be able to attach to extracellular matrix components and to other cells (of the same or different type). These interactions either among cells (cell-cell) or between cells and substratum components (cell-matrix) are mediated by adhesion molecules, such as integrins, CD44, immunoglobulin superfamily (IgSF) and cadherins (Hood and Cheresh 2002).

1.2.1.2.1 Integrins

Integrins are transmembrane receptors that bind to a variety of ECM molecules including laminin, fibronectin and collagens and have an essential role in the invasion process. Integrins are formed from 18α and 8β subunits, which dimerise to yield at least 24 different integrin heterodimers, each with distinct ligand binding and signalling properties (Okado and Hawley 1995, Lu *et al.* 2002). These heterodimers are integral cell membrane receptors that form focal adhesion contacts with various ECM-ligands, such as collagens, fibronectin, laminin, vitronectin. Ligand binding specificity depends on the specific α and β chains present in the heterocomplex. Changes in the expression pattern of integrins such as α2β1, α5β1 and αVβ3 in breast cancer have been reported in several studies (Hood and Cheresh 2002).

1.2.1.2.2 CD44

The CD44 protein is a type I transmembrane glycoprotein receptor involved in cell-to-cell interactions, cell adhesion and migration (Zutter *et al.* 1990 & 1993, Holler 2005, Sloan *et al.* 2006). The principal ligand of CD44 is hyaluronic acid, an integral component of the ECM. Other CD44 ligands include osteopontin, serglycin, collagens, fibronectin and laminin. Activation of CD44 by one of its various ligands results in intracellular signalling involving Rho/Ras and activated tyrosine kinase pathways which plays a significant role in cancer and invasion (Nagano *et al.* 2004).

1.2.1.2.3 Immunoglobulin superfamily (IgSF)

The immunoglobulin of adhesion superfamily of adhesion receptors is composed of numerous molecules of the cell surface which contain the typical immunoglobulin-like domains in the extracellular portion of the molecule (Ponta *et al.* 2003). The most important member of the IgSF, vascular cell adhesion molecule-1 (VCAM-1), has been associated with invasion and malignant progression via binding to its integrin ligand, $\alpha 4\beta 1$ (VLA-4) (Hunkapiller and Hood 1989). The alterations in the expression of some other cell adhesion molecules, like neural cell adhesion molecule (NCAM), have been observed to facilitate invasion and disseminating metastasis in tumour (Zimmerman *et al.* 1992, Cartwright *et al.* 1995).

1.2.1.2.4 E-cadherin

A number of cadherin molecules have been identified and characterized, but there are three main members of this family: E-cadherin (epithelial), N-cadherin (neural) and P-cadherin (placental) (Skubitz 2002). Of these, it is E-cadherin which has the most obvious involvement in cancer development since more than 90% of human cancers are carcinomas arising from epithelial tissues.

1.2.2 Proteolysis of extracellular matrix

The process of invasion is an active and dynamic process that requires protein synthesis and degradation. Tumour cells secrete proteolytic enzymes to degrade the extracellular matrix barriers in order to intravasate successfully. Matrix degrading proteases can be classified into the four major classes (matrix metalloproteinases, serine proteinases, cysteine proteinases and aspartyl proteinases), depending on the nature of the active sites.

1.2.2.1 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a family of zinc-binding enzymes that cleave ECM components. The expression levels of MMPs are often correlated with invasiveness and therefore hold promise as therapeutic targets in the treatment of cancer (Takeichi 1991, Behrens *et al.* 1992). MMPs are secreted as proenzymes that require extracellular activation. Currently there are over 20 known members, which share functional and structural characteristics and can be categorised into the collagenases, gelatinases, stromelysin and membrane-type MMP sub families (Table 1.1).

Table 1.1 Family of matrix metalloproteinases (MMPs)

Group	**Enzyme name**
Stromelysins	MMP-3, MMP-7, MMP-10, MMP-11, MMP-12
Collagenases	MMP-1, MMP-8, MMP-13
Gelatinases	MMP-2 , MMP-9
Membrane-type MMPs	MMP-15,MMP-14, MMP-16, MMP-17, MMP-24
Other MMPs	MMP-18, MMP-19,MMP-20, MMP-21, MMP-23

Members of all classes of the MMP family are inhibited by endogenous inhibitors called tissue inhibitors of metalloproteinases (TIMPs). Specific cellular mechanisms have evolved allowing for activation of MMP proenzymes and also confining MMP activity to the peritumoural microenvironment. These mechanisms comprise (1) the expression of membrane-type MMPs; (2) the binding of soluble MMPs by membrane-tethered receptors; and (3) cell surface receptor-mediated activation of MMP proenzymes (Stetler-Stevenson *et al.* 1996).

1.2.2.2 Serine proteinases

Members of this class of endopeptidases are characterised by a serine residue at the active site and are produced in an inactive pro-form. Important members of the family include trypsin, thrombin, plasmin, cathepsin G and urokinase type plasminogen activator (uPA). uPA, which is the most extensively studied member of this group, catalyses the conversion of inactive plasminogen to the highly potent, broad-spectrum protease plasmin. Plasmin can degrade a wide variety of ECM components and also is

able to activate several latent proteases (including pro-collagenases) (Chen and Wang 1999, Deryugina and Quigley 2006).

1.2.2.3 Cysteine proteinases

Cathepsin B and L, characterised by a cysteine residue at their active site, are the two cysteine proteases most implicated in cancer metastasis. These are lysosomal proteases that can degrade a variety of ECM components at a neutral pH, but whose optimal activity is at an acid pH. Cathepsin B can activate certain MMPs and also receptor-bound uPA (Testa and Quigley 1990). It displays preferential binding to cell membranes in malignant tumours, but it is unknown if it has a membrane-bound receptor similar to uPA.

1.2.2.4 Aspartyl proteinases

Cathepsin D, like Cathepsin B, is a lysosomal protease whose optimum activity is at an acid pH. It degrades a large variety of endocytosed proteins and has been implicated in cancer invasion and metastasis, where relapse and metastatic disease correlate with high levels of Cathepsin D expression (Sloane 1990).

1.2.3 Cell migration

Active tumour cell motility, coupled with proteolysis, is required for the penetration of basement membranes and interstitial stroma during the transition from in-situ to invasive carcinoma. Certain factors contribute to metastasis by stimulation of motility. The factors that are secreted by the tumour cells themselves are autocrine motility factors. At least 11 autocrine motility factors have been identified, although only 3 have been cloned and purified to homogeneity: hepatocyte growth factor/scatter factor (HGF/SF), insulin-like growth factor II (IGF-II), and autotaxin (ATX). HGF/SF normally acts as a paracrine growth factor, but in tumour cells it can act as an autocrine motility factor (Ohri *et al.* 2008, Johnson *et al.* 1993). The receptor for HGF/SF is c-Met oncogene which could induce phosphorylation of PI-3 kinase, GAP, PLCγ, src, ras, and MAP kinase (Gherardi and Stoker 1991, El-Badry *et al.* 1990, Stracke *et al.* 1992, Gupta *et al.* 2008, Liou *et al.* 2007). The ATX autocrine motility factor is a potent stimulator of motility (Naldini *et al.* 1991, Garcia-Guzman *et al.* 2000). ATX has been demonstrated to exhibit phosphodiesterase activity, although it is not known how this enzymatic activity translates into cell motility (Santos *et al.* 1993, Nam *et al.* 2000).

Other tumour motility factors are host-secreted growth factors such as insulin-like growth factor-I (IGF-I), interleukin-8 (IL-8) and histamine. Many of these factors also act as mitogens for the tumour cells in which they cause motility.

1.3 Breast Cancer

Breast cancer is the second leading cause of cancer deaths in women (after lung cancer) and is the most common cancer among women. It is a disease of the middle and late ages of life as 75% of breast cancer is diagnosed in women over the age of 50. Although breast cancer is less common at a young age, younger women tend to have a more aggressive form of the disease than older women.

1.3.1 Mammary gland and breast cancer

Breast cancer develops from uncontrolled growth of the mammary gland and almost all instances of breast cancer originate in the lobules or ducts of the mammary glands. The breast contains two compartments: the glandular portion, which is involved in production and transportation of milk, and the stromal and connective tissues. The glandular part of the mammary gland has 15–20 lobes, and within each lobe there are many smaller lobules ending in dozens of tiny bulbs that can produce milk. The lobules are all linked by thin tubes called ducts, and all ducts lead to the nipple. The cells forming the ducts and lobules are epithelial cells whose main function is to produce and to secrete the various constituents of milk. In addition, epithelial cells are surrounded by a layer of myoepithelial cells, attached on a basal membrane, whose role is to maintain the tubular structure of ducts and lobules. Surrounding the lobules and ducts, connective and fat tissues are composed of fibroblasts, with their abundant extracellular matrix, and adipocytes. In addition, both blood vessels and lymph vessels irrigate the mammary gland, and nerve fibers, mostly sensory and sympathetic, are also present (Murata *et al.* 1994).

To acquire their functionality, the epithelial cells must receive proper signals from hormones (estrogens, progesterone) as well as from nearby cells and components of its microenvironment (growth factors). Before epithelial cells can produce milk, they develop into lobuloalveoli (functional units) through morphogenesis involving cell proliferation, invasion, and differentiation (Hondermarck 2003). The cyclic development of the mammary gland reflects the fact that it is only needed during well-

defined periods of life. Thus, mammary epithelium undergoes repeated cycles of growth, differentiation, and regression. Continuous cycling leads to the formation of a ductal tree. Multiplication of breast epithelial cells occurs constantly, stimulated by estrogens during menstrual cycle and pregnancy. After parturition, functional differentiation of the epithelial cells takes place. During lactation, these cells produce large amounts of milk, and after weaning, the gland regresses by a process of extensive cell death and tissue remodeling. Therefore, the mammary gland is a cellular ecosystem in which each represented cell type is subject to a constant turnover. This is particularly the case for the epithelial cells, which are subject to various hormone and growth factor stimulation throughout their lifetime, with correlative changes in morphology and metabolism. Most breast tumours are of epithelial origin (Sternlicht 2006), and therefore the large majority of malignant breast tumours are classified as carcinomas (malignant epithelial tumours). Sarcomas (malignant tumours arising from connective tissue) are rarely observed in the breast.

1.3.2 Classification of Breast cancer

Breast cancer broadly can be divided into non-invasive and invasive cancer. (Refer to "Pathology Reporting in Breast Cancer Screening", National Coordinating Group for Breast Screening Pathology. NHSBSP publications, Third edition, 2005)

1.3.2.1 Non-invasive breast cancer

Non-invasive breast cancer has been divided into two main classes of *in situ* carcinomas, ductal carcinomas *in situ* (DCIS) and lobular carcinomas *in situ* (LCIS).

1.3.2.1.1 Ductal carcinomas *in situ* (DCIS)

Ductal carcinoma *in situ* (DCIS) is a proliferation of epithelial cells with cytological features of malignancy within parenchymal structures of the breast and refers to the most common type of non-invasive breast cancer in women. DCIS presents with a range of architectural forms, with differing growth rates, patterns, and cytological features.

1.3.2.1.2 Lobular carcinoma in situ (LCIS)

Lobular carcinoma in situ (LCIS) occurs within the lobules located at the end of the breast ducts. LCIS arises when the entire cell population of the lobular units consist of characteristic small rounded cells with granular or hyperchromatic nuclei,

inconspicuous nucleoli and high nucleo-cytoplasmic ratio. Most cancer experts do not consider lobular carcinoma in situ to be a cancer. Rather, it is considered an area of abnormal tissue growth that signals an increased risk of developing invasive breast cancer later on.

1.3.2.2 Invasive breast cancer

The invasive breast cancers have been divided into following categories

1.3.2.2.1 Invasive (infiltrating) ductal carcinoma

Invasive or infiltrating ductal carcinoma is the most common form of invasive breast cancer. This type of breast cancer is characterized by its solid core, which is usually hard and firm on palpation. Invasive ductal carcinoma commonly spreads to the regional lymph nodes and carries the poorest prognosis of the various ductal carcinomas.

1.3.2.2.2 Invasive lobular carcinoma

This form of breast cancer is relatively uncommon, comprising only 5–10% of invasive breast tumours. Invasive lobular carcinomas are characterized by a greater proportion of multicentricity in the same or contralateral breast. The lesions tend to have ill-defined margins, and occasionally the only evidence is subtle thickening or induration. Patients with infiltrating lobular carcinoma are especially prone to having bilateral carcinoma. Stage by stage, invasive lobular carcinoma has a similar prognosis to infiltrating ductal carcinoma.

1.3.2.2.3 Tubular carcinoma

Tubular carcinoma comprises about 5% of all invasive breast cancers, although the incidence is increasing due to earlier detection through screening. It usually presents as a small, ill-defined or spiculated mass on mammogram. Tubular carcinoma is a well-differentiated tumour characterized by well-defined, open, angulated glands that invade through breast parenchyma. Lymph node metastasis is rare and the prognosis is good (around 90% at 5 years).

1.3.2.2.4 Medullary carcinoma

Medullary carcinoma accounts for around 5% of invasive breast cancers and is the most common in younger women; it tends to appear as a distinct, well-circumscribed mass on mammography. The diagnosis is based on the finding of high-grade tumour cells arranged in a syncytial pattern with a pushing border, with a prominent lymphocyte infiltrate. The prognosis is generally better than for invasive ductal cancer.

1.3.2.2.5 Mucinous carcinoma

Mucinous carcinoma, also known as colloid carcinoma, is a rare form of invasive breast cancer. It has a good prognosis in the pure form, but if it gets mixed with infiltrating ductal cancer, it takes on the prognosis of the worst component. The tumour presents as a well-circumscribed mass, generally in older women, and may be mistaken for a fibroadenoma. Histologically, mucinous carcinoma is characterized by abundant extravasated mucin that dissects throughout the breast parenchyma.

1.3.2.2.6 Inflammatory breast carcinoma

Inflammatory breast carcinoma is uncommon, accounting for 1–3% of invasive breast cancers. It is characterized by diffuse skin edema, skin and breast redness, and firmness of the underlying tissue without a palpable mass. The clinical manifestation is primarily due to tumour cells embolizing in the dermal lymph channels, with associated engorgement of superficial capillaries.

1.3.2.2.7 Invasive cribriform carcinoma

These carcinomas are composed of masses of small regular cells, as seen in tubular carcinoma. The invasive islands, however, exhibit a cribriform rather than a tubular appearance. Apical snouting is often present. Nuclei should not show high grade degrees of atypia. More than 90% of the lesion should exhibit the cribriform appearance except in cases where the only coexistent pattern is tubular carcinoma, when over 50% must be of the cribriform appearance in order to be classified as an invasive cribriform type.

1.3.2.2.8 Clinically relevant subgroups

Efforts have been made to use gene expression patterns to classify breast tumors into clinically relevant subgroups (luminal A, luminal B, basal, ERBB2-overexpressing, and

normal-like) (Young *et al.* 2004). Recently the molecular taxonomy has been confirmed by protein expression profiling (Sorlie *et al.* 2001).

1.3.2.2.8.1 Luminal

They are estrogen receptor (ER)-positive, progesterone receptor (PR)-positive, cytokeratin 18-positive and have a good prognosis (even though subtype B which has lower ER and a higher proliferative profile, has a poor prognosis in comparison to subtype A). This subtype comprises the majority of breast cancers (≈85%) (Abd El-Rehim *et al.* 2005).

1.3.2.2.8.2 Basal

They are estrogen receptor (ER)-negative, progesterone receptor (PR)- negative, cytokeratin 18-negative and cytokeratin 5-positive. This subtype considered as poor prognosis subtypes and comprising 3–15% of breast cancers (Horwitz *et al.* 2008). The subgroup of "basal-type" breast cancer is Triple-negative which does not express the genes for estrogen receptor (ER), progesterone receptor (PR) or HER2. This subtype of breast cancer is clinically characterised as more aggressive and less responsive to standard treatment and associated poorer overall patient prognosis (Goncalves *et al.* 2008).

1.3.2.2.8.3 ERBB2-overexpessing

ERBB2, also known as HER-2/neu (HER-2), estrogen receptor (ER)-negative, include tumours which show increased expression of HER-2 gene/protein. This represents 15% to 25% of invasive breast carcinomas and is associated with a worse clinical outcome (Perou *et al.* 2000).

1.3.2.2.8.4 Normal-like

The normal-like types have high expression of genes characteristic of basal epithelial and adipose cells such as keratin 5, keratin 17, integrin-β4 and laminin, and the low expression of genes characteristic of luminal epithelial cells such as keratins 8/18 and transcription factors (Bundred 2001, Brenton *et al.* 2005).

1.4 Breast cancer biomarkers

Biological markers (biomarkers) have been defined as "cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells or fluids" (Perou *et al.* 2000) Recently, the definition has been broadened to include biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Hulka 1990). In practice, biomarkers include tools and technologies that can aid in understanding the prediction, cause, diagnosis, progression, regression or outcome of treatment of disease. There are three types of disease biomarkers: screening, diagnosis and disease progression. The potential uses of these biomarkers include: 1) identification of individuals destined to become affected or who are in the "preclinical" stages of the illness, 2) reduction in disease heterogeneity in clinical trials or epidemiologic studies, 3) reflection of the natural history of disease encompassing the phases of induction, latency and detection, and 4) target for a clinical trial.

The breast cancer biomarkers that are currently recommended for use to certain degrees in the clinical setting have been discribed in Table 1.2.

Table 1.2: Serum and tissue biomarker for breast cancer

Origin	**Biomarkers**
Serum biomarkers	CA 15-3,CA 27.29, CEA
Tissue biomarkers	ER, PR, HER2, uPA and PAI-1
Other potential biomarkers	BRCA1, BRCA2, Cyclin E and p53

1.4.1 Serum Biomarkers

Serum biomarkers including members of the MUC1 family of mucin glycoproteins (CA 15-3 and CA 27.29, MCA, CA549) and carcinoembryonic antigen (CEA) have been described for breast cancer.

1.4.1.1 CA 15-3 and CA 27.29

The members of the MUC1 family are large glycosylated molecules and are the most widely used serum tumour biomarkers in breast cancer (Naylor 2003). The physiological functions of MUC1 are still unclear but it has been implicated in cell

adhesion, immunity and metastasis. Two members, CA 15-3 and CA 27.29, are the most useful serum biomarkers for breast cancer (Bieglmayer *et al.* 1991, Price *et al.* 1998). The sensitivity of these markers is significantly higher in patients with advanced disease (Bieglmayer *et al.* 1991, Price *et al.* 1998). Therefore, CA 15-3 and CA 27.29 cannot be recommended for screening or early diagnosis (Gion *et al.* 2001, Safi *et al.* 1991). The main applications of CA 15-3 and CA 27.29 include the prediction of surveillance of patients with diagnosed breast cancer and monitoring therapy in advanced disease (Molina *et al.* 2005).

1.4.1.2 Carcinoembryonic antigen (CEA)

CEA is a secreted glycoprotein and was identified in 1965 as the first human cancer-associated antigen and serological tumour biomarker (Bieglmayer *et al.* 1991). Many studies have also shown that the concentration of CEA is increased in a variety of cancers including breast cancer (Gold and Freedman 1965). Therefore, it is an important tumour marker for the diagnosis and monitoring of the therapy of breast cancer (Ebeling *et al.* 1999, Sajid *et al.* 2008, Lee *et al.* 1991). CEA is elevated in about 50-60% of patients with metastatic breast cancer (Ebeling *et al.* 1999). Therefore similar to mucin family members, CEA cannot be recommended for screening or early diagnosis, but serial levels may be useful in the early diagnosis of distant metastases (Esteban *et al.* 1994). However, the use of CA 15-3 and CA 27.29 with CEA could provide additional complementary information and for this reason the combination of at least one MUC1 biomarker with CEA is the recommended serum biomarker panel in patients with breast cancer (Molina *et al.* 2005).

1.4.2 Tissue Biomarkers

While serum markers in breast cancer are mostly used for monitoring patients with diagnosed disease, tissue-based markers are primarily measured in order to determine prognosis and predict response to therapy. Clinically, the most useful tissue-based markers in breast cancer are estrogen receptor (ER), progesterone receptor (PR) and HER-2 (also known as c-erbB-2 or neu). Although not yet in widespread clinical use, urokinase plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1) are potential markers for determining prognosis in lymph node-negative breast cancer patients.

1.4.2.1 Estrogens and Progesterone receptors (ER and PR)

Estrogen and progesterone receptors (ER and PR) are transcriptional factors which mediate the actions of estrogens and progesterone, respectively. ER and PR are powerful mitogens for breast epithelium cells and breast cancer cells. ER and PR are measured on every primary invasive breast cancer (Molina *et al.* 2005). In both pre- and post-menopausal women, steroid hormone receptor status is used to identify patients most likely to benefit from endocrine forms of therapy in both early breast cancer and metastatic disease (Molina *et al.* 2005, Taucher *et al.* 2003, Allegra *et al.* 1980). ER and probably PR positivity are associated with a favourable diagnosis because in both early and advanced disease, hormone receptor-positive patients have a significantly greater probability of responding to hormone therapy than patients lacking receptors (Taucher *et al.* 2003, Osborne 1998). ER and PR also have prognostic value in breast cancer. Generally, ER-positive patients have a better outcome than ER-negative patients for the first 4–5 years after diagnosis (Molina *et al.* 2005, Taucher *et al.* 2003, Duffy 2005). Although less work has been carried out on the prognostic impact of PR, patients with tumours expressing PR also tend to have a better prognosis than those lacking this receptor (Hayes *et al.* 2001). Currently immunohistochemistry is the most widely used technique to measure ER and PR status of breast tumours, however this is semi-quantitative technique and therefore limits the findings.

1.4.2.2 HER-2

HER-2 is a member of the epidermal growth factor receptor (EGFR) family. The HER-2 gene is either amplified or over expressed in 15–30% of invasive breast cancers (Duffy 2005). Amplification of the HER-2 gene means that instead of having only 2 copies of the gene per cell, there may be 50–100 gene copies per cell. The number of HER-2 proteins per cell can then increase from 20,000–50,000 to as high as 2 million (Molina *et al.* 2005). HER-2 has a number of potential uses in breast cancer, including determining prognosis, predicting relative resistance to both hormone and adjuvant CMF therapy (combination of three drugs cyclophosphamide, methotrexate and 5-fluorouracil), selecting for enhanced response to adjuvant anthracycline-based therapy and identifying patients for treatment with Herceptin (trastuzumab) (Slamon and Pegram 2001). Herceptin is a humanized MAb that binds with high affinity to the extracellular domain of HER-2, thereby blocking its role in signal transduction (Molina *et al.* 2005). HER-2 expression can be measured using a number of different ways; by

immunohistochemistry (IHC) on either frozen or paraffin embedded tissue sections using monoclonal or polyclonal antibodies, gene amplification by fluorescence *in situ* hybridization (FISH), and by Enzyme-linked immunosorbent assay (ELISA) or western blot of circulating HER-2 extracellular domain (ECD) levels. There are limitations to HER-2; over expression of HER-2 is not always associated with resistance to hormone and adjuvant CMF therapy and secondly a small number of patients have been considered for HER-2 investigations.

1.4.2.3 Urokinase plasminogen activator (uPA) and plasminogen Activator Inhibitor 1 (PAI-1)

uPA is a serine protease implicated in cancer growth, invasion and metastasis and PAI-1 is an endogenous inhibitor of uPA, but paradoxically is also involved in tumour progression (Harries and Smith 2002). uPA and PAI-1 have been shown to be potent and independent prognostic factors in breast cancer (Andreasen *et al.* 1997). The main use of uPA and PAI-1 lies in selecting lymph node-negative patients, who do not need or are unlikely to benefit from adjuvant chemotherapy. However, over expression of uPA and/or PAI-1 has been consistently related to poor prognosis in early stage and node-negative breast cancer (Duffy *et al.* 1999, Duffy 2002, Schneider *et al.* 2003). The IHC and ELISA assays are commonly used to monitor the levels of uPA and PAI-1, however the outcomes do not always correlate with patient's outcome.

1.4.3 Others potential biomarkers

There are number of biomarkers for breast cancer currently in the pipeline. The evaluation of these biomarkers could improve the efficiency of diagnosis and prognosis process. These novel biomarkers include BRCA1 and BRCA2, cyclin-E and p53.

1.4.3.1 BRCA1 and BRCA2

BRCA1 and BRCA2 are putative tumour suppressor genes located on chromosome 17q21. Many tumours with germline BRCA1 and BRCA2 mutations display loss of heterozygosity at this locus, which uniformly involves loss of the wild-type alleles indicating the role of BRCA1 and BRCA2 as tumour suppressor gene (Grondahl-Hansen *et al.* 1993). However, previous studies have also suggested that complete loss of the BRCA1 and BRCA2 proteins correlate with poor prognostic markers (Smith *et al.* 1992, Egawa *et al.* 2001) and their expression levels predict distant metastasis of

sporadic breast cancers (Lee *et al.* 1999, Egawa *et al.* 2002). Therefore, in depth investigation of these proteins could provide an additional prognostic parameter for breast cancer.

1.4.3.2 Cyclin E

Cyclin E is a regulator of the cell cycle transition in breast-cancer cells. In normal dividing cells, cyclin E regulates the transition from the G_1 phase to the S phase (Egawa *et al.* 2001). A high level of the cyclin E protein accelerates the transition through the G_1 phase (Dou *et al.* 1993). The cyclin E gene is amplified and the cyclin E protein is often constitutively expressed in breast-cancer cell lines (Resnitzky *et al.* 1994). Some of these lines over express not only the full-length 50-kD cyclin E protein, but also up to five low-molecular-weight isoforms of cyclin E (ranging in size from 34 to 49 kD) (Keyomarsi and Pardee 1993, Keyomarsi *et al.* 1994, Buckley *et al.* 1993, Sgambato *et al.* 1997). These isoforms, which lack the amino terminus, are hyperactive, as compared with the full-length protein, in phosphorylating substrates and inducing progression from the G_1 phase to the S phase (Keyomarsi and Pardee 1993, Keyomarsi *et al.* 2002, Keyomarsi and Herliczek 1997, Porter *et al.* 2001). Recently the levels of total cyclin E and low-molecular-weight cyclin E in tumour tissue were observed to correlate strongly with survival in patients with breast cancer (Porter *et al.* 2001). Cyclin E has been observed to be an independent prognostic marker for lymph node–negative breast cancer patients (Keyomarsi *et al.* 2002). Therefore these investigations indicate that cyclin E could serve as a potential biomarker for breast cancer diagnosis and prognosis.

1.4.3.3 p53

p53, a tumour suppressor, is involved in regulating cell proliferation, inducing apoptosis, and promoting chromosomal stability (Sieuwerts *et al.* 2006). Disruption of these functions has an important role in carcinogenesis (Toledo and Wahl 2006). The abrogation in the p53 gene is associated wtih breast cancer progression (Ho *et al.* 2001, Gasco *et al.* 2002). Mutations in the p53 gene (Malkin *et al.* 1990), changes in its upstream regulators (Bartek *et al.* 1990), changes in its transcriptional targets (Bell *et al.* 1999), and modifications in its coactivators (Ferguson *et al.* 2000) have been extensively observed in breast cancer disease. The presence of p53 mutations has been observed to correlate with worse survival in aggressive breast cancer (Samuels-Lev *et al.* 2001). Similar results were observed in a comprehensive meta-analysis of the effect

of somatic p53 mutations on prognosis in breast cancer (Mazars *et al.* 1992). Although mutant p53 has been correlated with poor prognosis of breast cancer (Pharoah *et al.* 1999, Norberg *et al.* 2001), the investigation of p53 and its associated proteins could enable the identification of improved biomarker and drug targets.

In summary, CA 15-3, CA 27.29, carcinoembryonic antigen, estrogen receptor, progesterone receptor, urokinase plasminogen activator and plasminogen activator inhibitor 1 are the breast cancer biomarkers that are being used clinically to some extent. However, other markers (BRCA1, BRCA2, Cyclin E and p53 and HER2) are being investigated for their clinical value.

1.5. Proteomics and breast cancer

The term 'proteome' refers to all the proteins in a cell, tissue, or organism and 'Proteomics' refers to the study of the proteome. Because proteins are involved in almost all biological activities, the proteome is a rich source of biological information. Proteomic studies of mammary glands have progressed enormously in recent years and a number of potential biomarkers for breast cancer have been identified using proteomics tools (Table 1.3).

1.5.1 Cell culture-based investigations

Breast cancer cell lines have been the most widely used models to investigate how proliferation, apoptosis and migration become deregulated during the progression of breast cancer (Linderholm *et al.* 2001). The reason for this lies in the fact that the biological fluids and tissue represent a high degree of complexity and variability and limited number and amounts of samples. Cell line models are easy to maintain for longer times and are reproducible in terms of sample generation. Moreover it has been observed that different cell lines from same tissue origin may also represent a high degree of heterogeneity in expression of genes. Therefore, in the initial discovery phase for novel cancer biomarkers, a less complex sample, such as cell lines, is essential (Lacroix and Leclercq 2004). To gain more information regarding the cancer and invasion in breast, different fractions such as whole cell lysate, secreted proteins in conditioned media and membrane & membrane-associated proteins are being investigated using proteomics-based techniques.

A. Table 1.3 List of potential biomarkers identified using proteomics tools for cancer (**A**) and invasion (**B**) in breast cancer disease.

Protein name	**Potential biomarkers for breast cancer (Regulation in cancer)**	**Sample**	**Reference**
Maspin	Down-regulated	Conditioned media and tissue	Lacroix and Leclercq 2004, Kulasingam and Diamandis 2008
Cathepsin D	Up-regulated	Conditioned media and tissue	Vecchi *et al.* 2008, Maass *et al.* 2001a & b, Toillon *et al.* 2007
14-3-3 protein	Down-regulated	Cell lysate and tissue	Deng *et al.* 2006, Westley and Rochefort 1980
HSP60	Up-regulated	Tissue and serum	Vercoutter-Edouart *et al.* 2001b, Umbricht *et al.* 2001, Hondermarck *et al.* 2001, Rui *et al.* 2003
HSP27	Up-regulated	Tissue and serum	Desmetz *et al.* 2008, Fransson *et al.* 2006, Franzen *et al.* 1996
MUC-1	Up-regulated	Tissue and serum	Rui *et al.* 2003, Gharbi *et al.* 2002, Song *et al.* 2005, Kabbage *et al.* 2008
HSP70 and HSP90	Up-regulated	Cell lines and tissue	Bieglmayer *et al.* 1991, Seuma *et al.* 2008
Peroxiredoxin-2	Down-regulated	Tissue and serum	Hondermarck *et al.* 2001, Vercoutter-Edouart *et al.* 2001a, Somiari *et al.* 2003
Tropomyosin-1	Up-regulated	Tissue	Somiari *et al.* 2003, Hamrita *et al.* 2008

eIF-4E	Up-regulated	Tissue	Bhattacharya *et al.* 1990, Ismail *et al.* 2008
Annexin 1	Down-regulated	Cell lines	Hudelist *et al.* 2004, Anthony *et al.* 1996, DeFatta *et al.* 1999
L-plastin	Up-regulated	Plasma	Shen *et al.* 2006, Shen *et al.* 2005, Ou *et al.* 2008
K5, K6, K7, K14 and K17	Down-regulated	Cell lines	Pitteri *et al.* 2008, Lapillonne *et al.* 2000
K8, K18 and K19	Up-regulated	Cell lines	Trask *et al.* 1990
Prohibitin	Up-regulated	Cell lines and tissue	Trask *et al.* 1990, Moll *et al.* 1982

B.

Protein name	**Potential biomarkers for invasion**	**Sample**	**Reference**
Nm23	Down-regulated	Tissue	Hamrita *et al.* 2008, Jupe *et al.* 1996, Dowling *et al.* 2007b
Cathepsin D	Up-regulated	Cell lines and tissue	Ismail *et al.* 2008, Peihong and Perry 2007, Niu *et al.* 2002
Cofilin1	Up-regulated	Cell lines	Johnson *et al.* 1993, Hahnel *et al.* 1993, Bazel and Alhadeff 1999
Vimentin	Up-regulated	Cell lines and tissue	Dowling *et al.* 2007b, Imai *et al.* 2008, Keshamouni *et al.* 2009
Galectin-1	Down-regulated	Cell lines	Korsching *et al.* 2005, Thomas *et al.* 1999, Lin *et al.* 2005, Liang *et al.* 2009
14-3-3 protein	Down-regulated	Cell lines and tissue	Imai *et al.* 2008
Gelsolin	Down-regulated	Tissue	Umbricht *et al.* 2001, Imai *et al.* 2008

1.5.1.1 Cell lysates

Molecular analysis of cells in their native tissue environment provides the most accurate picture of the in vivo disease state. However, tissues are complicated three-dimensional structures, composed of large numbers of interacting cell populations therefore it can be complicated to prepare protein samples (Kim *et al.* 2008, Winston *et al.* 2001, Tanaka *et al.* 2006). Therefore a considerable part of research on breast carcinomas is based on *in vitro* studies performed with breast cancer cell (BCC) lines. The cell lysates from cell lines provide an unlimited source of homogenous, self-replicating material which is free of contaminating stromal cells, and often easily cultured in simple standard media (Liotta and Petricoin 2000) . Investigations using cell lysates provide an overall view of cellular alterations in cells in respose to specific conditions and therefore have been considered valuable reseach material for indentification of biomarkes and drug targets.

In a study using two-dimensional PAGE and MALDI-ToF MS, the expression of 14-3-3 sigma was observed to be down-regulated in primary breast carcinomas cell lines, MCF-7 and MDA-MB-231, and in primary breast carcinomas as compared with normal breast epithelial cells (Lacroix and Leclercq 2004). 14-3-3 has been shown to interact with cyclin-dependent kinases and to control the rate of entry into mitosis. Hypermethylation of 14-3-3 sigma was observed in early stage in breast cancer indicating that inactivation or depletion of 14-3-3 sigma is directly associated with carcinogenesis in breast cells (Vercoutter-Edouart *et al.* 2001b). Recently a study using a proteomic approach complemented by immunohistochemistry showed that the loss of 14-3-3 sigma protein is a frequent event in breast cancer, as out of the 68 breast tumours, only 3 represented cross reactivity for 14-3-3 sigma during immunohistochemistry (Umbricht *et al.* 2001). Therefore 14-3-3 sigma could be an attractive biomarker for breast cancer diagnosis identified from breast cancer cell lines.

An luminal epithelial cell line expressing ErbB-2 was investigated using 2D-gel electrophoresis as ErbB-2 has been observed over expressed in breast cancer and therefore the changes associated with ErbB-2-over expression could help to understand the process of transformation from a normal cell to a cancer cell (Moreira *et al.* 2005). Members of plastins (T and L plastin), HSPs (HSP27 and 70) and 14–3-3 (14–3-3b) family were observed to be differentially regulated indicating their potential role in breast cancer (Gharbi *et al.* 2002). Recently, four isogenic cell lines representing non-metastatic, mild-metastatic, moderately metastatic and aggressive metastatic phenotypes were investigated using an isobaric peptide labeling approach (iTRAQ) coupled with

NanoLC-MS/MS (Gharbi *et al.* 2002). SH3GLB1 was down regulated whereas SUB1, SND1 and TRIM28 were up regulated in the metastatic cell model compared to normal cell and similar expression patterns were confirmed using immunohistochemistry which suggests their potential role as potential drug targets and/or biomarkers for breast cancer metastasis (Ho *et al.* 2008).

The protein profiles from seven breast-cancer cell lines (MDA-MB-231, HCC1428, AU565, MDA-MB-468, SK-BR-3, MCF7 and BT-474) and one normal cell (HMEC) generated using fluorogenic derivatization–liquid chromatography/tandem mass spectrometry (FD-LC-MS/MS) were compared to identify proteins that may be involved in cancer (Ho *et al.* 2008). Tropomyosin-1 was highly expressed only in the normal cell lines (HMEC), whereas it was undetectable in all seven breast cancer cell lines used in this investigation. Tropomysins are critical to stabilize the cytoskeletal filaments and therefore loss of tropomyosin-1 could be associated with the development of metastatic breast cancer (Imai et al. 2008). Annexin-2 and galectin-1 were highly expressed in the normal cell line (HMEC) compared to all seven breast cancer cell lines. This suggests that cytoskeletal proteins, such as tropomyosin-1, annexin-2 and galectin-1, could serve as potential biomarkers for the breast cancer. On the other hand, Ran, thioredoxin-1 and RhoGDI were highly expressed in breast cancer cell lines compared to the normal cells. Thioredoxin-1 and RhoGDI have already been associated with cancer (Bhattacharya et al. 1990) while the role of Ran in cancer is still unknown. In another study, the comparison of genomic (microarray) and proteomic (2D-PAGE) expression profiles of breast cancer cell lines (MCF-7 and HCC-38) with normal breast cells (CCD-1059Sk) revealed that structural proteins including, annexin-1 (ANXA1) and α-basic-crystallin protein (CRAB), were down regulated while 6-phosphogluconolactonase (6PGL) and F-actin capping protein α-2 subunit (CAZ2) were up regulated specifically in the breast cancer cell lines compared to normal cells (Zhang et al. 2005, Berggren et al. 2001). The expression patterns of ANXA1, CRAB, 6PGL and CAZ2 were confirmed by immunohistochemistry analysis and therefore suggested as potential biomarkers for breast cancer (Ou et al. 2008).

Because phosphorylated cytoskeletal proteins and chaperones mediate cell motility and apoptotic resistance, phosphoproteomic changes in the MCF10AT model of breast cancer progression were studied using a combination of phosphotyrosyl affinity enrichment, iTRAQ technology, and LC-MS/MS (Ou *et al.* 2008). A number of tyrosine

kinases, phosphatases, and other signaling proteins including SLC4A7 were detected to undergo differential phosphorylation during disease progression. SLC4A7 is a bicarbonate transporter and was observed to be down-regulated in 64% of the 25 matched normal and tumour clinical samples in further investigations (Chen *et al.* 2007a). This demonstrates that starting with a cell culture based cellular proteome, which exhibits a lower level of complexity, facilitates the identification of relevant proteins that can be validated in tumours.

1.5.1.2 Conditioned media

Secreted proteins can act locally and systemically in the body and play important roles in the regulation of cell physiology (growth and apoptosis, etc) and pathophysiology (invasion and motility). Therefore, the secretome reflects the functionality of a cell in a given environment (Chen *et al.* 2007a). Biological fluids, such as serum, may contain the molecules of interest (biomarker or drug target) in too low concentrations which cannot be easily measured or purified, unless specific immunological reagents and highly sensitive ELISA methods are used (Hathout 2007). Moreover, the secretome in a tumour microenvironment contains the secreted proteins such as cytokines, chemokines, growth factors and proteases as well as the extracellular matrix, constituted by proteins, receptors and adhesion molecules, and this makes biological fluids a complex mixture to investigate and may mask the potential biomarkers (Kulasingam and Diamandis 2008).

Increased expression of a secreted cathepsin D was identified using 2D-gel electrophoresis in estrogen-receptor positive human breast cancer cell lines, MCF7, ZR75 and T47D (Chen *et al.* 2008b). Cathepsin D has been observed to be involved in the regulation of breast cancer and invasion phenotype (Westley and Rochefort 1980). Recently, an extensive comparative proteomic analysis of supernatants from 3 breast cancer cell lines, MCF-10A, BT474 and MDA-MB-468 was performed and a number of proteins, including proteases, receptors, protease inhibitors, cytokines and growth factors were identified using LC-MS/MS (Hahnel *et al.* 1993). Three kallikreins (KLK5, KLK6, and KLK10) and HER2 were observed to be differentially regulated in conditioned media from BT474 and MDA-MB-468 cells. KLK5 and KLK10 have already been used as prognostic markers for breast carcinoma (Kulasingam and Diamandis 2007).

The normal (HTB-125) and malignant (HTB-126) breast cell lines have also been investigated using 'stable isotope labelling with amino acids in cell culture' (SILAC) coupled with mass spectrometry (Luo *et al.* 2002, Yousef *et al.* 2002). A number of growth factors were observed to be differentially regulated between normal and malignant cell lines. In this study, approximately 37% of proteins identified were observed to belong to ECM and cytoskeletal proteins, including collagen, vimentin, laminin, fibronectin, ECM protein 1, annexin, filamin, lumican, perlican, and plectin. There is increasing evidence to suggest that the ECM proteins participate in the control of successive stages of breast tumors, from appearance to progression and metastasis (Liang *et al.* 2009). Tumour necrosis factor (TNF), pigment epithelial-differentiating factor (PEDF) and stem-cell growth factor precursor showed decreased expression in breast-cancer cell lines, whereas Inhibin-β and macrophage migration inhibitory factor show increased expression. Interestingly, protease inhibitors, including plasma protease (C1) inhibitor, PZP precursor, and SERPINE2 were significantly down-regulated in the cancer cell lines as were angiostatic factors from extracellular matrix (ECM) such as endorepillin. Members of the SERPIN family are already well known to have tumour suppressor activity (Lochter and Bissell 1995). Furthermore, the C-terminal fragment of type XVIII collagen, endostatin, a potent angiostatic factor, was reported to be down-regulated as well whereas extracellular collagens and osteoblast-specific factor 2 (OSF-2), were reported to be up-regulated in this investigation. This indicates that the tumour cells differ from normal cells significantly in their secretome.

Another interesting study used tandem mass spectrometry to investigate the conditioned media from 4 isogenic breast cancer cell lines differing in aggressiveness includes non-tumourigenic (MCF10A), pre-malignant/tumourigenic (MCF10AT), tumourigenic/locally invasive (MCF10 DCIS.com) and tumourigenic/metastatic (MCF 10CA cl. D.) cells, and a number of differentially expressed proteins were identified in this investigation (Shi *et al.* 2001). The most apparent changes were observed for alpha-1-antichymotrypsin and galectin-3-binding protein which were secreted extensively from MCF10 DCIS.com and MCF10CA cl. D. cells compared to MCF10A and MCF10AT cell lines. Galectin-3-binding protein has already been associated with aggressive prostate and colon cancer suggesting its importance in the regulation of aggressiveness of breast cells (Mbeunkui *et al.* 2007). More recently, another group created the secreted protein map by analyzing the conditioned media of human mammary epithelial cells (HMEC) and identified ~900 proteins using LC-MS/MS

(Mbeunkui *et al.* 2006, Ulmer *et al.* 2006). Of these identified proteins, ~150 have already been known to be extracellular in localization and were comprised from growth factors and MMPs.

The MMPs could enable tumour cells to degrade basement membrane and thus to metastasize and therefore a number of small-molecule and peptidic MMP inhibitors were investigate for their therapeutic value (Jacobs *et al.* 2008). These inhibitors were unsuccessful in treating cancer disease in the phase III clinical trials because at that time only three MMPs were recognized to be involved in metastasis, however the full range of their substrates and other biological functions are unknown (Bissett *et al.* 2005, Coussens *et al.* 2002). Therefore a breast cell line expressing membrane type-1 MMP-14 was treated with an anti-MMP-14 drug (prinomastat) and the membrane-associated and secreted proteins from both treated and non-treated cells were investigated for their differential expression using isotope-coded affinity tag (ICAT) labeling and tandem mass spectrometry (Overall and Kleifeld 2006). In this investigation, 12 unknown substrate molecules, including CD59 and uPAR, were identified to be the potential substrates for MMP-14, of which 7 proteins were specifically observed in the conditioned media and includes ALCAM and melanotransferrin. ALCAM, CD59 and uPAR are already well known for their involvement in the regulation of cancerous and invasive phenotypes in breast (Butler *et al.* 2008).

1.5.1.3 Membrane and membrane-associated fraction

Membrane and membrane-associated proteins in cancer have been observed to be targets of a number of new drug and antibody cancer therapeutics such as Gleevec (abl-kinase), herceptin (her2neu), Panorex (Ep-CAM), and IRESSA (EGF1 receptor) (Cartwright *et al.* 1995, Andreasen *et al.* 1997). Furthermore many other membrane-associated proteins such as small GTPases, kinases, and catenins are implicated in carcinogenesis (Brenner and Adams 1999, Abicht and Lochmuller 2000, Ranson *et al.* 2002). Thus a comprehensive definition of cancer cell membrane-associated proteins can reveal further proteins involved in cancer biology which may themselves represent new therapeutic targets. However, a limited numbers of studies have been carried out to date to investigate the role of membrane and membrane-associated proteins in breast cancer.

Recently a database of cancer membrane-associated proteins was created by identifying more than 500 proteins from multiple human breast carcinoma cell lines with different

molecular pathologies MDA-MB-468, T-47D, BT-474, and MCF-7 (Ranson *et al.* 2002, Adam *et al.* 2003, Liang *et al.* 2006). In this investigation, three novel breast cancer membrane proteins, BCMP11, BCMP84, and BCMP101, were specifically observed to be associated with the cancer cell membrane compared to normal breast epithelial cells. Similarly, in an another effort, more than 830 cancer membrane-associated proteins were identified from culturing normal and malignant breast cancer cells isolated from a 74-year-old female with breast carcinoma using SILAC coupled with mass spectrometry (Adam *et al.* 2003). Among these, more than 40 proteins were found up-regulated or down-regulated by greater than 3-fold. A number of proteins that are already known to be associated with carcinogenesis in breast cells such as cathepsins, integrins and annexins were observed to be differentially regulated (Liang *et al.* 2006).

The protein profiles of cell surface proteins between the human breast cancer cell line MDA-MB-231 and its highly osteotropic B02 subclone have also been analysed using cell surface biotinylation and a mass spectrometric approach, and Class I HLAs were observed to be down-regulated, whereas αv-β3 integrins were up-regulated in B02 cells compared to MDA-MB-231 cells (Liang *et al.* 2006). The reduced HLAs class I expression has already been found in metastatic cells from the bone marrow of patients with squamous cell carcinoma of the head and neck region (Kischel *et al.* 2008). Similarly, αv-β3 integrins confer to breast tumor cells a greater propensity to metastasize to bone (Andratschke *et al.* 2003) and can even promote spontaneous metastasis of breast cancer cells to bone (Pecheur *et al.* 2002). This indicate that analysis of the cell surface proteome of parental cancerous cell lines and derived organ-specific metastatic cell lines provides an effective approach for the identification of potential biomarkers of breast cancer disease.

The investigation of membrane-associated and secreted proteins from MDA-MB-231 expressing MMP-14 and cells (MMP-14 expressing) treated with an anti-MMP-14 drug (prinomastat) was carried out using ICAT labeling coupled with tandem mass spectrometry (Sloan *et al.* 2006). In this investigation, 16 unknown substrate molecules, including CD59 and uPAR, were identified to be the potential substrates for MMP-14, of which 7 proteins were specifically observed in the membrane fraction and includes integrin-α3. Integrins, CD59 and uPAR are already well known for their involvement in the regulation of the cancerous and invasive phenotypes in breast (Butler *et al.* 2008).

This suggests that the knowledge of MMP-14 target proteins localized in membrane could help to understand the cell signaling induced by MMP-14 to facilitate metastasis.

1.5.2 Serum-based investigation

It has been realized that an extensive, complex and functionally significant cross talk between different molecules in the microenvironment does occur and that these interactions determine to a large extent, the malignant phenotype of the cells (Andreasen *et al.* 1997, Felding-Habermann 2003, Han *et al.* 1999). Secreted proteins are an important part of microenvironment. These microenvironmental molecules could influence tumour progression by three major non-mutually exclusive mechanisms; by further increasing the genetic instability of tumour cells (Park *et al.* 2000, Liotta and Kohn 2001), by inducing signalling cascades in tumour cells via tumour-associated receptors thereby controlling gene expression in these cells (Yuan and Glazer 1998), and by exerting selective pressures on the cells. Microenvironmental factors exert, in many cases, opposing effects on tumour cells and on tumour progression. Therefore, investigations for alterations in the microenvironment due to secreted proteins could help us to understand the cancerous and invasive behaviour of breast cells (Bissell *et al.* 1999).

One of the sources to mine for potential secreted biomarkers is serum or plasma of breast cancer patients. In a proteomics study of breast cancer patient serum, two proteins were differentially regulated; HSP27 was observed to be increased while 14-3-3 sigma protein was found to be decreased in cancer serum compared to normal serum using 2D-PAGE coupled with MALDI-ToF MS (Liotta and Kohn 2001). The comparison of the expression patterns of these two proteins correctly classified 97% of the controls as not cancer, and 100% of cancer samples as malignant. This result yielded 100% sensitivity. The positive predictive value for this sample set was 98%. MALDI-ToF MS profiles have also been used to discriminate diseased samples from normal samples (Rui *et al.* 2003). A total of 72 peaks identified from MALDI-ToF MS profiles of 48 breast cancer patients and 28 controls were observed to identify breast cancer patients with approximately 85% sensitivity and specificity (Callesen *et al.* 2008). In another study, 2D-DIGE analysis of serum samples obtained from 39 patients with breast cancer and 35 controls revealed that proapolipoprotein A-I, transferrin, and hemoglobin were up-regulated, and three proteins, apolipoprotein A-I, apolipoprotein C-III, and haptoglobin a2 were down-regulated in cancer patients (Callesen *et al.* 2008).

A serological proteomics-based approach (SERPA) has also been applied to identify the novel tumour antigens that may induce a humoral immune response in sera from patients with breast carcinomas (Huang *et al.* 2006). Tumor antigens elicit a humoral immune response in patients. In SERPA, cellular proteins from tumour (breast) cell lines are separated by 2-DE and transferred onto PVDF membranes. Using these membranes, sera from cancer (breast) patients and controls, as a source of primary antibody, are screened individually by western blot analysis for antibodies that react against separated tumour-derived proteins on PVDF membranes. An RNA-protein interaction protein, RS/DJ-1, was identified using cellular proteins from a breast cancer cell line (SUM-44), sera from 30 breast cancer patients, 116 patients with other cancer and 42 control individuals (Hamrita *et al.* 2008, Le Naour *et al.* 2001). Similarly, 26 tumour antigenic proteins were identified using MCF-7 cellular proteins and sera from 40 invasive breast cancer patients and 42 control individuals (Le Naour *et al.* 2001). Among these, HSP60, prohibitin, β-tubulin, peroxiredoxin-2, hnRNPK, Mn-SOD and F1-ATPase represented significant immunoreactivity in specifically with patients sera, whereas cytokeratins 8 and 18, and F1- actin were observed in both control and breast cancer patients sera (Hamrita *et al.* 2008). Many of these proteins such as HSP60, prohibitin and hnRNPK have already been correlated with breast cancer (Hamrita *et al.* 2008). The autoantibodies for HSP60 has been observed in the 16/49 (31%) early stage breast cancer and 18/58 (32.6%) DCIS patients, compared to 4/93 (4.3%) healthy subjects (Desmetz *et al.* 2008, Jupe *et al.* 1996, Fink 1999, Bukau and Horwich 1998, Moumen *et al.* 2005). In particular, autoantibodies were present in 11/23 patients (47.8%) with high-grade DCIS, compared to 5/26 (19.2%) with low-grade DCIS and expression of HSP60 was also observed using immunohistochemisty to increase gradually from normal through DCIS to invasive tissues (Desmetz *et al.* 2008). Based on these findings, the SERPA approach offers great potential to discover novel biomarkers as well as drug targets for breast cancer disease.

A pattern of three serum biomarkers (two up-regulated at 8.1 kDa and 8.9 kDa and one down-regulated at 4.3 kDa) were identified from the SELDI profiles of 169 serum samples from 103 breast cancer patients, 41 healthy women, and from 25 benign breast cancer patients that distinguished patients from controls (Desmetz *et al.* 2008). The sensitivity and specificity after cross-validation within the sample group (bootstrapping), using a random subset of the data to build the model and testing it with the remaining data were found to be 93% and 91%, respectively. However, a new set of

samples in a later study confirmed the up-regulation of the 8.1 kDa and 8.9 kDa biomarker species, and subsequently the 8.9 kDa species was identified to be a complement component of C3a (desArg) and the 8.1kDa species as a C-terminal-truncated form of C3a (desArg) (Li *et al.* 2002). In a later study also using SELDI-ToF MS, the combination of an independent cancer biomarker Ca 15.3 with the serum biomarkers, 4.2 kDa and 4.3 kDa possibly corresponding to the 4.3 kDa peak identified by Li *et al.* (Li *et al.* 2005) and 8.9 kDa and 8.9 kDa possibly corresponding to the 8.9 kDa peak also identified by Li *et al.* (Li *et al.* 2002), significantly improved breast cancer diagnosis. In another study, four peaks, CA1 (17.3 kDa), CA2 (26.2 kDa), CA3 (5.7 kDa), and CA4 (8.9 kDa), were chosen as potential biomarkers from the SELDI profiles of 49 breast cancer patients, 51 patients with benign breast diseases, and 33 healthy women to build a prediction model using artificial neural networks and discriminant analysis (Li *et al.* 2005). 100% sensitivity and specificity were observed in a training set while a blind test set showed 76.47% sensitivity and 90% specificity. Another study utilized this technology as a prognostic tool on a pool of 81 serum samples from high risk early breast cancer patients, collected in a retrospective way after primary surgical resection and before starting any adjuvant chemotherapy. Forty-eight patients showed metastatic relapse while 33 were long-term metastasis-free survivors. SELDI-ToF MS analysis produced a pattern of 40 peaks which allowed the development of a multi protein index that was able to correctly predict outcome in 83% of patients with a sensitivity of 87% and a specificity of 76% (Hu *et al.* 2005).

Glycosylation, one of the most abundant post-translational modifications required by proteins to perform its specific biological functions, has been associated with secreted proteins (Goncalves *et al.* 2006). However, very little information is available for the role of these secreted glycoproteins in cancer disease, as they are typically not detected due to their low concentration in biological fluids, such as serum. Recently, lectin-based and hydrophilic interaction chromatography (HILIC) using resin-based affinity techniques have been successfully applied to enrich the glycoproteins and glycopeptides in serum samples from breast cancer patients to identify these glycosylated proteins using MALDI and ESI mass spectrometry (Calvano *et al.* 2008). A total of 45 proteins were identified in this investigation and includes SERPINS, integrins and Kallikrein which are already well known for their role in the breast cancer.

1.5.3 Plasma-based investigations

The plasma samples from mice bearing breast tumours have been compared with normal mice by combining isotopic labelling, extensive intact protein separation (2D-HPLC) and mass spectrometry (LTQ-Orbitrap mass spectrometer coupled with a NanoLC-1D MS) (Calvano *et al.* 2008). A total of 133 proteins were observed to be increased in the plasma samples from the tumour bearing mice compared to the normal mice (Pitteri *et al.* 2008). Of these, 49 proteins including fibronectin, enolase 2, perlecan and L-plastin, were already known to be up regulated in breast cancer cell lines compared to normal cells (Pitteri *et al.* 2008). Osteopontin and fibulin-2 have been observed as a plasma biomarker for breast tumours, whereas osteopontin was observed to be capable of early disease detection in the mouse (Kulasingam and Diamandis 2007, Patwardhan *et al.* 2005, Hou *et al.* 2007). In another study, the plasma samples from MCF7-xenografted tumour model (mouse) and normal mice were investigated using multilectin affinity chromatography (M-LAC) to identify tumour-secreted glycoproteins (Whiteaker *et al.* 2007). A large set of known murine-secreted proteins was identified in this study, including several signalling molecules such as HER-2, interleukin-6 receptor, protein-kinase C, and phosphatidylinositol kinase which changed in plasma levels relative to tumour-free animals. Whereas, 17 human tumour-derived proteins, that were involved in wide variety of cellular functions such as cell signalling, immune response, and transcriptional regulation, were also identified in this investigation. This suggests that glycoproteins could be investigated using enrichment techniques and could enable us to identify potential drug targets and/or biomarker for cancer disease.

1.5.4 Nipple aspirate fluid-based investigation

Nipple aspirate fluid (NAF) has been identified as a potential source of biomarkers for early diagnosis of breast cancer (Orazine *et al.* 2008). Therefore NAF samples from 23 women with stage I or II unilateral invasive breast carcinoma and five healthy female volunteers were investigated using SELDI Protein-chip arrays (WCX2 and IMAC3-Cu++), and protein expression was analyzed using time-of-flight MS (Sauter *et al.* 1997, Liu *et al.* 2000b). Two peaks (952.59 and 2310.87 *m/z*) that were over expressed in breast cancer patients and one peak (3284.74 *m/z*) that was down regulated in breast cancer patients showed a distinct expression pattern in normal, stage I and stage II samples and could be useful for breast cancer screening and diagnosis. NAF have also been investigated using 2D-gel electrophoresis. 52 NAF samples from breast cancer

patients and 53 NAF samples form normal were investigated using 2D-gel electrophoresis and a total of 41 different proteins were identified using MALDI-ToF MS (Pawlik *et al.* 2005), of which 25 proteins, including cystic disease fluid protein (GCDFP)-15 and α-1-acid glycoprotein (AAG), were known to be secretary in nature. The expression of GCDFP-15 and AAG was observed to correlate with presence and stage of breast cancer disease. The NAF protein profiles from 18 women with I or II unilateral invasive breast carcinoma and 4 healthy volunteers were analyzed using ICAT labeling, sodium dodecyl sulfate-polyacrylamide gel (SDSPAGE), liquid chromatography and MS (Alexander *et al.* 2004). Alpha2HS-glycoprotein was observed to be down regulated, while lipophilin B hemopexin and vitamin D-binding protein precursor were over expressed in NAF from tumor-bearing breast compared to healthy women in this investigation suggesting these proteins as potential plasma biomarkers for breast cancer disease. Recently, NAF proteomic profiles have been observed not to vary substantially during the menstrual cycle and therefore NAF proteomic profiles for different stages in the menstrual cycle could be compared to identify potential biomarkers and/or drug targets for breast cancer disease (Pawlik *et al.* 2006).

1.5.6 Tissue based investigations

Tissue biopsies are the ideal source for biomarkers and/or drug target discovery for breast cancer disease as they represent a similar state of disease as observed *in vivo*. However, their use is limited due to limited number (and amount) of available samples representing various stages (early or late) of disease and complexity of specific tissues. Tissue sample have been used to validate the potential drug targets and/or biomarkers as well as for their discovery. The proteomic analysis of ductal carcinoma in situ (DCIS) has revealed 57 proteins that show differential expression between normal cells and DCIS (Noble *et al.* 2007). Although the differential expression was predominantly due to differences in overall abundance, there was also evidence of posttranslational modification of the proteins. Proteins such as transgelin and the voltage-dependent anion channel protein (VDAC) showed evidence of posttranslational modification (Wulfkuhle *et al.* 2002). A subset of proteins including, Annexin V, profilin and HSP90 showing differential expression on 2D gels have been confirmed by IHC, a further evidence that current proteomic strategies have sufficient sensitivity (qualitatively and quantitatively) to detect clinically relevant changes and therefore can be successfully

used to explore protein expression trends and discover novel clinically relevant protein expression portraits previously unconnected to breast cancer. The proteomic analysis of infiltrating ductal carcinoma (IDCA) of the breast from different stages (Stage I (normal), IIA, IIB and IIIA) by 2D-DIGE revealed a number of proteins that appeared to be differentially expressed between breast IDCA and matching normal tissue (Wulfkuhle *et al.* 2002). Approximately, 69–85% of the proteins detected were found not to be significantly different between the normal and diseased samples. A total of 27 differentially expressed proteins were identified in this investigation using LC-MS/MS, however the functional role or how the majority of these identified proteins mediate the highly complex tumour processes are largely unknown. Examples of the proteins detected as differentially expressed were carbonic dehydratase, disulfide isomerase, gelsolin and fibrinogen beta (Somiari *et al.* 2003). The protein expression profile and observed trend for a number of the proteins is consistent with information in literature for some of the proteins. For example, fibrinogen gamma-chain and fibrinogen beta-chain fragments have been identified in various solid tumour types at the protein level, and fibrinogen gamma-chain dimmer cross linked by transglutaminase were detected in tumour patients but not in controls (Somiari *et al.* 2003). It is suggested that the elevation of β-fibrinogen correlates with tumour-associated fibrin deposition. Carbonic dehydratase, disulfide isomerase, gelsolin and fibrinogen beta are over expressed in IDCA whereas gelsolin, which is known to bind to β-actin, is less abundant in IDCA compared to normal tissue (Jones *et al.* 2006).

The investigation of protein profiles of primary epithelial cells from 7 metastatic breast cancer patients and 16 non-malignant breast cancer patients using 2D-DIGE coupled with LC-MS/MS revealed that the expression of nucleoplasmin was observed to be increased and the expression of glutathione peroxidise 1 and 2,3-trans-Enoyl-CoA isomerase were reduced in metastatic breast cancer patients compared to non-malignant breast cancer patients (Gerner *et al.* 2001). The nucleoplasmin and glutathione peroxidise 1 are already known to be involved in cancer whereas the roles of 2,3-trans-Enoyl-CoA isomerise are still unknown (Vydra *et al.* 2008).

In an another study, 8 ER^+ and 8 ER^- breast tumours were investigated using 2D-PAGE coupled with MALDI-ToF MS (Lei *et al.* 2007, Zhang 2004). Progesterone receptor membrane component 1 (PGRMC1) was identified to be differentially expressed at three different spots on the 2D-gel due to phosphorylation of this protein and of these 3 spots, 2 spots were more abundant in ER^- tumours compared to the ER^+ tumours.

Abrogated activity of PGRMC1 was observed to result in reduced peroxide-induced cell death in MCF7 breast cancer cells. PGRMC1 abundance and phosphorylation was expected to be involved in the regulation of clinical consequences of tumours, potentially maintaining not only cell migration and tissue morphogenesis but also improved survival and tissue homeostasis. A pool of 4 differentially expressed peaks identified in the comparison of protein profiles from 61 ER^+ breast tumours and 56 ER^- breast tumours using laser capture microdissection and MALDI-ToF were observed to identify ER^+ breast tumours compared to ER^- breast tumours with 66.1% accuracy in the testing cohort with a sensitivity of 53% and a specificity of 87.5% (Neubauer *et al.* 2008). The analysis of protein profiles from 122 invasive mammary carcinomas and 167 normal mammary epithelium cells generated using laser capture microdissection and MALDI-ToF was also performed and this identified a set of 14 peaks that could be used to discriminate diseased samples from normal tissue (Sanders *et al.* 2008). These 14 peaks were observed to identify breast cancer tissue with 94% accuracy from normal samples with a high sensitivity (89%) and specificity (98%).

Some studies have been carried out to classify breast tumours on paraffin-embedded samples at the protein level. 166 breast cancer tissue samples were evaluated by immunohistochemistry, using 15 different antibodies (Sanders *et al.* 2008). Cytokeratin 5/6 (Ck 5/6)-positive breast carcinomas were in general negative for estrogen receptor and progesterone receptor and were highly proliferating whereas Ck 5/6-negative breast carcinomas revealed a lower tumour proliferation rate (Korsching *et al.* 2002). Another study classified tumour in two distinct groups based on ER-negative and ER-positive status (Korsching *et al.* 2002). The ER^- and ER^+ groups were further divided into two subgroups based on the VEGF status, thus forming four subgroups, ER-/$VEGF^+$, ER-/$VEGF^-$, ER^+/$VEGF^+$ and ER^+/$VEGF^-$. Interestingly, the ER^-/$VEGF^-$, ER^+/$VEGF^+$ and ER^+/$VEGF^-$ phenotypes were either p53 positive or p53 negative, whereas the ER^-/$VEGF^+$ tumours were distinguishable as either c-erbB2 positive or c-erbB2 negative (Zhang *et al.* 2003).

In another effort, SELDI proteomic profiles were compared with a previous cDNA expression analysis using whole tissue lysates of 105 breast carcinomas (Zhang *et al.* 2003). SELDI-ToF MS analysis provided similar clustering of tumours to those identified by the cDNA expression profiles (Brenton *et al.* 2005). The first subgroup was luminal subtypes (A and B), which makes up the hormone receptor-expressing breast cancer and has expression patterns reminiscent of the luminal epithelial

component of the breast (including luminal cytokeratin 8/18 and cyclin D_1 genes). The second subgroup was called the HER2/*neu* subtype, but should not be confused with HER2/*neu*-positive tumours identified by immunohistochemistry or fluorescence *in situ* hybridization because not all clinically HER2/*neu*-positive tumours show the RNA expression changes that define this subtype (Brenton *et al.* 2005, Brozkova *et al.* 2008). SELDI-ToF MS analysis also identified differential protein peaks in primary breast cancers that predict the presence and number of axillary lymph node (ALN) metastases and non- sentinel lymph node (SLN) status (Brenton *et al.* 2005, Brozkova *et al.* 2008). Two metal-binding polypeptides at 4,871 and 8,596 Da were identified to be associated with significant risk factors for nodal metastasis in this investigation. Recently, SELDI-ToF MS analysis of breast tissue has identified the carboxyl terminus-truncated ubiquitin as a marker for luminal-like and S1009 for basal type (Nakagawa *et al.* 2006). Therefore the investigation of cancer tissue using proteomics based approaches could reveal potential drug targets and/or biomarkers for breast cancer diagnosis and prognosis.

1.5.7. Summary

It is clear that there is a limited number of established biomarkers and/or drug targets for the diagnosis and prognosis of breast cancer in the early phase of disease. To date, only a few studies have investigated the expression of proteins that may have a role in the regulation of breast cancer and invasion. Therefore, investigation of differential expression of cellular, secreted and membrane & membrane-associated proteins in breast cancer cell models could enable us to identify proteins that could be potential biomarkers and/or drug targets for breast cancer disease.

1.5.8 Selection of cell line models for this study

Although cell line model based investigation has significantly contributed to develop new therapies for cancer disease, currently there are only few studies on breast cancer invasion using cell lines. Therefore four cell lines (HMEC, MCF-10A, MCF-7 and BT-20) representing normal, cancerous, non-invasive and invasive phenotypes were investigated in this study to identify cancer and invasion-associated proteins. HMEC cells are normal human mammary epithelial cells that are derived from the basal subtype and are invasive. MCF-10A cells are basal subtype with intact p53 that were

derived by spontaneous immortalization of breast epithelial cells from a patient with fibrocystic disease (Goncalves *et al.* 2008). These cells do not survive when implanted subcutaneously into immuno-deficient mice (Soule *et al.* 1990). MCF-10A cells are therefore immortalized normal cells and have been used extensively as a non-invasive normal control in breast cancer studies. MCF-7 cells are 'luminal epithelial-like' subtype established from a pleural effusion obtained from a 69-year-old caucasian woman and express estrogen receptor (Soule *et al.* 1990). MCF-7 cells are the most common example of estrogen-responsive breast cancer cells and are non-invasive. The BT-20 cell lines are derived from an advanced invasive ductal carcinoma during a mastectomy in 1958 (Dickson *et al.* 1986, Charafe-Jauffret *et al.* 2006). These cells were obtained by slicing the primary tumour into thin sections and collecting the cells that had detached from the stroma. It is a basal subtype and expresses estrogen receptor and is invasive.

A panel of breast cell lines including non-invasive (MCF-7, MCF-10A, T47D and UACC 812) and invasive (HMEC, SKBR-3, MDA-MB 231, HCC 1937, BT20 and Hs578T) were also included to screen identified protein targets which have potential role in invasion. This panel of cell lines covered a broad range of origin (basal, luminal and normal-like), i.e. basal (MCF-10A, MDA MB 231, HCC-1937 and Hs578T), luminal (MCF-7, T47D, UACC 812 and SKBR-3) and normal-like (MCF-10A). Thus, these cell lines represented heterogenity of samples which often occurs in *in-vivo* models and was therefore could lead to the identification of real targets associated with invasion in breast cells.

1.6 Melanoma

Melanoma is a malignant tumour originating from melanocytes, the cells that produce the pigment melanin. During the first trimester of fetal life, precursor melanocytes arise in the neural crest (ectoderm). As the fetus develops, these cells migrate to different body areas, such as the skin, uvea, leptomeninges and mucous membranes (e.g. upper esophagus, vulva, anus). Accordingly, melanoma can arise in all these sites, although cutaneous melanoma is by far the most frequent type of melanoma. Melanoma is the most lethal skin cancer and accounts for about 75% of all deaths from skin tumours (Lasfargues and Ozzello 1958). Melanoma originates from the melanocytes of the epidermis. Melanoma mostly occurs in skin but may occur in eye or other places where

melanocytes occurs. The etiologic factors leading to melanoma are unknown, although a few factors, i.e. nature of skin (caucasians or black), exposure to sun light and hereditary factors have been correlated with the development of melanoma (Liotta *et al.* 2003). Over the past two decades, substantial preclinical advancements have been made in the understanding of melanoma molecular biology (development, progression, resistance to medical therapy). Nevertheless, the clinical implementation of molecular medicine principles (e.g. the use of melanoma-specific prognostic biomarkers and molecular therapies) is just in its infancy, and much work remains to be done in order to develop a tumour-targeted patient-tailored therapeutic approach to this disease.

1.6.1 Types of Melanoma

Four main morphological types of melanoma have been classically recognized: superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM), and acral lentiginous melanoma (ALM). Furthermore, several unusual variants of melanoma such as desmoplastic melanoma and nevoid melanoma have been recently characterized (Setlow 1999).

1.6.1.1 Superficial Spreading Type

This type of melanoma is more commonly found on the trunk, upper arms, and thighs, and is the most common form of melanoma in white races. It begins as a small pigmented macule that is asymmetric, has irregular borders, and has colour variations. This type of melanoma remains in the flat phase for a shorter period of time than the lentigo maligna type before it penetrates into the deeper levels of the skin.

1.6.1.2 Nodular Type

This type of melanoma can occur on any skin surface but is found more commonly on the trunk, upper arms, and thighs. The nodular type of melanoma has a very short flat phase before it forms a raised nodule and penetrates into the deeper levels of the skin. This type of melanoma may ulcerate and present as a non-healing skin ulcer.

1.6.1.3 Lentigo maligna melanoma (LMM)

LMM typically occurs in older people, on sun-exposed areas (mostly head and neck). LMM was considered to have a better prognosis than the other forms of melanoma, but recent studies have suggested that the different biologic behaviour may be related to an

inferior thickness at the time of diagnosis. This variant of melanoma is histologically characterized by a confluent growth of atypical melanocytes along the dermal-epidermal junction frequently extending downwards the cutaneous appendages. The invasive component may be composed of spindle cells or may be associated to a desmoplastic reaction.

1.6.1.4 Acral-Lentiginous Type

This type of melanoma is more commonly found on the hands, feet, and nail beds. It is seen in all races, but is most frequently found in dark-skinned races. It is similar to the lentigo maligna and superficial spreading type in that it has a relatively long flat phase before it penetrates into the deeper levels of the skin.

1.7 Serum biomarkers for Melanoma

Biomarkers enable the clinical diagnosis and prognostic classification of various types and stages of cancers. Biomarkers are usually proteins that may be produced by cancer cells or by normal cells (Magro *et al.* 2006). They may be intracellularly or on the surface membrane, from where they can be released in to body fluid. There are some promising serum markers for melanoma currently being studied:

1.7.1 S100

S100 was originally extracted from bovine brain and its name is derived from its solubility in 100% saturated ammonium sulphate at neutral pH. The S100 protein is a low molecular weight (21 kDa), acidic, thermolabile, calcium-binding protein. It is a dimer consisting of two subunits, α and/or β, with three combinations αα, α β and β β cells. The beta subunit is expressed in cells of the central nervous system as well as in cells of the melanocytic lineage (Liotta *et al.* 2003). In melanoma, the β-subunit is positively correlated with the invasiveness of the tumour and was considered as a prognostic marker in stage I to IV melanoma patients (Jackel *et al.* 1999, Moore 1965).

1.7.2 Melanoma inhibitory activity

Melanoma inhibitory activity (MIA) is an 11-kDa soluble protein identified as an autocrine-secreted tumour cell growth inhibitor (from which it derived its name) isolated from the supernatant of the human HTZ-19 melanoma cell line culture (Henze *et al.* 1997). This protein showed the reduction of melanoma cell attachment to the

extracellular matrix, because it binds to fibronectin and laminin, thereby preventing cell–matrix interaction (Bogdahn *et al.* 1989). MIA was shown to be useful prognostic marker in melanoma patients with metastatic malignant melanoma (Apfel *et al.* 1992).

1.7.3 Lactate dehydrogenase

The strongest prognostic serum biomarker in advanced metastatic melanoma is lactate dehydrogenase (LDH), an unspecific marker indicating high tumour load in a variety of tumour entities, including melanoma. Studies comparing LDH, S100-beta and MIA using multivariate data analysis showed LDH to be the strongest independent prognostic factor in stage IV melanoma patients (Deryugina and Quigley 2006). Due to its high prognostic significance, coupled with its easy, cost-efficient and widely distributed detection methodology, serum LDH is the only molecular marker that has been included in the current melanoma staging and classification system of the AJCC (Deichmann *et al.* 1999).

1.7.4 Cytokines and cytokine receptors

Cytokines are small and secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. The cytokine family consists mainly of smaller, water-soluble proteins and glycoproteins with a mass between 8 and 30 kDa. Increased serum levels of IL-6 and IL-8 were found increased in the serum of stage IV patients and there was a correlation with tumour burden. Serum levels of IL-6 were also higher in patients who were non-responsive to therapy, regardless of tumour burden (Balch *et al.* 2001). IL -10 was also found increased in patients with metastatic malignant melanoma and correlated with poor survival (Mouawad *et al.* 1996, Scheibenbogen *et al.* 1995, Tartour *et al.* 1994). Elevated levels of sIL-2R have been reported in melanoma, even in early stage disease and a correlation between the sIL-2R level and melanoma progression has also been reported (Dummer *et al.* 1995, Nemunaitis *et al.* 2001). Although these substances have been demonstrated in melanoma patients, the exact role in tumour immunology is not yet understood.

1.7.5 Cell adhesion molecules

Cell Adhesion Molecules (CAMs) are proteins located on the cell surface involved with the binding with other cells or with the extracellular matrix (ECM) in the process called cell adhesion. Increased intercellular adhesion molecule-1 (sICAM-1), and decresed L-

selectin (sL-selectin) levels were generally associated with the progression of malignant melanoma (Ottaiano *et al.* 2006). Elevated levels of VCAM-1 in serum (sVCAM-1) have been observed in some, but not all of the previous reports on melanoma (Yamada *et al.* 2005).

1.7.6 5-S-cysteinyldopa (5-S-CD)

5-S-cysteinyldopa (5-S-CD) is a precursor of melanin. 5-S-CD is produced by melanocytes and melanoma cells, and is detectable in sera of patients. Elevated serum levels of 5-S-CD have been reported in melanoma patients and have correlated its expression with the prognosis of stage IV melanoma patients (Franzke *et al.* 1998, Miller *et al.* 1997).

1.7.7 Glypican-3 (GPC3) and secreted protein acidic and rich in cysteine (SPARC)

Glypican-3 is a cell surface heparan sulfate proteoglycans. GPC3 may be involved in the suppression/modulation of growth in the predominantly mesodermal tissues and organs. Secreted protein acidic and rich in cysteine (SPARC), also called osteonectin or BM-40, is a matricellular glycoprotein that modulates cellular interaction with the extracellular matrix during tissue remodeling. One study reported glypican-3 (GPC3) as a novel tumour marker but could diagnose only 40% of melanomas (Banfalvi *et al.* 2002). They also detected SPARC in the sera of melanoma patients at higher concentrations than in healthy donors. Indeed, SPARC was detected in the sera of 33% of all melanoma patients, irrespective of the clinical stages and even in the sera of patients with stage 0 *in situ* melanoma. Moreover, the combined use of secreted protein acidic and rich in cysteine (SPARC) and GPC3 will thus make it possible to diagnose melanoma, especially in the early stages (0-II) (Nakatsura *et al.* 2004).

1.7.8 Cytoplasmic melanoma-associated antigen (CYT-MAA)

Cytoplasmic melanoma-associated antigen (CYT-MAA) is a cytoplasmic protein composed of 4 noncovalently associated polypeptides of 94, 75, 70 and 25 kDa molecular weight. While elevated levels of the marker do not correlate to the stage of the disease, being equally high at all AJCC stages (II–IV), elevated serum levels correlate significantly with disease recurrence and progression, and was found to be an accurate marker when used to monitor response to treatment in patients with AJCC stages IIb–IV. 90% of patients that were initially CYT-MAA positive, showed a

decrease in CYT-MAA serum levels during the treatment and those with elevated marker levels during the treatment were around three times more likely to recur or suffer disease progression (Ikuta *et al.* 2005).

1.7.9 High-molecular-weight melanoma-associated antigen (HMW-MAA)

A high-molecular weight melanoma-associated antigen (HMW-MAA), also known as melanoma-associated chondroitin sulphate proteoglycan (MSCP) is found expressed in high density on the surface of cultured melanoma cells. HMW -MAA is a serum marker for residual melanoma in patients with resected disease (Reynolds *et al.* 2006).

1.7.10 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF-C, VEGF-D) are multifunctional, homodimeric peptide cytokines. Elevated levels of VEGF in sera of patients with melanoma have been correlated with progression of melanoma (Vergilis *et al.* 2005). One recent study reported that VEGF-C might be involved in the deep lymphatic dissemination and progression of melanoma metastasis (Ascierto *et al.* 2004). Another study reported significantly increased serum levels of VEGF, bFGF, interleukin-8 and angiogenin in melanoma patients with advanced stage disease and tumour burden (Vihinen *et al.* 2007). Elevated levels of endostatinin in serum of melanoma patients IV disease have also been reported (Ugurel *et al.* 2001a).

1.7.11 L-Dopa/L-tyrosine and Tyrosinase

The melanin biosynthetic pathway is critically regulated at the enzymatic level by tyrosinase in melanocyte-specific copper-containing organelles called melanosomes. Tyrosinase is a key factor in melanogenesis since it catalyzes the rate limiting step in melanin biosynthesis, i.e. L-tyrosine to L-DOPA, and subsequent oxidation to DOPAquinone. DOPAquinone is oxidized to DOPAchrome which gives dihydroxyindoles. These dihydroxyindoles are further oxidised to produce eumelanina (brown/black pigment) (Kurschat *et al.* 2007). A high level of serum L-dopa/L-tyrosine ratio (an index of tyrosinase functional activity) in melanoma patients correlates with the tumour burden and in some cases predicted disease progression in metastatic melanoma patients (Letellier *et al.* 1997). Very few studies showed high tyrosinase activity in serum from patients with malignant melanoma (Stoitchkov *et al.* 2002).

1.7.12 soluble HLA-DR (sHLA-DR)

HLA-DR is a major histocompatibility complex, MHC class II, cell surface receptor encoded by the human leukocyte antigen complex. The complex of HLA-DR and its ligand, a peptide of 9 amino acids in length or longer, constitutes a ligand for the T-cell receptor (TCR). HLA-DR is also involved in several autoimmune conditions, disease susceptibility and disease resistance. Lower sHLA-DR levels in serum from melanoma patients have been reported and reduced amounts of sHLA-DR have been found associated with advanced disease stages and tumour burden (Agrup *et al.* 1989, Lugovic *et al.* 2007).

1.7.13 CRP (C-reactive protein)

C-reactive protein (CRP) is a plasma protein, an acute phase protein produced by the liver and by adipocytes. CRP is a member of the pentraxin family of proteins. Elevated levels of CRP in serum have been associated with shortened survival in metastatic melanoma patients and resistance to interleukin-2 therapy (Rebmann *et al.* 2002). High CRP levels to indicate progression of distant metastases during therapy could be useful serum markers for monitoring metastatic malignant melanoma (Tartour *et al.* 1996).

1.7.14 sFas/CD95

The membrane-bound type I protein Fas/CD95 and its ligand, FasL, play a key role in maintaining tissue homeostasis via induction of apoptosis. Besides regulation of lymphatic cells and tissues providing immunotolerance, limiting clonal expansion, and maintaining immunoprivileged sites of the organism, the Fas/CD95-FasL system has recently been described to exert important functions in the control of malignant proliferation. Elevated levels of serum sFas/CD95 had shown a prognostic relevance in melanoma patients (Deichmann *et al.* 2000). Limited studies on function and clinical significance of serum sFas/CD95 have been carried out to date.

1.7.15 YKL-40

YKL-40 is a growth factor for connective tissue cells and stimulates migration of endothelial cells. Cancer cells, macrophages, and neutrophils secrete YKL-40. Its function in cancer is unknown. Elevated serum levels of YKL-40 have been reported and could be an early biomarker of relapse and survival in patients with AJCC stage I and II melanoma (Ugurel *et al.* 2001b).

1.7.16 TA-90

TA-90 has been found on the outer surface of melanoma cells. TA-90 is also found in the serum of melanoma patients as a free antigen and as an immune complex (IC) with IgG antibody. In addition, immunoglobulin-M (IgM) and IgG-mediated antibody responses to TA90 can be detected in the sera of patients with melanoma. The presence of TA90-IC in the serum of patients with melanoma has been shown to correlate with occult lymph node metastases and decreased overall survival (Schmidt *et al.* 2006, Schmidt *et al.* 2006). Both the presence of TA90-IC and the absence of IgM antibody against TA90 (anti-TA90) after surgical resection of distant melanoma metastases (AJCC stage IV) are inversely correlated with outcomes even in patients who receive postoperative adjuvant treatment with a polyvalent vaccine (Kelley *et al.* 1998).

1.7.17 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMP) are a family of zinc-dependent neutral endopeptidases characterized by their ability to degrade extracellular matrix components as described in section 1.2.2.2.1. The serum levels of MMP-2 in patients with advanced melanoma was studied and found that serum MMP-2 does not have any role as a prognostic marker (Hsueh *et al.* 2000). On the other hand, the serum levels of MMP-2 were significantly higher in patients with metastatic melanoma than in patients with localized melanoma. In addition, somewhat higher MMP-2 levels were noted in patients with more advanced disease when MMP-2 levels were compared with different tumour stages. High serum levels of MMP-9 were found to be associated with poor overall survival. Elevated MMP-9 levels were found to correlate with multiple tumour sites. In some studies elevated levels of TIMP-1 and TIMP-2 were found that reflected the extent of metastatic melanoma lesions (Vuoristo *et al.* 2000).

In summary, serum S100-beta has been shown to be superior compared to MIA, as an early indicator of tumour progression, relapse or metastasis, and its distribution as a serum biomarker of melanoma, therefore, is the broadest. Both markers have been shown to be useful prognostic markers in melanoma patients with distant metastases (stage IV, classification system of the American Joint Committee on Cancer, AJCC). The strongest prognostic serum biomarker in advanced metastatic melanoma is lactate dehydrogenase, an unspecific marker indicating high tumour load in a variety of tumour entities, including melanoma. Studies comparing LDH, S100β and MIA using multivariate data analysis showed LDH to be the strongest independent prognostic

factor in stage IV melanoma patients. Although LDH, S100β and MIA have been shown to be useful prognostic markers of malignant melanoma, they failed to be prognostic in early-stage of melanoma. None of the other markers could be confirmed to be superior to S100-beta or LDH in reflecting the prognosis of patients in advanced disease stages, nor could any marker be shown to be of strong prognostic relevance in early stage tumour-free patients.

1.8 Proteomics and melanoma

Currently no protein marker is available for surveillance of melanoma progression in early-stage melanoma (Yoshino *et al.* 2008). Only little work has been reported to date concerning the presence of abnormal proteins or peptides in patients bearing melanoma. The secretome of uveal malignant melanoma (UM) was investigated using 2D-gel electrophoresis coupled with LC-MS/MS spectrometer in an attempt to detect tumour specific proteins liberated into the tumour surroundings (Wilson *et al.* 2004, Caputo *et al.* 2005). Cathepsin D, melanoma-specific antigen gp100, and adapter protein mda-9/syntenin 1 (melanoma differentiation-associated protein) were found to be positive in all the UM secretomes compared to normal cells indicating these proteins as potential biomarkers (Pardo *et al.* 2007). There are a few reports in the literature on profiling serum from melanoma patients using SELDI-ToF MS. Serum specimens of 49 early stage (American Joint Committee on Cancer (AJCC) stage I and II) patients including 25 patients with melanoma recurrence and 24 without evidence of disease following resection were profiled using SELDI-ToF MS (Pardo *et al.* 2007). The differential patterns of peaks among patients with recurrence and without recurrence were used for predicting the chances of melanoma recurrence and it resulted in a sensitivity of 72% and a specificity of 75% that was significant (Wilson *et al.* 2004). Similarly 205 serum samples from 101 early-stage (AJCC stage I) and 104 advanced stage (AJCC stage IV) melanoma patients were analysed by SELDI-ToF and MALDI-ToF mass spectrometry (Wilson *et al.* 2004). SELDI profiles were used to train artificial neural networks (ANN). Based on these ANN algorithms, 88% of stage assignment was correctly predicted. Moreover, 80% of stage III samples could be correctly predicted as progressors and 82% of stage III progressors were correctly identified. The prediction accuracy was much improved compared to the cases predicted by the conventional marker S-100β (Mian *et al.* 2005) where only 21% of the stage III progressors were

detected. In other study, plasma peptide components (PPC) from ten melanoma (Mel) and healthy individuals were examined by a combination of RP-HPLC, SELDI-ToF MS and tandem mass spectrometry (Mian *et al.* 2005). They found that three peak patterns (2023, 2039, 2053.5 m/z) were primarily present, whereas fibrinogen alpha (2554.3 m/z) and inter-α-trypsin inhibitor heavy chain H4 fragments (2272.0 m/z) were absent in melanoma tumour samples compared to normal samples (Caputo *et al.* 2005). In another attempt, 17 samples of sera from patients with malignant cutaneous melanoma at various stages and 14 samples from healthy subjects were analysed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Caputo *et al.* 2005). In samples from melanoma patients, the peaks for the low molecular weight proteins (m/z 2500–3500) were observed whereas it was completely absent in spectra from healthy subjects. Moreover, the presence and abundance of low molecular weight proteins was increased as cells progressed from stage 1 to stage 2 of the disease indicating that these peaks could be used in the diagnosis of melanoma (Ferrari *et al.* 2000).

Recently differential expression of proteins in the patient primary and metastatic melanoma cell lines WM-115 and WM-266-4, respectively, has been investigated using 2DE to understand the processes regulating tumour progression (Ferrari *et al.* 2000). Increased expression of 131 proteins and down regulation of 15 proteins was observed in the metastatic compared to the primary melanoma cell line. These proteins included galectin-1 and 14-3-3 that are already known to be involved in cancer other than melanoma (Al-Ghoul *et al.* 2008) and therefore could be useful biomarkers for melanoma. Galectin-1 has also been reported to be abundant in the fraction of detergent-resistant membrane proteins in early stage (radial growth phase) melanoma cells compared to those of metastatic cells (Rubinstein *et al.* 2004, Qi *et al.* 2005). A potentially novel protein, cyclophilin A, was observed to be expressed at higher levels in metastatic melanoma compared to the primary melanoma and normal fibroblasts suggesting it as potential drug target and biomarker for melanoma in this study (Baruthio *et al.* 2008). In an another effort, the proteome of two clear cell sarcoma (CCS) cell lines (soft tissue melanoma) from a patient were compared with the corresponding xenografts after xenotransplantation of those cells into the mice (Al-Ghoul *et al.* 2008). A total of 124 proteins, including vimentin, nestin and S100, were reported common between the cell lines and the xenografts, whereas 249 proteins were specific to the cell lines and 178 proteins were specific to the xenografts. This knowledge could help to identify potential drug targets or biomarkers for melanoma.

The solid melanoma tumours from a mice (fresh and stored at -80 °C for one month) have also been investigated using phosphoproteomics approach coupled with high resolution mass spectrometry to understand the signalling mechanism in melanoma (Dimas *et al.* 2008). A detailed phosphoproteome map describing a total of 5600 phosphorylation sites on 2250 proteins was created and it was also observed in this investigation that one month storage at -80 °C did not significantly decrease the number of identified phosphorylation sites in tumour samples. Of the identified proteins, 31 proteins, such as ERK, Jun and AKT, have already been reported to be phosphorylated in cancer and kinase proteins were the most abundantly identified proteins in this investigation suggesting the dominance of kinases-induced signalling cascades in melanoma.

It is clear that very limited investigations have focused their interests in investigating the proteins that may have role in regulation of cancer and invasion in melanoma disease and no established protein biomarker is available to date for diagnosis of melanoma cases in early stage of disease. Therefore, the investigation of secreted proteins from melanoma cell lines using proteomics based approaches could reveal potential biomarkers and/or drug targets for melanoma diagnosis and prognosis.

1.9 Proteomics technologies

Cancer is a complex disease that reflects the genetic as well as protein changes within a cell. Gene expression data gives us limited relevant information since proteins are the main functional units performing all biological process in the cell or organism and may have post-transcriptional event(s) and post-translational modification(s) that contribute towards biological activity of proteins. Protein expression patterns are also changed specifically and significantly in response to every disease (Zanivan *et al.* 2008). The first protein cancer marker, carcinoembryonic antigen (CEA), was identified in 1965 for the detection of colorectal cancer in patient serum (Banks *et al.* 2000). Other biomarkers discovered in the 1970s and 1980s include prostate-specific antigen (PSA) for prostate cancer, CA-19 for colorectal and pancreatic cancer, CA-15-3 for breast cancer and CA-125 for ovarian cancer. However, not all biomarkers are effective in all clinical situations. For example, PSA is well established in clinical practice, but approximately one third of patients with an elevated PSA level often undergo unnecessary medical procedures because they do not have a malignant form of prostate

cancer (Gold and Freedman 1965). Identification of new tumour biomarkers with predictive value is needed to allow early detection and treatment of cancer. It is also necessary to distinguish between poor and good prognosis and being able to suggest patient-tailored therapy, thus improving treatment. The recent advances in mass spectrometry technology - a technique for separating and identifying molecules based on mass - have highlighted this possibility and it has become an important tool for proteomic studies. Several proteomics technologies including Two Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE), Surface Enhanced Laser Desorption/Ionisation Time of Flight Mass Spectrometry (SELDI-ToF MS), Protein Arrays, Isotope Coded Affinity Tags (ICAT), iTRAQ, Multidimensional Protein Identification Technology (MudPIT) and SILAC are the approaches being implemented in cancer research. 2D-PAGE and SELDI-ToF are the main technologies used to date in serum cancer research, however other technologies such as protein arrays, ICAT, iTRAQ and SILAC also offer great potential for future biomarker discovery in cancer. We used Two Dimensional differential Gel Electrophoresis (2D-DIGE) couples with MALDI-ToF and LC-MS/MS and Surface Enhanced Laser Desorption/Ionisation Time of Flight (SELDI-ToF) in this thesis.

1.9.1 Two-dimensional Differential Gel Electrophoresis (2D-DIGE)

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the most widely used proteomics technique to study the proteome as well as cancer biomarkers (U.S. Preventive Services Task Force 2008). 2D-PAGE remains challenging mainly because of its low sensitivity and reproducibility. Modified 2D electrophoresis by fluorescent tagging to proteins, Differential gel electrophoresis (DIGE), offers increased throughput, ease of use, reproducibility, and accurate quantitation of protein expression differences (Gharbi *et al.* 2002, Somiari *et al.* 2003, Lilley *et al.* 2002, Yu *et al.* 2005). This system enables the separation of two or three fluorescently labelled protein samples (Cy2, Cy3 and Cy5) on the same gel. Differential analysis software identifies the differentially expressed protein targets that can be trypsin- digested and readily identified using mass spectrometry by generating peptide mass fingerprints (PMF) using Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF MS), a technique that is both relatively easy to use and reasonably sensitive for identifying proteins. Additionally other mass spectrometry techniques such

as electrospray ionization (ESI-MS/MS) are capable of providing amino acid sequence information on peptide fragments of the parent protein (Unlu *et al.* 1997).

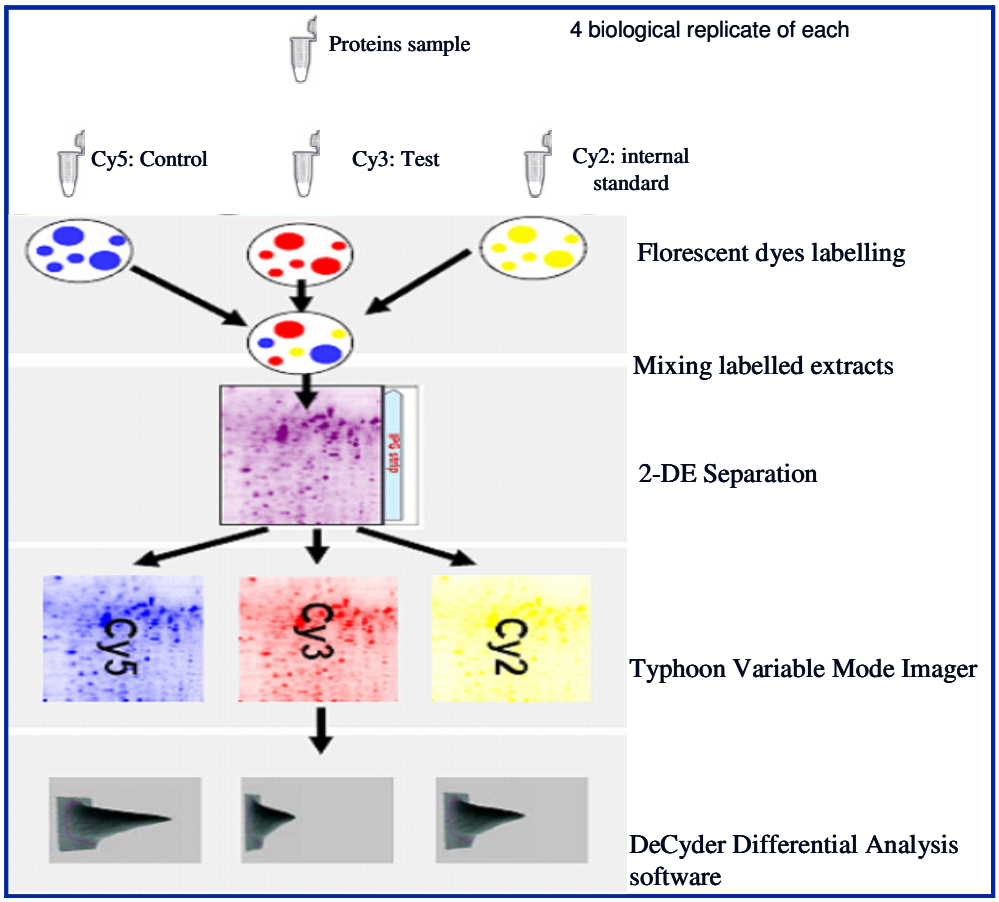


Figure 1.2: Work flow of 2D-DIGE experiment

Although 2D-PAGE based techniques have a reasonable level of throughput, however there are a number of difficulties inherent to the technique such as separation of low abundant proteins as it is difficult to enrich for these proteins. Membrane proteins are also difficult to separate due to poor solubility. Efforts have been made to overcome these limitations. For example, low abundant proteins can be identified using higher protein concentrations, and applying fractionation methods. Moreover, membrane proteins can be identified to some extent by using commercially available mild detergents such as oligooxyethylene, sulfobetaine, dodecyl maltoside, and decaethylene glycol mono hexadecyl, as the use of strong detergents like SDS, interfere with the isoelectric focusing of proteins (Mann *et al.* 2001). Additional problems with 2D electrophoresis include insufficient resolution to separate multiple species originating from a single protein with post-translational modifications, such as those with

carbohydrates, difficulties in detecting proteins with molecular masses >120000 Da and those with pI values <4 or >9, low visualisation of less-abundant proteins, and co-migration of proteins to same spots (Luche *et al.* 2003). Conventional 2-D electrophoresis shows only protein expression and cannot detect protein-protein interactions and protein function without using particular methods such as affinity electrophoresis (Gygi *et al.* 2000).

1.9.2 Matrix assisted Laser Desorption/Ionisation Time-of-flight Mass spectrometry (MALDI-ToF MS)

Matrix assisted Laser Desorption/Ionisation Time-of-flight Mass Spectrometry (MALDI-ToF MS) involves the precipitation of sample molecules with an excess of matrix material (α-cyano-4-hydroxycinnamic acid or dihydroxybenzoic acid). The precipitant is then bombarded with laser pulses and imparts energy. The matrix materials have absorbances at the wavelength of the laser and are subject to desorption and ionisation accompanied by fragmentation. The MS measures the mass-to-charge ratio (m/z) of the protein, peptide or peptide fragments. The time-of-flight (ToF) analyzer separates ions according to their m/z ratios by measuring the time it takes for ions to travel through a field free region known as the flight or drift tube. The laser is set in such a way that it always generates single charged ions and therefore m/z represents the approximate molecular weight of the protein target. The smaller ions possess a higher velocity relative to larger/heavier ions. Separated ion fractions arriving at the end of the drift tube are detected by an appropriate recorder that produces a signal upon impact of each ion group. The ToF mass spectrum is a recording of the detector signal as a function of time. This peptide mass fingerprint can then be used to search databases using MASCOT and ProFound against SWISS-PROT and NCBI to identify the protein (Kameshita *et al.* 1998).

1.9.3 Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS)

LC-MS/MS combines the liquid chromatographic separation with mass spectrometric detection. The combination of these two powerful techniques enables us to analyze virtually any molecular species including thermally labile, non-volatile, and high molecular weight species. Most of the analyte species are amenable to separation with liquid chromatography. Tandem mass spectrometry (LC-MS/MS) is capable of

providing structure, molecular weight, empirical formula, and quantitative information about a specific analyte. Therefore LC-MS/MS has become a major tool for the study of biological processes. Biological samples are generally highly complex mixtures of biomolecule(s). The complexity of these samples is usually reduced in a series of separation steps in LC-MS/MS analysis. Whole proteins often first undergo an enzymatic digestion with trypsin. The resulting mixture of peptides and other compounds is transferred into a chromatographic column. The retention period of the individual components in the mixture depends on their interaction with the column material, and consequently, different peptides are likely to elute at different retention times. The separated substance zones are continuously transferred into a mass spectrometer, where they are ionized and separated by their mass/charge ratio. The mass spectrometric data from an LC-MS/MS experiment consists of a sequence of spectra (or scans). Each scan gives a snapshot of the peptides eluting from the column during a fixed interval. It consists of a series of data points, each described by m/z and ion count (intensity). The set of scans from one LC-MS/MS sample constitutes an LC-MS/MS data set (or LC-MS/MS map). The atoms contained in a peptide exist in different isotopic variants which occur in a fairly constant distribution in nature. This gives rise to a characteristic pattern of adjacent peaks in the mass spectrum. Analysis of distances between peaks could enable us to identify amino acids in the peptide. The LC-MS/MS maps can be searched in to the database of known proteins using SEQUEST and MASCOT against SWISS-PROT and NCBI to identify protein/peptide. Therefore LC-MS/MS is so sensitive that it can generate the sequence information for the protein/peptide under investigation.

1.9.4 Surface Enhanced Laser Desorption/Ionization time-of-flight mass spectrometry (SELDI –ToF MS)

This technique allows proteins/peptides to be profiled from different biological samples, on a variety of chemically (anionic, cationic, hydrophobic, hydrophilic, metal affinity capture, etc.) or bio-chemically (immobilized antibody, receptor, DNA, enzyme, etc.) defined chromatographic surfaces. A small amount of sample of interest is loaded onto ProteinChip^{TM} arrays that selectively bind different subset of proteins in crude samples by adsorption, partition, electrostatic interaction or affinity chromatography according to their surface chemistries. After a short incubation period, unbound proteins and unspecific substances are washed away with an appropriate buffer and water. The ToF

reader records the time of flight and calculates the accurate molecular weight of proteins/peptides in the form of a spectral map containing mass to charge ratios (*m*/*z*) and intensities corresponding to each bound proteins/peptides. Biomarker wizard software analyses the spectral map and detects differentially expressed protein/peptides with statistical significance. SELDI –ToF MS produces a characteristic profile for the source material analysis of serum, plasma, intestinal fluid, urine, cell lysates and cellular secretions focusing on the discovery and identification of potential biomarkers for various diseases. SELDI-ToF MS utilizes the different chromatographic properties of ProteinChip® arrays to bind different subsets of peptides and proteins for subsequent analysis (Blackstock and Weir 1999). Further advantages of this MS -based technology is the small amount of sample required and its ability to detect low molecular weight proteins (<15 KDa) which are usually missed by other techniques (like, for example, 2–D gel Electrophoresis) and on which there is an increasing interest of researchers (Merchant and Weinberger 2000). SELDI-ToF MS has been extensively used in the field of biomedical research including breast and melanoma cancer (section 1.5 and 1.8). However, there is some controversy over this technology such as its reproducibility, the bioinformatics used, the possibility of over-fitting, the potential biasness in the samples as well as how this could possibly fit into a routine diagnostic lab (Petricoin and Liotta 2004).

Aim of thesis

The goals of this work were the identification of differentially expressed proteins/peptides between normal non-invasive, normal-invasive, cancerous-non-invasive and cancerous-invasive cell lines using proteomic profiling techniques, which could possibly help to understand the molecular mechanisms of cancer invasion and lead to the discovery of biomarkers for melanoma and breast cancer.

The specific aims of this work were

- To identify key cellular proteins, followed by functional analysis using siRNA knockdown, in order to investigate the mechanisms of N+C invasion and proliferation in human breast cancer cell lines.

- To investigate the changes in expression profiles of secreted proteins in the conditioned media from breast cell lines to identify cancer and N+C invasion-associated proteins.

- To investigate differential protein expression mapping of the membrane fraction of breast cell lines to identify cancer-specific and N+C invasion-specific proteins and potential targets for cancer invasion.

- To identify differentially expressed proteins/peptides in conditioned media from the melanoma cell line MDA-MB-435S-F and its drug resistant variants (paclitaxel-resistant cell line MDA-MB-435S-F/ Taxol10p4pSI and doxorubicin-resistant cell line MDA-MB-435S-F/Adr-10p10pSI) followed by further analysis to establish cancer specificity and/or role in invasion.

- To discover low molecular weight biomarkers for melanoma in conditioned media from melanocytes and melanoma cells.

Section 2

Materials & Methods

2.1 Ultrapure water

Ultrapure water was used in the preparation of all media and solutions. For ultra-purification, the water was initially pre-treated which involved activated carbon, pre-filtration and anti-scaling. This water was then purified by a reverse osmosis system (Elga USF Maxima Water Purification System) to a standard of 12 - 18 MΩ/cm resistance.

2.2 Glassware

All glassware and lids were soaked in a 2% (v/v) solution of RBS-25 (VWR International) for at least 1 hour (hr). This is a deproteinising agent, which removes proteineous material from the bottles. Glassware was scrubbed and rinsed several times in tap water. The bottles were then washed by machine using Neodisher detergent, an organic, phosphate-based acid detergent. The bottles were then rinsed twice with distilled water, once with ultrapure water and sterilised by autoclaving.

2.3 Preparation of cell culture media

Ultrapure water (UHP) and glassware were autoclaved before use in cell culture as described in Section 2.1 & 2.2. All 1X basal media were prepared in-house as follows: 10X media were added to sterile UHP water, buffered with HEPES (N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) (Sigma, H-9136) and $NaHCO_3$ (BDH, 30151) as required and adjusted to pH 7.5 using sterile 1.5N NaOH or 1.5N HCl. The media was then filtered through sterile 0.22µm bell filters (Gelman, 12158) and stored in sterile 500mL bottles at 4°C. Sterility checks on all media bottles for bacterial, yeast and fungal contamination were made using Trypto Soya Broth (TSB, Oxoid, CM0129) and Thioglycolate broths (Oxoid, CM173). All samples for sterility checks were then incubated at both 25°C and 37°C. Basal media were stored at 4°C for up to three months in the dark.

Complete media was then prepared by adding serum as required (Table 2.1). An aliquot of complete medium was kept in a T-25cm^2 flask in an incubator over 5-7 days to ensure that the complete medium is free of contamination at the time of use. Complete media was also stored at 4°C for a maximum of one month in the dark.

Table 2.1. Details of cell lines and complete growth media components

Cell Lines	**Growth media components**
HMEC	MEGM basal media, plus growth factor kit (bovine pituitary extract (2mL/500mL), epithelial growth factor (0.5mL/500mL), insulin (0.5mL/500mL), hydrocortisone (0.5mL/500mL), transferrin (0.5mL/500mL) (Lonza, CC-3150)
MCF10A	MEGM basal media, plus growth factor kit (bovine pituitary extract (2mL/500mL), epithelial growth factor (0.5mL/500mL), insulin (0.5mL/500mL), hydrocortisone (0.5mL/500mL), transferrin (0.5mL/500mL) (Lonza, CC-3150), plus 50µg/mL cholera toxin (Sigma, C8052)
MCF-7	DMEM Media, 10% (v/v) fetal calf serum (Sigma, F7524)
BT20	RPMI-1640, 10% (v/v) fetal calf serum
MDA-MB231	RPMI-1640, 10% (v/v) fetal calf serum, 1% (v/v) Sodium pyruvate (Gibco, 11360-035)
HCC1937	RPMI-1640, 10% (v/v) fetal calf serum
Hs578T	DMEM, 10% (v/v) fetal calf serum, 10µg/mL bovine insulin (Sigma, 10516)
SK-BR-3	RPMI, 10% (v/v) fetal calf serum
TD47D	DMEM, 10% fetal calf serum
UACC-182	L15 media (Sigma, L-5520) , 15% (v/v) fetal calf serum
NHEM	Melanocyte growth medium M2 (487.7mL) + Supplement Mix (PromoCell, C-24300) (12.3mL)

SKMEL-5	RPMI 1640, 10% (v/v) fetal calf serum
SKMEL-28	RPMI 1640, 10% (v/v) fetal calf serum
Malme-3M	RPMI 1640, 10% (v/v) fetal calf serum
HT144	McCoy's 5a, 10% (v/v) fetal calf serum
M14	RPMI 1640, 10% (v/v) fetal calf serum
Lox-IMVI	RPMI 1640, 10% (v/v) fetal calf serum
DLKP	ATCC (1:1 (v/v) mixture of DMEM and Hams F12), 5% (v/v) fetal calf serum
H1299	RPMI 1640, 1mM sodium pyruvate (Gibco, 11360-035), 5% (v/v) fetal calf serum
MDA-MB 435S	RPMI 1640, 10% (v/v) fetal calf serum
MDA-MB435S /taxol10p4p	RPMI 1640, 10% (v/v) fetal calf serum
MDA-MB435S /Adr10p10p	RPMI 1640, 10% (v/v) fetal calf serum

2.4 Routine management of cell lines

2.4.1 Safety Precautions

All cell culture work was carried out in a class II laminar air-flow (LF) cabinet (Holten). Before and after use, the LF cabinet was cleaned with 70% industrial methylated spirits (IMS) for 15 minutes. Any items brought into the cabinet were also swabbed down with IMS. At a time, only one cell line was used in the LF cabinet and upon completion of work with any given cell line, 15 minutes clearance was given to eliminate any possibilities of cross-contamination between the various cell lines. The cabinet was cleaned weekly with Virkon (Antech International, P0550) and IMS.

2.4.2 Cell Lines

The cell lines used during the course of this study, their sources and detailed information are listed in Table 2.2. Cells were maintained in 25cm^2 flasks (Costar, 3050), 75cm^2 flasks (Costar, 3075) or 175cm^2 flasks (Nulge Nunc, 156502) at 37°C and were fed every 2nd or 3th day or as required in order to maintain active cell growth in culture.

Table 2.2 Details of cell lines used in this investigation.

Cell Lines	Source	Detailed Information
HMEC	Lonza	Normal mammary epithelial
MCF10A	ATCC#	Non-tumorigenic mammary epithelial
MCF-7	NICB*	Breast adenocarcinoma
BT20	NICB*	Breast invasive ductal carcinoma
MDA-MB231	NICB*	Breast invasive ductal carcinoma
HCC1937	NICB*	Breast primary ductal carcinoma
Hs578T	NICB*	Breast carcinosarcoma
SK-BR-3	NICB*	Breast invasive ductal carcinoma
TD47D	NICB*	Breast invasive ductal carcinoma
UACC-182	NICB*	Breast invasive ductal carcinoma
NHEM	PromoCell	Normal Epithelial Melanocytes
SKMEL-5	NICB*	Malignant melanoma
SKMEL-28	NICB*	Malignant melanoma
Malme-3M	NICB*	Malignant melanoma
HT144	NICB*	Malignant melanoma
M14	NICB*	Malignant amelanotic melanoma
Lox-IMVI	NICB*	Malignant amelanotic melanoma
DLKP	NICB*	Poorly differentiated lung squamous carcinoma
H1299	NICB*	Large cell lung carcinoma
MDA-MB 435S	NICB*	Metastatic carcinoma, melanoma
MDA-MB435S /taxol10p4p	NICB*	Metastatic carcinoma, melanoma, paclitaxel-resistant variant
MDA-MB435S /Adr10p10p	NICB*	Metastatic carcinoma, melanoma, doxorubicin-resistant variant

ATCC - American Type Tissue Culture Collection, * NICB- National Institute for Cellular Biotechnology

2.4.3 Subculturing of cancer cell lines

Exhausted cell culture medium was removed from the tissue culture flask and discarded into a sterile bottle. The flask was then rinsed out with 2mL of trypsin solution (0.25% (v/v) of trypsin (Gibco, 043-05090) and 0.01% (v/v) of EDTA (Sigma, E9884) solution

in PBS (Oxoid, BRI4a)) to ensure the removal of any residual media. Depending on the size of the flask, 2-5mL of trypsin was then added. Cells were then incubated at 37°C for approximately 2-5 minutes until all of the cells detached from the inside surface of the flask. This was monitored by microscopic observation. An equal volume of complete media was added to the flask to deactivate the trypsin. The cell suspension was removed from the flask and placed in a sterile universal container (Sterilin, 128a) and centrifuged at 250g for 5 minutes. The supernatant was then discarded from the universal and the pellet was suspended gently in fresh complete medium. A cell count was performed as described in section 2.4.6 and an aliquot of cells was used to seed a flask at the required density. All cell waste or media exposed to cells was autoclaved before disposal.

2.4.4 Subculturing of normal cells (HMEC and Melanocytes)

HMEC cells were subcultured using the subculture reagent kit supplied by Lonza (CC-5002) and melanocytes were subcultured using the subculture reagent kit supplied by PromoCell (C-41210). For subculture, cells were rinsed with 5mL of room temperature HEPES Buffered Saline Solution from the kit. The cells were covered with 5mL of room temperature Trypsin/EDTA. The cells were monitored using an inverted microscope. The trypsinization process was allowed to continue until approximately 90% of the cells had rounded up. The process takes about 2-6 minutes. At this point, the flask was rapped against the palm of hand to release the majority of the cells from the culture surface. If only a few cells detached, trypsinization was allowed to continue for a further 1-2 min. The flask was then given a further rap with the palm of the hand. This process was repeated until majority of cells had detached. The trypsin in the flask was immediately neutralized by addition of 5mL of room temperature Trypsin Neutralization Solution from the kit. The cell suspension was removed from the flask and placed in a sterile universal container (Sterilin, 128a) and centrifuged at 250g for 5 minutes. The supernatant was then discarded from the universal and the pellet was suspended gently in fresh complete medium. A cell count was performed as described in Section 2.4.6 and an aliquot of cells was used to seed a flask at the required density. All cell waste or media exposed to cells was autoclaved before disposal.

2.4.5 Subculture of MCF-10A

Exhausted cell culture medium was removed from the tissue culture flask and discarded into a sterile bottle. The flask was then rinsed out with 2mL of trypsin solution to ensure the removal of any residual media. Depending on the size of the flask, 2-5mL of trypsin was then added to the flask, which was then incubated at 37°C, for approximately 5 minutes, until all of the cells detached from the inside surface of the flask monitored by microscopic observation. Since the culture medium for MCF-10A does not contain serum (trypsin inhibitor), an equal volume of soybean trypsin inhibitor (1mg/mL) (Sigma, T6522) was added to the flask to deactivate the trypsin. The cell suspension was removed from the flask and placed in a sterile universal container (Sterilin, 128a) and centrifuged at 250g for 5 minutes. The supernatant was then discarded from the universal and the pellet was suspended gently in complete medium. A cell count was performed and an aliquot of cells was used to seed a flask at the required density. All cell waste or media exposed to cells was autoclaved before disposal.

2.4.6 Assessment of cell number and viability

Prior to cell counts, cells were prepared by subculturing as detailed in sections 2.4.3-5. An aliquot of the cell suspension was then added to trypan blue (Gibco, 525) at a ratio of 1:1 (v/v). The mixture was incubated for 2-3 minutes at room temperature. An aliquot (10µL) was then applied to the chamber of a glass coverslip-enclosed haemocytometer. For each of the four grids, cells in the 16 squares were counted. Non-viable cells stained blue, while viable cells excluded the trypan blue dye as their membrane remained intact, and remained unstained. The average number of viable and dead cells per 16 squares was multiplied by a factor of 10^4 (volume of the grid) and the relevant dilution factor to determine the average cell number per mL in the original cell suspension. Using the data for viable and non-viable cells, percentage viability was calculated.

2.4.7 Cryopreservation of cells

Cells for cryopreservation were harvested in the mid-log phase of growth and counted as described in section 2.4.6. Cell pellets were resuspended in a suitable volume of cold serum. An equal volume of filter sterilized solution of 10% (v/v) DMSO in serum was

added dropwise with mixing into the cell suspension. 1mL of cell suspension was then aliqoted into the cryovials (Greiner, 122278) and immediately placed in the -20°C freezer for 1hr and then placed in a -80°C freezer for four hours or overnight. The cryovials were then transferred to liquid nitrogen tank for long term storage (-196°C).

2.4.8 Thawing of cryopreserved cells

A volume of 5mL of fresh complete culture medium was added to a sterile universal and into a T-25cm^2 flask and incubated at 37^{0}C for ~1hr. The cryopreserved cells were removed from the liquid nitrogen and thawed at 37°C as quickly as possible. The cells were removed from the vials and transferred into the pre-warmed aliquoted media in the universal. The resulting cell suspension was centrifuged at 250g for 5 minutes. The supernatant was removed and the pellet was resuspended in the pre-warmed culture medium from T-25 cm^2 flask. This cell suspension was then transferred to T-25 cm^2 flask and allowed to attach and grow overnight in incubator. The following day, the culture media was replaced with fresh complete culture medium to remove any non-viable cells and floating cells.

2.5 *Mycoplasma* analysis of cell lines

Mycoplasma testing was carried out for all cell lines for possible *Mycoplasma* contamination in house by Ms. Shane and Mr. Michael Henry at the NICB following the protocols described in sections 2.5.1 and 2.5.2. All cell lines are found to be *Mycoplasma* free.

2.5.1 Indirect staining procedure for *Mycoplasma* analysis

Mycoplasma negative NRK (Normal rat kidney fibroblast) cells were used as indicator cells for this analysis. The cells were incubated with supernatant from the cell lines in question and then examined for *Mycoplasma* contamination. A fluorescent Hoechst stain was used in this analysis. This stain binds specifically to DNA (i.e. the nucleus of the cell and any *Mycoplasma* present). *Mycoplasma* infection was indicated by fluorescent bodies in the cytoplasm of the NRK cells.

2.5.2 Direct staining procedure for *Mycoplasma* analysis

Direct staining for *Mycoplasma* analysis involved inoculating samples on to a *Mycoplasma* culture broth (Oxoid, CM403). This was supplemented with 16% (v/v) serum, 0.002% (v/v) DNA (BDH, 42026), 2µg/mL fungizone (Gibco, 042 05920), $2x10^3$ units penicillin (Sigma, Pen-3) and 10mL of a 25% yeast extract solution. Incubation was carried out at 37°C for a period of 48 hours. Samples of this broth were then streaked onto plates of *Mycoplasma* agar base (Oxoid, CM401) which had been supplemented as described above. The plates were incubated for three weeks at 37°C and examined microscopically every 7 days. The appearance of small oval shaped colonies indicated the presence of *Mycoplasma* infection.

2.6 Invasion assay

2.6.1 Preparation of invasion chambers

Invasion assays were carried out using BD BioCoat™ MATRIGEL™ Invasion Chambers (BD Biosciences, 354483). Inserts were rehydrated with serum-free basal media for 2 hours at 37°C in incubator containing 5% CO_2 atmosphere. Cell suspensions were prepared in serum-free basal media at a concentration of $2x10^5$ cells/mL. A volume of 750µL of complete media was added to the lower chamber of the insert in BD Falcon™ TC Companion Plate. A volume of 500µl of the cell suspension was then added into the insert. The invasion assays were then incubated for 48 hours at 37°C in 5% CO_2 atmosphere.

2.6.2 Staining of invasive cells

After 48 hours incubation, the non-invading cells were removed from the upper surface of the membrane. The inner side of the insert was wiped with a PBS-soaked swab while the outer side of the insert was stained with 0.25% crystal violet for 10 minutes and then rinsed in ultrapure water and allowed to dry. Inserts were then analyzed using the microscope.

2.6.3 Counting of invading cells

Cell counting was facilitated by photographing the insert-membrane using an inverted microscope. The cells were observed at 200X magnification. Cells in the central fields

of membranes were counted and an average count calculated from 5 counts per chamber within a grid at 20x objective. The average count was multiplied by the conversion factor 140 (growth area of membrane divided by field of viewed area at 200 X magnification) to determine the total number of invading/migrating cells. All assays were performed in triplicate.

2.7 Proteomic analysis

2.7.1 Protein sample preparation

2.7.1.1 Lysates

Cells were seeded in four biological replicates in T-75cm^2 flasks. Cells pellets were obtained by trypsinization as described in section 2.4.3-5, once culture reached 70-80% confluency. The cell pellets were dissolved in cold PBS and centrifuged at 250g for 5min. This was repeated again to remove residual medium or trypsin from cell pellet. Cell pellets were then washed twice with sucrose buffer (10nM sucrose, 100mM Tris, pH 8.0). Cell pellets were then reconstituted with 0.5mL of the lysis buffer (Table 2.3).

Table 2.3 Preparation of lysis buffer

Contents	Recipe	Required weight/volume for making 25mL of lysis buffer
Urea	7M	10.5 g
Thiourea	2M	3.8 g
CHAPS	4%	1 g
Tris	30mM	0.091 g
Magnesium acetate	5mM	0.027 g
Water		Up to 25mL
pH		8 – 9
Aliquot in 1 mL/eppendorf and store at -20^{0}C		

The lysate was then homogenised by passing though a 21-gauge needle 6 times using a 1mL syringe. After incubation, lysates were centrifuged on a bench-top centrifuge at 3500g for 15 minutes at 4°C. The middle layer of the supernatant containing extracted protein was transferred to a fresh chilled eppendorf tube and its pH was checked by spotting 3µL onto a pH indicator strip to ensure that it lies between pH 8.0-9.0. A small aliquot of sample was used for protein estimation. The sample was then divided into smaller aliquots and stored at -80^0C.

2.7.1.2 Conditioned Media preparation

Cells ($3x10^6$) were seeded in four biological replicates in T-175cm^2 flasks. Cells were allowed to grow until 50-60% confluent. Cells were washed three times with 10mL of serum-free (SF) basal media and then incubated in 15mL of SF basal media for 60 min. After this time, cells were washed again twice with SF basal media. 15mL of SF basal media was added to the cells and incubated for 72hrs. After such time, conditioned media (CM) was collected, centrifuged for 15min at 250g, and stored at –80°C. At the time of analysis conditioned media was concentrated using 5,000 dalton (Da) molecular weight cut-off concentrators (Millipore, UFC 900524). A volume of 15mL was concentrated to 500µL by centrifuging at 1000g at 4°C.

Before 2D-DIGE analysis proteins were precipitated using acetone precipitation method. 1:5 volumes of sample and cold acetone were incubated overnight and centrifuged at 1000g for 20 minutes. Samples were reconstituted with 100-500µL of the lysis buffer. The pH of samples was roughly maintained to pH 8-9 by addition of HCl (acidic pH) or NaOH (alkaline pH). A small aliquot of sample was used for protein estimation. The sample was then divided into smaller aliquots and stored at –80°C.

2.7.1.3 Membrane fractionation

Cells were seeded in four biological replicates in T-175cm^2 flasks. Cells pellets were obtained by trypsinization as described in section 2.4.3-5, once the culture reached 50-60% confluency. The cell pellets were washed twice with cold PBS as described in section 2.7.1.1.

The fractionation process for the isolation of hydrophobic proteins from the cell lines were carried out using ReadyPrep™ Protein Extraction Kit (Membrane I) (Biorad, 163-2088). The kit separates the membrane proteins from other proteins using

temperature-dependent phase partitioning (Bordier 1981, Santoni and Kieffer 2000). For this, cells were lysed by incubating for 15 minutes in ice in a solution containing 2% precondensed Triton X-114 in TBS (150mM NaCl, 10mM Tris-HCl, pH 7.6). Samples were then vortex 4–5 times, 60 second each. The tubes were maintained in an ice for 30–60 second between each vortexing step and the solution was collected and incubated at 37^0C for 30 minutes. The cloudy solution obtained was again centrifuged at 10,000x g for 10 minutes at room temperature. This caused the formation of two liquid phases. The lower liquid phase that is enriched in hydrophobic proteins was collected. Before 2D-DIGE analysis proteins were precipitated using the acetone precipitation method as described in section 2.7.1.2.

2.7.2 Protein quantification

2mg/mL of bovine serum albumin (BSA) solution (Sigma, A9543) was used as a known standard. Dilutions of BSA stock for 0.125, 0.25, 0.5 and 1.0mg/mL was prepared and used for generating a protein standard curve. 240µL/well of thiourea-compatible Bradford protein assay reagent (Bio-Rad, 500-0205) was added to a 96-well plate. 10µL of protein standard dilution or sample (diluted 1:10) was added to the 96-well plate. All samples were assayed in triplicate. After 5 minutes incubation, the absorbance was assessed at 595nm. The concentration of the protein samples was determined from the plot of the absorbance at 595nm versus the concentration of the protein standard.

2.7.3 Two-dimensional difference gel electrophoresis (2D-DIGE)

2D-DIGE is an advanced version of classical two-dimensional gel electrophoresis (2D-PAGE). In 2D-DIGE, protein samples are labeled with three different fluorescent dyes (Cy2, Cy3 and Cy5) and then separated by 2D-PAGE. Labeling of different protein samples with different fluorescent dyes and then mixing together allows samples to be separated on the same gel and this minimizes gel-to-gel variation. The following steps were carried out to perform 2D-DIGE experiment in this investigation.

2.7.3.1 Labelling of proteins

2.7.3.1.1 Preparation of CyDye dyes for minimal-DIGE protein labelling

The three CyDye DIGE Fluor Minimal dyes (Cy3, Cy5 and Cy2 (GE Healthcare, 25-8010-65) were thawed from –20^{o}C to room temperature for 5 minutes. To each

microfuge tube dimethylformamide (DMF) (Sigma, 22,705-6) was added to a concentration of 1 nmol/µL. Each microfuge tube was vortexed vigorously for 30 seconds to dissolve the dye. The tubes were then centrifuged for 30 seconds at 3500g in a microcentrifuge. The reconstituted dyes were stored at –20^{o}C for up to 3 months.

2.7.3.1.2 Preparation of 10 µL working dye solution (200 pmol/µL)

On thawing, the dye stock solutions were centrifuged in a microcentrifuge for 30 seconds. To make 10 µL of the three working dye solutions, 8 µL of DMF was added to three fresh eppendorfs labelled Cy2, Cy3 and Cy5 and 2 µL of Cy dye (1 pmol/µL). A 200 pmol/µL volume of each of the reconstituted dye stock solutions was added to their respective tubes. The dyes were stored at -20^{o}C in tinfoil in the dark for 3 months.

2.7.3.2 Protein sample labeling

The 2D-DIGE experiment was carried out using four biological replicate samples. The protein samples equivalent to 50µg (or 25µg for the membrane fraction study) was placed into eppendorf tubes and HMEC and MCF-7 samples were labeled with the Cy3 and MCF-10A and BT20 samples with Cy5 minimal dyes. The Cy2 pool for each gel (50µg) (or 25µg for the membrane fraction study) contained an equal concentration aliquot of each of the protein samples from HMEC, MCF-7, MCF-10A and BT20. To this, 1µL of each dye (200 pmol/µL) was added 50µg (or 25µg for the membrane fraction study) of protein sample. Each tube was mixed by vortexing, centrifuged and then left on ice for 30 minutes in the dark. To stop the reaction, 1µL of 10 mM lysine (per 200pm of dye) was added. The tubes were mixed, centrifuged briefly and left on ice for 10 minutes in the dark. An equal volume of 2X sample buffer (rehydration buffer stock solution (7M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer pH 4-7 (GE Healthcare, 17-6000-86), 50mM DTT (Sigma, D9163)) was added to the labeled protein samples. The mixture was left on ice for at least 10 minutes then applied to Immobiline DryStrips for isoelectric focussing. The mixture was left on ice for at least 10 minutes and then applied to Immobiline DryStrips for isoelectric focussing.

2.7.3.3 First dimension separation - isoelectric focussing

2.7.3.3.1 Strip rehydration

Isoelectric focussing of all samples was carried out using immobiline pH gradient (IPG) strips (18cm, 4-7pH) (GE Healthcare, 17-1233-01). The protective lid was removed

from the Immobiline Dry Strip Reswelling tray. The tray was levelled using the spirit level. 350µL of rehydration buffer was slowly pipetted into the centre of each slot. All air bubbles generated were removed. The cover film from the IPG strip (GE Healthcare) was removed and positioned with the gel side down and lowered. To ensure the entire strip was evenly coated with rehydration buffer, the strip was gently lifted and lowered onto the entire surface of the solution avoiding trapping air bubbles. Each strip was overlaid with about 3mL IPG Cover Fluid (GE Healthcare, 17-1335-01) starting on both ends of the strip and moving to the centre. The protective lid was then replaced and the strips were left at room temperature to rehydrate overnight (or at least 12 hours).

2.7.3.3.2 Isoelectric focussing

Following the rehydration procedure, the Manifold (GE Healthcare) was placed onto the IPGphor unit (GE Healthcare) by inserting the "T" shape into the hollow provided. A 9 mL of Cover Fluid was placed into each of the twelve lanes in the tray in order to cover the surface. Two wicks (GE Healthcare, 80-6499-14) per strip were placed on tinfoil and 150 µL of UHP was pipetted onto each one to rehydrate them. The rehydrated strips were placed in the correct orientation (+ to anode) and aligned just below the indented mark, to allow for the wicks to overlap the strip. The rehydrated wicks were then placed over both the cathodic and anodic ends of all the strips. The wicks were checked to ensure they were positioned over the gel portion of the strip and avoiding the indent in the lane so as to guarantee a good contact with the electrodes. The sample cups (GE Healthcare, 80-6498-95) were then positioned approximately 1 cm from the cathodic end of the strip and an insertion tool was used to securely "click" the cups into place. The electrodes were then fitted with their "Cams" open and in direct contact with the wicks. The amount of protein loaded per strip was 150µg for DIGE or 400µg for spot picking. The protein samples were prepared by centrifuging to remove any insoluble material and the appropriate volume was loaded with a pipette tip placed just beneath the surface of the cover fluid. The cover of the IPGphor unit was closed and the desired programme selected. The temperature was set for 20^{o}C with 50µA/strip. The IEF parameters are as follows: step 1: 300 volts for 3 hours (step-and-hold), step 2: 600 volts for 3 hours (gradient), step 3: 1000 volts for 3 hours (gradient), step 4: 8000 volts for 3 hours (gradient). The IEF was hold at 500 volts until ready for SDS-PAGE step. step 5: IEF was refocused for 1 hour at 8000 volts, step 6: On completion of the IEF

run, the strips were drained of the cover fluid and stored in glass tubes at –80^{o}C or used directly in the second dimension.

2.7.3.4 Second Dimension – SDS polyacrylamide gel electrophoresis

2.7.3.4.1 Casting gels in the ETTAN Dalt-12 gel caster

The 12.5 % acrylamide gel solution was prepared in a glass beaker (acrylamide/bis 30%, 1.5 M Tris pH 8.8, 10 % SDS). Prior to pouring, 10 % ammonium persulfate and neat TEMED were added.

Two types of plates were used, low fluorescent for DIGE experiments and hinged for preparative and silver stained gels. All plates (both normal hinged and low fluorescent) and casting equipment were inspected to ensure they were clean. The gel caster frame was placed on a level bench leaning on its "legs" so that the back of the caster was open and facing the operator. The plates were assembled so that the front and back plates were evenly aligned and all seals and hinges in place. A thin spacer was placed in the gel caster unit followed by an assembled plate and then a thin spacer and then another plate. The plates were positioned in the caster unit so that the lower, front plate was the furthest away from the operator and the spacers packed with their curved edges to the top. This layering was repeated until all 14 plates and spacers were in place. All plates and spacers were checked to ensure they were packed tightly together so as to minimise any gaps and air pockets. When the desired amount of plates had been added, the thicker spacers were placed next to bring the level marginally over the edge of the back of the caster. The backing plate was then added to the caster frame and screwed into place with the 6 screws provided. The silicone tubing was added to the outlet of the glass beaker and the glass tube was inserted to the other end of the silicone tubing. The glass tube was inserted into the inlet of the reservoir and the glass beaker containing the gel solution was then clamped to a retort stand. The gel solution was held in place using arterial clamps on the top tube and the tube running down from the reservoir to the caster chamber. The top tube was unclamped and the gel solution was allowed to fill the tubing and the reservoir drain. Air bubbles that had been generated were dislodged by flicking the tube. When all air bubbles had been removed the bottom clamp was released allowing the gel solution into the gel caster. When the gel solution reached the indicator line across the top of the caster, the bottom and top tubes were reclamped. The displacement solution (0.375M Tris-Cl 1.5M pH8.8, 30% glycerol, UHP and bromophenol blue) was added to the reservoir and the glass tubing was slowly removed

from the reservoir inlet. The clamp was removed from the bottom tube allowing the displacement solution into the tube and forcing the remaining gel solution into the gel caster. The gels were overlaid with 1 mL of saturated butanol or sprayed with 0.1 % SDS solution. The gels were left to set for at least three hours at room temperature. Following this, the caster was gently unlocked and the gels removed and rinsed with distilled water. If the gels were not used immediately they were stored for up to four days in 1X running buffer at 4^{o}C.

If gels were to be used for "spot picking" the plates were silanised to stick the acrylamide gel to the plates. A volume of 2mL of working bind-silane solution (8mL ethanol, 200µL glacial acetic acid, 10µL bind-silane (GE Healthcare) and 1.8mL UHP) was pipetted over the glass plate and wiped over with a lint free cloth. This was left to air dry for 15 minutes, after which 2mL ethanol and 2 mL UHP were each pipetted over the plate and wiped off respectively. The plate was left to air dry for approximately 1 hour 30 minutes.

2.7.3.4.2 Preparing the ETTAN DALT-12 electrophoresis unit

The electrophoresis chamber was prepared by adding 6.48 litres of UHP and 720 mL of 10X SDS running buffer. The pump was then turned on to cool the system to 10^{o}C.

2.7.3.4.3 Equilibration of focussed Immobiline DryStrips

The SDS equilibration buffer (30% glycerol, 6M urea, 50nm 1.5M Tris-Cl pH 8.8, 2% SDS, bromophenol blue and UHP) which had been prepared, aliquotted into 30 mL volumes and frozen at –20^{o}C was allowed to thaw to room temperature. Two SDS equilibration buffer solutions with DTT (65 mM) or iodoacetamide (240 mM) (Sigma, I1149) were then prepared. Using a forceps, the IPG strips were removed from the IPGphor unit, the cover fluid was drained off by holding the strips at an angle and they were placed into individual glass tubes with the support film toward the wall. Equilibration buffer (10 mL containing DTT) was added to each tube and incubated for 15 minutes with gentle agitation using an orbital shaker. During this equilibration step, the gel cassettes were rinsed with UHP and then the tops rinsed with 1X running buffer. After the first equilibration, DTT containing equilibration solution was removed and 5 mL of the iodoacetamide containing equilibration buffer was added. The strips were incubated for 15 minutes with gentle agitation. During this equilibration step, the

agarose overlay solution (0.5% agarose in running buffer) was prepared and 50 mL of 1X running buffer was placed in a glass tube. If the strips had been frozen at this stage they were left at room temperature to thaw before the DTT-containing equilibration solution was added.

2.7.3.4.4 Loading the focussed Immobiline DryStrips

Using a forceps and holding the anode end, the IPG strips were rinsed in 1X SDS electrophoresis running buffer and placed between the two glass plates of the gel. The strip was pushed down gently using a thin plastic spacer until it came in contact with the surface of the gel. Any air bubbles trapped between the gel surface and the strip were gently removed. Approximately 1mL of the 0.5 % agarose overlay solution was applied over the IPG strip to seal it in place.

2.7.3.4.5 Loading gels into the Ettan DALT-12 electrophoresis unit

When the running buffer reached the desired temperature (10^{o}C) the loaded gel cassettes were wetted with UHP and inserted into the tank through the slots provided in the same orientation. When all 12 slots in the upper chamber were filled, 2X running buffer was added to the upper chamber until the mark on the side of the chamber was reached. The cover of the unit was replaced and the required running conditions selected. The unit was run for 18–24 hours at 1.5 Watts per gel at 10°C or until the bromophenol blue dye front reached the bottom of the gel. When the run was completed, the gel cassettes were removed from the tank one at a time using the DALT cassette removal tool and rinsed with UHP to remove the running buffer.

2.7.3.5 Method for scanning DIGE labeled samples

The Typhoon Variable Mode Imager (GE Healthcare) was turned on and left to warm up for 30 minutes prior to scanning. The scanning control software was opened and the fluorescence mode was selected. The appropriate emission filters and lasers were then selected for the separate dyes (Cy2 520 BP40 Blue (488), Cy3 580 BP30 Green (532) and Cy5 670 BP30 Red (633)). The first gel was placed in the scanner and pre-scanned at a 1000 pixel resolution in order to obtain the correct photo multiplier tube (PMT) value (to prevent saturation of the signal from high abundant spots). Once the correct PMT value was found (500-600), the gel was scanned at 100 pixel resolution, resulting in the generation of three images, one each for Cy2, Cy3 and Cy5. Once the scanning

was completed, the gel images were imported into the Image Quant software. All gels were cropped identically to facilitate spot matching in the Decyder BVA module.

2.7.3.6 Analysis of gel images

Scanned fluorescent gel images were analysed using the Biological Variation Analysis (BVA) module of DeCyder 6.5 software (GE Healthcare). Images were loaded into DeCyder software using the image loader function. The Batch Processor Module of DeCyder was used. The experiment design was set up to compare the cancerous cells (MCF-7+BT20) with normal cells (MCF-10A+HMEC), normal and cancer invasive cells (HMEC+BT20) with normal and cancer non-invasive cells (MCF-10A+MCF-7), cancer invasive (BT20) with normal invasive, normal non-invasive and cancer non-invasive cells (HMEC+MCF-10A+MCF-7). The spots on the gels were then matched across all gels in the experiment. The software standardises the relative spot intensity of the Cy5 image and Cy3 image in the same or different gels using the intensity of the Cy2 image. The standardised spot intensity was then averaged across the four replicate gels. The BVA module calculates the degree of difference in the standardized protein abundance between 2 spots from different groups and expressed these differences as average ratio. The values by the software are displayed in the range of $-\infty$ to -1 for a decrease in expression and +1 to $+\infty$ for an increase in expression. For example, a two-fold increase and decrease is represented by +2 and -2, respectively (not by 2 and 0.5). The 'average ratio' has been termed as 'fold change' in this thesis. The software also calculates the consistency of the differences between samples across all the gels and applies statistics to associate a level of confidence (p-value) for each of the differences. The spots with statistically significant changes in protein expression (±1.5 fold with p-value ≤ 0.05) were considered as differentially expressed proteins. These differentially expressed proteins observed using DeCyder were designated "proteins of interest" and placed in a pick-list to pick with the ETTAN Spot Picker (GE Healthcare) for identification using MALDI-ToF MS (GE Healthcare).

2.7.3.7 Staining Methods

2.7.3.7.1 Silver staining

This method is used to screen protein samples prior to labelling with the Cy Dyes. After 2-DE, the gels were removed from the plates by very carefully placing a ruler between the two plates at the top right hand corner and the top plate removed gradually. Using

the ruler, the side borders of the gel were cut away in line with the ends of the IPG strip at the top of the gel. The strip and agarose were then removed and the gel was lifted gently and placed in a gel box containing fixing solution (50mL ethanol, 12.5 mL acetic acid (Lennox) and 62.5 mL UHP). The gel boxes were placed on an orbital shaker and fixed for at least 1 hour (usually overnight). After fixing, the solution was drained from the gels. The gels were then washed three times with 150 mL of UHP for 5 minutes each time and drained. The gels were next sensitised (60 mL ethanol, 13.6 g sodium acetate, 0.4 g sodium thiosulfate and UHP in 200 mL) for 30 minutes on the orbital shaker.

The gels were then washed three times (for 10 minutes). Following the washes, 200 mL of silver staining solution (0.5 g silver nitrate, 80µL formaldehyde and 200mL UHP) was added and the boxes returned to the orbital shaker. After 20 minutes the silver solution was drained and the gels were washed twice for 5 minutes each with UHP. After the last wash, 200 ml of developer (5 g sodium carbonate, 40 µL formaldehyde and 200mL UHP) was added to each of the boxes. The gels were placed on the orbital shaker and allowed to develop. When the desired amount of spots appeared the developer was drained into the silver containing 5 L drum (this precipitated out the silver) and 200 mL of stopping solution (2.92g EDTA and 200 mL UHP) was added. The gels were left on the orbital shaker for at least 10 minutes. The gels were then scanned at 300dpi resolution.

2.7.3.7.2 Brilliant blue G Colloidal Coomassie staining

After electrophoresis, the smaller lower plates that are attached with preparative-gels were placed in the boxes containing fixing solution (7% glacial acetic acid in 40% (v/v) methanol) for at least one hour. During this step a 1X working solution of Brilliant Blue G colloidal coomassie (Sigma, B2025) was prepared by adding 800 mL UHP to the stock bottle. When the fixing step had nearly elapsed a solution containing 4 parts of 1X working colloidal coomassie solution and 1 part methanol was made, mixed by vortexing for 30 seconds and then placed on top of the gels. The gels were left to stain for 2 hours. To destain, a solution containing 10% acetic acid in 25% methanol was poured over the shaking gels for 60 seconds. The gels were then rinsed with 25% methanol for 30 seconds and then destained with 25% methanol for 24 hours. The glass surface was dried and two reference markers (GE Healthcare, 18-1143-34) were attached to the underside of the glass plate before scanning. The resulting image was

imported into the ImageMaster software (GE Healthcare) and the spots were detected, normalised and the reference markers selected. While keeping the shift key depressed, all spots of interest were manually selected. The resulting image was saved and exported into the Ettan Spot Picker software (GE Healthcare).

2.7.3.7.3 Ruthenium (II) tris bathophenanthroline Bisulponate (RuPBS) staining

For the synthesis of RuPBS, 0.5g of potassium pentachloro aquo ruthenate (Ru, 26.9%) was dissolved in 50mL of boiling water and resulting in deep red-brown solution. 3M of 1, 10-phenthroline monohydrate was added. The solution turned to deep greenish brown. Meanwhile, a reducing solution containing 12mM of sodium hydroxide and 8mM of phosphinic monohydrate was added to the refluxing mixture and refluxing was continued for another 20 min. The solution turned to a deep orange-brown. After cooling, the pH was adjusted to 7 with NaOH or HCL and the volume was adjusted to 65mL with water. This gives a 20mM stock solution, which can be stored in the fridge for ~6 months.

Low fluorescence plates were used for running preparative 2D-gels for RuPBS staining. After electrophoresis, the smaller lower plates that are attached with preparative-gels were placed in the boxes containing fixing solution (7% glacial acetic acid in 40% (v/v) methanol) for at least one hour. 600mL of RuPBS staining solution was prepared by adding 123mL of 0.2M acetic acid, 37mL of 0.2M sodium acetate, 120mL of ethanol, 120mL of glycerol, 3mL of tween, 15µL of RuPBS stock and water. Staining solution was poured on to the gel and allowed to stain for at least 6hrs or overnight under gentle agitation. The destaining solution (600mL) was composed of 123mL of 0.2M acetic acid, 17mL of 0.2M sodium acetate, 120mL of ethanol and water. Destaining was performed using 2 washes with destaining solution for 4hrs and one for overnight. Two reference markers were fixed on the back of the low fluorescence plates and gels were scanned at excitation wavelength of 488nm and emission wavelength of 605nm using the Typoon 9400 scanner. Images were imported into ImageQuant software and cropped appropriately. A single gel DIA file was created for the detection of spots and reference marker location were assigned. Spot maps were exported and imported into the BVA file created for the DIGE experiment for which the preparative gel was part of. Matching mode was selected in the BVA experiment file and landmark mode was activated. Highly abundant spot patterns were matched manually in this mode across the gels as landmarks. The software was then instructed to match image to experiment

using these landmarks. Pick spots were displayed in matched mode gel images by alteration of image preferences. Matching of these proteins spots between DIGE images preparative 2D-gel images were also checked individually and proteins that were matched incorrectly were corrected. Where the correct spot could not be found the spot was left unmatched. The pick list was then exported to the Ettan Spot Picker (GE Healthcare) software.

2.7.3.8 Spot picking

Spots that showed differential protein expression in DeCyder analysis were picked with the ETTAN Spot Picker (GE Healthcare). The stained gel was placed in the tray of the Ettan Spot Picker with reference markers (GE Healthcare) aligned appropriately and covered with UHP. The imported pick list was opened, the syringe primed and the system was set up for picking the spots from the pick list. The spots were robotically picked and placed in 96-well plates, which was stored at 4^{o}C until spot digestion.

2.7.3.9 Spot digestion

The 96-well plate was placed in the Ettan Digester (GE Healthcare, 18-1142-68) to digest the protein as follows: Step 1- the gel plugs were washed three times for 20 minutes each with 50µL of 50mM ammonium bicarbonate (Sigma, A6141) in 50% methanol. Step 2- the gel plugs were washed twice for 15 minutes with 50µL of 70% acetonitrile (Sigma, 34967). The gel plugs were left to dry for at least 60 minutes. After drying, the individual gel pieces were rehydrated in 10µL digestion buffer (12.5ng sequence-grade trypsin (Promega, V5111) per µL of 10% acetonitrile, 40mM ammonium bicarbonate). Exhaustive digestion was carried out overnight at 37^{o}C. After digestion, the samples were transferred as follows: Step 1 – A volume of 40µL of 0.1% trifluoroacetic acid (Sigma, 302031) in 50% acetonitrile was added to the wells, mixed and left for 20 minutes. A volume of 50µL of this solution was transferred to a fresh 96-well plate. Step 2 - A volume of 40µL of 0.1% trifluoroacetic acid in 50% acetonitrile was added to the wells, mixed and left for 20 minutes. A volume of 40µL of this solution was transferred to a fresh 96-well plate. The liquid in the plate was dried using a Maxi-Dry-Plus vacuum-dryer (Medical Supply Co.). After drying, the 96-well plate was placed in the Ettan Spotter (GE Healthcare, 18-1142-67) for spotting the peptides onto the target plates. A volume of 3µL of 0.5% trifluoroacetic acid in 50% acetonitrile was added to the concentrated peptides and mixed 5 times. A volume of 0.3µL of this

mixture was spotted onto the target plate followed by a volume of 0.3µL matrix solution (7.5mg/mL a-cyano-4-hydroxycinnamic acid (LaserBio labs, 28166-41-8) in 0.1% trifluoroacetic acid in 50% acetonitrile). A sample mix, Pep4 (Laser Biolabs) was also spotted onto target slides and used as an external calibrant.

2.7.3.10 Identification of proteins with MALDI-ToF

Mass spectra were recorded in the positive ion, reflectron mode using the Ettan MALDI-ToF Pro mass spectrometer (GE Healthcare) equipped with delayed extraction and a standard nitrogen laser (337 nm). Mass spectra were recorded using the MALDI TOF instrument operating in the positive reflector mode at the following parameters: accelerating voltage 20 kV; and pulsed extraction: on (focus mass 2500). Spectra were acquired by selective accumulation of 250 individual laser shots and processed using Ettan MALDI evaluation software. The spectra were internally calibrated with trypsin enzyme autolysis peptide peaks at m/z 842.51 and m/z 2211.10, and externally calibrated with Pep4 mix with the five individual peaks covering the 500-3500 Da mass range and include bradykinin fragment 1-5 (573.315), angiotensin II human (1046.5424), neurotensin (1672.9176) and insulin B chain oxidised (3494.6514). Known contaminant peaks were removed from the resulting mass spectra and remaining sample-related peaks were used for database searching. The identification sequencing method were set for the mass range 0-300kDa, pI 1-14, missed cleavage 1. The artificial modifications of peptides (carbamidomethylation of cysteines and partial oxidation of methionines) were also considered. Protein identification was achieved using Ettan MALDI-ToF Pro evaluation software (GE Healthcare) incorporating the ProFound database search engine for peptide mass fingerprints. The sequence database searched was the NCBI-nr database (2007/12/02) using subset species *Homo sapiens* and NR_NICB_Human070308. Protein identifications were accepted if they could be established at greater than 99% probability and had at least 3 identified peptides. The unidentified protein spot that had promising spectrum to get identification were desalted and concentrated using C-18 Zip-Tips (Millipore) and were reanalysed by MALDI-ToF MS to get identification. For this, the mixtures of tryptic peptides from individual samples were desalted using Millipore C-18 Zip-Tips (Millipore) and eluted onto the sample plate with the matrix solution (5 mg/mL CHCA in 50% ACN/0.1% TFA v/v) and analysed with MALDI-ToF.

2.7.3.11 Identification of proteins with LC–MS/MS

Tryptic digest of proteins (for those which were more than 3 fold differentially expressed) were also analysed by 1-D LC-MS using the Ettan™ MDLC system (GE Healthcare) in high-throughput configuration directly connected to a Finnigan™ LTQ™ (Thermo Electron). Samples were concentrated and desalted on RPC trap columns (Zorbax™ 300SB C18, 0.3 mm65 mm, Agilent Technologies) and the peptides were separated on a nano-RPC column (Zorbax 300SB C18, 0.075 mm 6100 mm, Agilent Technologies) using a linear ACN gradient from 0 to 65% acetonitrile (Sigma, 34967) over 60 min. All buffers used for nano-LC separation contained 0.1% formic acid (Fluka, 94318) as the ion pairing reagent. Full scan mass spectra were recorded in profile mode and tandem mass spectra in centroid mode. A scan time of ~0.15 s (one microscan with a maximum ion injection time of 10ms) over an m/z range of 300-2000 was used followed by MS/MS analysis of the 3 most abundant peaks from each scan which were then excluded for the next 60 seconds followed by MS/MS of the next next three abundant peaks which in turn were excluded for 60 seconds and so on. A "collision energy"setting of 35% was applied for ion fragmentation and dynamic exclusion was used to discriminate against previously analysed ions (data dependent analysis).

Protein identification search was performed using the Turbo-SEQUEST algorithm in the BioWorks 3.1 software package (Thermo Electron) and the Swiss-Prot human database (Swiss Institute of Bioinformatics, Geneva, Switzerland). The identified peptides were further evaluated using charge state versus cross-correlation number (XCorr). The criteria for positive identification of peptides was XCorr > 1.5 for singly charged ions, XCorr > 2.0 for doubly charged ions, and XCorr > 2.5 for triply charged ions. The distinct peptides with p-value ≤ 0.05 were considered for protein identification and also had at least 2 peptides.

2.8 Western blot Analysis

2.8.1 Gel electrophoresis

Proteins for analysis by Western blotting were resolved using 12% NuPAGE Bis-Tris Gels (Invitrogen, NP0341BOX) in XCell SureLock™ Mini-Cell (Invitrogen, EI0001) running instrument. Western blotting samples (2µg/µL) were prepared by diluting samples with water and then an equal volume of 2X loading buffer (Sigma, S3401). 10-

40µg of protein and 5µL of molecular weight marker (ISIS, P7708S) were loaded onto gels. The samples were electrophoretically separated at 200V and 45mA using a MOPS/SDS buffer (MOPS 1M (Fluka, 69949), 1M Tris base (Sigma, T8404), 2% SDS (Sigma, 23771), 20mM EDTA, pH 7.7 (Fluka, 03609)), until the bromophenol blue dye reached the bottom of the gel.

2.8.2 Western blotting

Once electrophoresis had been completed, the gel was equilibrated in transfer buffer (25mM Tris (Sigma, T8404), 192mM glycine (Sigma, G-7126), pH 8.3-8.5) for approximately 30 minutes. Five sheets of Whatman 3mm filter paper (Whatman, 1001824) were soaked in freshly prepared transfer buffer. These were then placed on the cathode plate of a semi-dry blotting apparatus (Bio-rad). Air pockets were then removed from between the filter paper. Nitrocellulose membrane (GE Healthcare, RPN3032D), which had been equilibrated in the same transfer buffer, was placed over the filter paper on the cathode plate. Air pockets were once again removed. The gels were then aligned onto the membrane. Five additional sheets of transfer buffer-soaked filter paper were placed on top of the gel, all air pockets removed and excess transfer buffer removed from the cathode plate. The proteins were transferred from the gel to the membrane at a current of 34mA at 15V for 30-40 minutes, until all colour markers had transferred. Following protein transfer, membranes were stained using Ponceau (Sigma, P7170) to ensure efficient protein transfer. The membranes were then blocked overnight using 1-5% Marvel (Cadburys; Marvel skimmed milk) in PBS at 4°C. The membranes were washed with PBS prior to the addition of the primary antibody. Membranes were incubated with primary antibody overnight at 4°C. Antibodies were prepared in 1-5% Marvel in PBS. The antibody dilutions are illustrated in Tables 2.4 and 2.5. The membranes were then rinsed 3 times with PBS containing 0.5% Tween 20 (Sigma P1379) for a total of 15-30 minutes. Relevant secondary antibody (1/1000 dilution of anti-mouse (Dako Cytomation, P0260) or anti-rabbit (Dako Cytomation, P0448) or anti-goat (Santa Cruz Biotechnology, Sc2098) IgG peroxidase conjugate in 2-5% Marvel-TBS) was added for 1 hour at room temperature (Table 2.4). The membranes were again washed three times thoroughly in TBS containing 0.5% Tween for 15 minutes.

Table 2.4 Primary antibodies and dilutions

No.	Primary Antibody	Dilution	Source	Details
1	3-PGDH	1/1000	Mouse	Abcam, ab57030
2	Vimentin	1/2000	Goat	Sigma, MMS-459S
3	Erp29	1/2000	Rabbit	Abcam, ab11420
4	QPRT	1/1000	Mouse	Abcam, ab57125
5	Nucleobindin-1 precursour	1/1000	Rabbit	Abcam, ab26093
6	Alpha-tubulin	1/1000	Mouse	Sigma, T6199
7	GAPDH	1/10000	Mouse	Abcam, ab8245
8	TXNRD1	1/1000	Goat	Santa Cruz Biotechnology,sc-18220
9	RAD23B	1/1000	Goat	Abcam, ab3835
10	Cathepsin B	1/1000	Mouse	Abcam, ab58802
11	Transaldolase-1	1/200	Goat	Santa Cruz Biotechnology,sc-51439
12	Keratin 8	1/1000	Mouse	Sigma, C5301
13	Nm23	1/1000	Mouse	Calbiochem, OP48
14	Bip	1/1000	Rabbit	Sigma, G8918
15	Maspin (SERPINB5)	1/1000	Rabbit	Abcam, ab22354
16	STIP-1	1/200	Goat	Santa Cruz Biotechnology,sc-15276
17	CLIC1	1/1000	Rabbit	Abcam, ab28722
18	PSME1	1/1000	Goat	**Abcam, ab60278**
19	CLP	1/1000	Mouse	Abnova, H00023406
20	Ubiquitin	1/500	Mouse	Sigma, U0508
21	Transferrin Receptor	1/1000	Mouse	Abcam, ab1086

Table 2.5 Secondary antibodies and dilutions

No.	Secondary Antibody	Dilution	Details
1	Anti-mouse	1/1000	DakoCytomation, P0260
2	Anti-rabbit	1/1000	DakoCytomation, P0448
3	Anti-goat	1/1000	DakoCytomation, E0466
4	IRDye 800CW Goat Anti-Mouse	1/10000	LI-COR Bioscences, 926-32210
5	IRDye 800CW Goat Anti-Rabbit	1/10000	LI-COR Bioscences, 926-32211

2.8.3 Enhanced chemiluminescence detection using autoradiographic films

Immunoblots were developed using an Enhanced Chemiluminescence kit (GE Healthcare, RPN2106), which facilitated the detection of bound peroxidase-conjugated secondary antibody. Following the final washing, membranes were subjected to ECL. A layer of parafilm was flattened over a glass plate and the membrane placed gently upon the plate. A volume of 3 mL of a freshly prepared 1:1 (v/v) mixture of ECL reagent A and B was used to cover the membrane. The ECL reagent mixture was completely removed after a period of one minute and the membrane were covered with transparent film. All excess air bubbles were removed. The membrane was then exposed to autoradiographic film (Roche, 11666916001) for various times (from 10 seconds to 30 minutes depending on the intensity of the signal). The exposed autoradiographic film was developed for 3 minutes in developer solution (Kodak, LX24, diluted 1:5 in water). The film was then washed in water for 15 seconds and transferred to a fixative solution (Kodak, FX-40, diluted 1:5 in water) for 5 minutes. The film was washed with water for 5-10 minutes and left to dry at room temperature.

2.8.4 Western blot signal detection using IRDye® infrared dyes conjugated secondary antibody

After protein transfer, the membranes were incubated with 10mL of PBS containing odyssey blocking buffer (1:1 (v/v), PBS: odyssey blocking buffer (LI-COR Bioscences-N0012)). Membranes were the incubated with primary antibody overnight in PBS at 4°C. Antibodies were also prepared in 10mL of 1:1 (v/v) of PBS: odyssey blocking

buffer. The primary antibody dilutions are illustrated in Table 2.8.1. The expression of CLIC1, CLP and ubiquitin in samples were analyzed using IRDye® infrared dyes conjugated secondary antibody in western blotting. The membranes were then rinsed 3 times with 10mL of 1:1 (v/v) of PBS: odyssey blocking buffer for 15-30 minutes each. Relevant secondary antibody (1/10000 dilution of anti-mouse or anti-rabbit conjugate with IRDye® infrared dyes in 10mL of 1:1 (v/v) of PBS: odyssey blocking buffer) was added on to the membrane for 1hrs at room temperature. The membranes were again washed three times thoroughly in 10mL of 1:1 (v/v) of PBS: odyssey blocking buffer for 15 minutes each. The membranes were then scanned by using infrared imaging system (LI-COR Bioscences, version 3) at resolution 80µm, intensity 5.0µm and wavelengths 800nm (for anti-mouse) and 700nm (for anti-rabbit).

2.9 RNA interference (RNAi)

RNAi using small interfering RNAs (siRNAs) was carried out to silence the expression of specific genes. The siRNAs used were purchased from Applied Biosystems. siRNAs were introduced into the cells using the transfection reagent, siPORT™ *Neo*FX™ (Applied Biosystems, AM4511).

2.9.1 Transfection optimization

The conditions for siRNA transfection into MCF-10A, MCF-7, SKBR-3 and BT20 cells were optimized in 96-well and 6-well plates.

2.9.1.1 96-well plate optimization

In order to determine the optimal conditions for siRNA transfection in 96-well plates, siRNAs for kinesin (Applied Biosystems, AM16704) were transfected into MCF-10A, MCF-7, SKBR-3 and BT20 cell lines using different cell densities and neoFX concentrations. Scrambled-treated cultures were considered as controls, however untreated cultures were also monitored. siRNAs for scrambled siRNA (Applied Biosystems, 17010) and kinesin siRNAs were incubated in 5µL of optiMEM (Gibco™, 31985) to achieve 30nM siRNA concentration in 100µL of volume. NeoFX solutions at a range of concentrations as described in Table 2.9.1 were also prepared in 5µL of optiMEM. siRNA and neoFX solutions were incubated at room temperature for 10 minutes. After incubation, either scrambled siRNAs or kinesin siRNAs solution were added to respective neoFX solutions. siRNA/NeoFX solutions were mixed well and

incubated for a further 10 minutes at room temperature. For neoFX-treated wells, 5µL of optiMEM was added to the tube containing 5µL of neoFX and incubated for 10min. 10µL of siRNA/neoFX or neoFX solution was added to respective each well. Cell suspension was added to each well to achieve the required cell density per well as described in table 2.6. This experiment was performed using biological triplicates. The plates were mixed gently and incubated at 37°C for 24 hours. After 24 hours, medium was replaced and cells were allowed to grow. The plates were assayed for changes in proliferation at 72 hours after transfection using the acid phosphatase assay (Section 2.9.3.1). Optimal conditions for transfection were determined as the combination of conditions that gave the greatest reduction in cell number after kinesin siRNA transfection and the least cell death in the presence of transfection reagent.

Table 2.6. Three different concentrations of cell density and NeoFX were used for optimization of conditions for siRNA transfection of MCF-10A, MCF-7, SKBR-3 and BT20 cell lines.

Cell lines	**Cell density/well (100µL)**	**NeoFX (µL)**		
		1	**2**	**3**
MCF-10A	$5x10^3$	0.4	0.6	0.8
	$7.5x10^3$			
	$1x10^4$			
MCF-7	$5x10^3$	0.4	0.6	0.8
	$7.5x10^3$			
	$1x10^4$			
SKBR-3	$1x10^3$	0.2	0.3	0.4
	$2x10^3$			
	$3x10^3$			
BT20	$2x10^3$	0.4	0.6	0.8
	$5x10^3$			
	$7.5x10^3$			

2.9.1.2 6-well plate optimization

After narrowing down the potential siRNA transfection conditions, optimization for siRNA transfection into MCF-10A, MCF-7, SKBR-3 and BT20 cell lines was performed in 6-well plate format using siRNA for GAPDH (Applied Biosystems, AM4605) to further ensure the efficiency of transfection conditions. Scrambled-treated

cultures were considered as controls, however untreated cultures were also monitored. Solutions of siRNAs for scrambled and kinesin at a final concentration of 30nM/mL were prepared in 50µL (per well) of optiMEM (Gibco^{TM}, 31985) and incubated at room temperature for 10 min. NeoFX was added into 50µL (per well) of optiMEM in parallel to achieve required final concentrations as mentioned in Table 2.9.2 and incubated at room temperature for 10 minutes. 50µL of the siRNAs were added to the respective 50µL of neoFX solution for another 10 min. For neoFX-treated wells, 50µL of optiMEM was topped up on the 50µL of neoFX and incubated for 10min. 100µL of siRNA/neoFX or neoFX solution was added to respective each well. Cells were added to achieve required cell density per well in 2mL of final working volume as mentioned in Table 2.7. The plates were mixed gently and incubated at 37°C for 24hrs. After 24hrs, the culture medium was replaced with fresh medium. After 72hrs (or after 96hrs in case of MCF-7), cells were collected for protein extraction as mentioned in section 2.7.1.1. The inhibition was investigated using Western blot analysis as described in section 2.8.

Table 2.7. Optimization of siRNA transfection conditions in 6-well plate format.

Cell lines	**Cell density/well (100µL)**	**NeoFX (µL)**		
		1	**2**	**3**
MCF-10A	$5x10^5$	4	6	8
MCF-7	$5x10^5$	4	6	8
SKBR-3	$1x10^5$	1	2	3
BT20	$5x10^5$	4	5	6

2.9.2 siRNA transfection of targets in MCF-10A, MCF-7, SKBR-3, BT20 and MDA-MB231

Three pre-designed *Silencer*® Select siRNA were chosen for each of the gene targets and transfected into cells. Two independent siRNA molecules were used for TXNRD1 and 3 independent siRNA molecules were used for 3-PGDH and RAD23B. The Silencer® *Select* siRNAs are chemically modified siRNAs that are meant to reduce overall off-target effects by up to 90% without compromising potency. For each set of siRNA transfections, scrambled siRNA treated cells were considered as control,

however un-treated cells (NT) and NeoFX treated cells were also monitored. Solutions of siRNAs for scrambled and kinesin at a final concentration of 30nM/mL were prepared in 50µL (per well) of optiMEM (Gibco^{TM}, 31985) and incubated at room temperature for 10 min. NeoFX was added into 50µL (per well) of optiMEM in parallel to achieve the required final concentrations as mentioned in Table 2.8 and incubated at room temperature for 10 minutes. 50µL of the siRNAs were topped on the respective 50µL of neoFX solution for another 10 min. For neoFX-treated wells, 50µL of optiMEM was topped up on the 50µL of neoFX and incubated for 10min. 100µL of siRNA/neoFX or neoFX solution was added to respective each well. Cells were added to achieve the required cell density per well in 2mL of final working volume as mentioned in Table 2.9.3. The plates were mixed gently and incubated at 37°C for 24hrs. After 24hrs, the culture medium was replaced with fresh medium. After 72hrs (or after 96hrs in case of MCF-7), cells were collected for protein extraction as mentioned in section 2.7.1.1. The inhibition was investigated using Western blot analysis as described in section 2.8. Table 2.9 outlines the list of siRNAs and IDs used in this thesis.

Table 2.8 Transfection conditioned used for MCF-10A, MCF-7, SKBR-3, MDA-MB 231 and BT20.

Target	**Transfected in**	**Cell density**	**siRNA (nM)**	**NeoFX (µL)**
TXNRD1	SKBR-3	$1x10^5$	30	3
	MDA-MB 231	$2x10^5$	50	4
RAD23B	MCF-10A	$5x10^5$	50	4
	MCF-7	$5x10^5$	50	4
3-PGDH	SKBR-3	$1x10^5$	30, 50	3
	MDA-MB 231	$2x10^5$	30, 125	4
	BT20	$5x10^5$	30	6

Table 2.9. List of siRNAs used and their Ambion ID. ***** denotes Silencer® *Select* siRNAs.

Name	**Ambion ID**
Scrambled	AM16708
Scrambled*	AM4390843
Kinesin	14851
GAPDH	4300
TXNDR1#1*	s755 (Validated)
TXNDR1#1*	s756 (Validated)
3-PGDH#1*	s515
3-PGDH#2*	S223685
3-PGDH#3*	S223686
RAD23B#1*	s11731
RAD23B#2*	s11732
RAD23B#3*	s11733

2.9.2.1 Invasion assays on siRNA transfected cells

After 48hrs of siRNA transfections for targets in 6-well plates, cells were trypsinized and used to set the invasion assays for investigating the effects of target knockdown on invasion status of cells. Invasion assays were carried out for a further 48hrs for MCF-10A, MDA-MB231 and SKBR-3 and for 72hrs for MCF-7as described in section 2.6. Scrambled treated cultures were considered as controls in this investigation.

2.9.2.2 Proliferation effects of siRNA transfection

Proliferation assay were carried out after 72hrs of transfection for each of the cell lines to see changes in proliferation of the cells due to inhibition of target genes. Kinesin was used as a negative control to assess the efficiency of the siRNA transfection. Kinesin plays an important role in cell division; facilitating cellular mitosis. Therefore, transfection of siRNA kinesin resulted in cell cycle arrest and confirmed efficient transfection. Scrambled treated cultures were considered as positive control in this investigation. The plates were assayed for changes in proliferation using the acid phosphatase assay (Section 2.9.3.2).

2.9.3 Assessment of cell number - Acid Phosphatase assay

2.9.3.1 Acid Phosphatase in 96-well plate

Following an incubation period of 72 hours, media was removed from the plates. Each well of the plate was washed with 100µL PBS. 100µL of freshly prepared phosphatase substrate (10mM *p*-nitrophenoln phosphate (Sigma 104-0) in 0.1M sodium acetate (Sigma, S8625), 0.1% triton X-100 (BDH, 30632) and pH 5.5) was added to each well. The plates were wrapped in tinfoil and incubated in the dark at 37°C for 1.5hrs. The enzymatic reaction was stopped by the addition of 50µL of 1M NaOH to each well. The OD of each was measured at 405nm. Low OD value indicates more cell death in culture.

2.9.3.2 Acid Phosphatase in 6-well plate

Following an incubation period of 72 hours, media was removed from the plates. Each well on the plate was washed with 1mL PBS. 2mL of freshly prepared phosphatase substrate was added to each well. The plates were wrapped in tinfoil and incubated in the dark at 37°C for 1-2hrs. The enzymatic reaction was stopped by the addition of 500µL of 1M NaOH to each well. The OD of each was measured at 405nm. Low OD value indicates more cell death in culture.

2.10 SELDI-ToF MS

SELDI-ToF MS is a highly sensitive proteomics tool being used to identify differential expression of proteins in a given sample. Protein samples from serum-free conditioned media of human cell lines were analyzed using SELDI-ToF MS to identify differentially expressed proteins.

2.10.1 Collection of serum-free conditioned media

Cells ($3x10^6$) were seeded in four biological replicates in T-175cm^2 flasks. Cells were allowed to grow until 50-60% confluent. Cells were washed three times with 10mL of serum-free (SF) basal media and then incubated in 15mL of SF basal media for 60 min. After this time, cells were washed again two times with SF basal media. 10mL of SF basal media was added to the cells and incubated for 72hrs. After such time, conditioned media (CM) was collected, centrifuged for 15min at 250g, and stored at –80°C.

2.10.2 Preparation of IMAC30 chip surface

IMAC30 array, 8-spot (Biorad, C730043) chip surface was activated by adding 5 µL of 100mM $CuSO_4$ (Sigma, 2091198) for a total of 30 minutes (2x15 minute applications) and then rinsed with high-performance liquid chromatography grade water (Sigma, 39295). The copper ions were charged by applying 50µL of 100mM sodium acetate (Sigma, S8625) for 5 minutes. The chip was placed in a bioprocessor (Biorad) and washed twice with 300µL of 250mM sodium chloride containing 0.1% Triton X-100. The protein sample was diluted in 300µL of 250mM sodium chloride containing 0.1% Triton X-100 and samples were applied to the spots of the array. The array was placed on a shaker and gently agitated for 90 minutes to allow for interaction with the array surface. After removing the sample, the array was washed x2 with 300µl of 250mM sodium chloride for 5 minutes, followed by a brief high-performance liquid chromatography grade water wash. 5mg of Sinapinic acid (Biorad, C730078) solution was prepared by dissolving in 200µL of 50% acetonitrile (Sigma, 34967) containing 0.5% trifluoroacetic acid (Sigma, 302031). After removing the array from the bioprocessor, a 0.8µL aliquot of saturated sinapinic acid was added to the spots, allowed to dry and repeated.

2.10.3 Preparation Q10 chip surface

Q10 array, 8-spot (Biorad, C730043) was placed in a bioprocessor and washed twice with 300µL of 50mM Tris (Sigma, T8404) pH 8.0. The protein sample was diluted in 300µL of 50mM Tris pH 8.0 and samples were applied to the spots of the array. The array was placed on a shaker and gently agitated for 90 minutes to allow for interaction with the array surface. After removing the sample, the array was washed x2 with 300µL of 50mM Tris pH 8.0 for 5 minutes, followed by a brief high-performance liquid chromatography grade water wash. After removing the array from the bioprocessor, a 0.8µL aliquot of saturated sinapinic acid was added to the spots, allowed to dry and repeated.

2.10.4 Preparation CM10 chip surface

CM10 array, 8-spot (Biorad, C530075) was placed in a bioprocessor and washed twice with 300µL of 20mM Tris pH 4.5. The protein sample was diluted in 300µL of 20mM Tris pH 4.5 and samples were applied to the spots of the array. The array was placed on a shaker and gently agitated for 90 minutes to allow for interaction with the array

surface. After removing the sample, the array was washed x2 with 300µL of 20mM Tris pH 4.5 for 5 minutes, followed by a brief high-performance liquid chromatography grade water wash. After removing the array from the bioprocessor, a 0.8µL aliquot of saturated sinapinic acid was added to the spots, allowed to dry and repeated.

2.10.5 Preparation H50 chip surface

H50 array, 8-spot (Biorad, C730065) was placed in a bioprocessor and washed twice with 10% acetonitrile/0.1% trifluoroacetic acid. The protein sample was diluted in 300µL of 10% acetonitrile/0.1% trifluoroacetic acid and samples were applied to the spots of the array. The array was placed on a shaker and gently agitated for 90 minutes to allow for interaction with the array surface. After removing the sample, the array was washed x2 with 300µL of 10% acetonitrile/0.1% trifluoroacetic acid for 5 minutes, followed by a brief high-performance liquid chromatography grade water wash. After removing the array from the bioprocessor, a 0.8µL aliquot of saturated sinapinic acid was added to the spots, allowed to dry and repeated.

2.10.6 Preparation NP20 chip surface

NP20 array, 8-spot (Biorad, C5730043) was washed twice with 2µL of high-performance liquid chromatography grade water. 1µL samples were applied to the spots of the array and incubated for 20 minutes to allow for interaction with the array surface. The array was washed x2 with high-performance liquid chromatography grade water. A 0.8µL aliquot of saturated sinapinic acid was added to the spots, allowed to dry and repeated.

2.10.7 Analysis of Protein chip array in ToF Reader

The IMAC-Cu^{2+} ,Q10, CM10 and H50 ProteinChip arrays were analyzed in a Ciphergen Series PBS-IIC ProteinChip® System, and time-of-flight (ToF) data was generated by averaging a total of 220 laser shots collected at a laser intensity of 200, a detector sensitivity of 8 and molecular mass range from 5–20,000 Da for low molecular weight range and 20,000-100,000 Da for high molecular weight range.

2.10.8 Analysis of differential expression of Proteins/peptides

Molecular weights were calibrated externally using an all-in-1 protein standard (Biorad, N76006). All data was analyzed using Biomarker Wizards Software, version 3.1 (Bio-

Rad). After automatic baseline noise correction, all of the spectra were normalized together by the ‘‘total ion content’’ method as described by the manufacturer, i.e. with an *m/z* between 5,000 and 100,000. The peaks with an *m/z* value < 3000 were excluded, as these peaks were mainly ion noise from the matrix (sinapinic acid). Peak clusters were generated by automatically detecting qualified mass peaks with a signal to noise ratio (S/N) >5 in the first pass, completed with a second-pass peak selection of S/N >3, with a 0.3% mass error for 5000-20,000 Da, and the same for 20,000-100,000 Da. Statistically significant peaks were considered to be those with $p < 0.05$.

2.10.9 Optimization of serum concentration

MDA-MB 435S-F and MDA-MB 435S-F/Taxol10p4p cells ($1x10^5$ cells/mL) were seeded in T-25cm^2 flask in 10% and 0.5% serum supplemented and serum-free media and incubated for 72 hours. Cells were grown in serum-free media using the same method as described in section 2.13.1. Conditioned media were collected and centrifuged at 250g for 15 minutes and then transferred to new universal. A volume of 1mL of conditioned media was concentrated to 100µL using a Maxi-Dry-Plus vaccum-dryer (Medical Supply Co.). Protein concentrations were estimated as described in section 2.7.2. 50µg of protein was analyzed on a Q10 chip surface as described in section 2.10.6.

2.10.10 Selection of chip surface

After 72 hours serum-free conditioned media from MDA-MB 435S-F and MDA-MB 435S-F/Taxol10p4p were collected and concentrated using 5000 MW (Millipore, UFC9 00524) and 10000 MW (Millipore, UFC901024) cut-off centrions to 500µL by centrifuging at 1000g at 4^0C. 10µg of protein sample was analyzed on IMAC30 (immobilized Metal Affinity capture) coupled with Cu^{++} ion, Q10 (Strong anion-exchange), CM10 (weak cation-exchange) and H50 (reversed-phage hydrophobic surface) protein chip arrays.

2.11 Protein purification and identification

2.11.1 Protein Purification

Serum-free conditioned media from the MDA-MB-435S-F/Taxol10p4pSI and SKMEL-28 were fractionated first using BioSepra IMAC-Cu^{2+} HyperCel spin columns (BioSepra, Cergy, C540-0027). Each of the fractions eluted from the spin columns

using 50 to 500 mmol/L imidazole in binding buffer containing 250mM sodium chloride was analyzed further on a hydrophilic NP20 ProteinChip Array (section 2.10.9) to monitor the elution and recovery of the protein of interest. The eluted fractions in which the marker was the most abundant were concentrated to 30µl using centricons.

2.11.2 Separation of protein

The 7.6kDa protein/peptide was separated on 12% NuPAGE Bis–Tris Gels (Invitrogen, NP0341BOX) and the 8.5kDa protein/peptide was separated on 16% Tricine gels (Invitrogen, EC6695BOX) as described in section 2.8.1.

16% Tricine gels were placed in the XCell SureLock™ Mini-Cell (Invitrogen, EI0001) running instrument. Samples were diluted in 2X loading buffer (Sigma, S3401). 40µg of protein was loaded onto a gel and electrophoretically separated using a 1X Tricine SDS buffer (Invitrogen, LC1675). Molecular weight peptide markers (Invitrogen, 17-501934) were loaded along side the samples in gels. The gels were run at 200V and 45mA until the bromophenol blue dye front was found to have reached the end of the gel, at which time sufficient resolution of the molecular weight markers was achieved.

2.12 Zymography

Zymography was used to assess the level of proteolytic activity of different proteinases in cell culture supernatants. The choice of substrate incorporated into the resolving gel depends on substrate specificity of the species of enzyme to be detected (Johansson *et al.* 1986). Gelatin as a substrate for matrix metalloproteinases (MMPs), serine and cysteine proteinases, was used in these experiments.

Serum-free conditioned media from MDA-MB 435S-F and MDA-MB 435S-F/Taxol10p4p cells collected and concentrated as discussed in 2.10.1. Protein was quantified using a Bio-Rad protein assay kit as discussed in section 2.7.2. Samples were mixed 3:1 with 4X sample buffer (20% glycerol; 0.25M Tris-HCl, pH 6.8; 0.1% (w/v) bromophenol blue) and were loaded onto the gel. A 5µl aliquot of boiled broad size range protein marker (New England Biolabs, 7708S) was also loaded onto the gel. The gelatin gels (Invitrogen, EC6175BOX) were run at 30 mA per gel in 1X running buffer (14.4g Glycine, 3.03g Tris and 1g SDS in 1L UHP) until the dye front reached the bottom of the gel. After electrophoresis, gels were soaked in 2.5% Triton X-100 at room temperature with gentle shaking for 30 min and incubated in substrate buffer (50mM

Tris-HCl, pH 8.0, 5mM $CaCl_2$) with ±EDTA, ±Aprotinin, ±Pepstatin A, ±E-64 or 1,10 Phenanthroline at 37°C for 18-24 hrs. Gels were then stained with 2.5 mg/ml Coomassie Blue for 2 hours and destained in a mixture of acetic acid: isopropanol: distilled water (1:3:6) until clear bands were visible. Gelatinase activity was determined as distinct, clear bands.

2.13 Treatment with MMP inhibitors

Cells were seeded at 2.0×10^5 cells/well in 6-well plates and grown to 50-60 % confluency in complete medium and then washed three times in serum-free medium. Cells were subsequently incubated in serum-free medium for 1 hour, washed two more times in serum-free medium, and incubated in 500µl of serum free media with 1µg of MMP2 and 1µM MMP9 inhibitor separately for 72 hours. Untreated cultures without MMP inhibitors were also maintained as controls for this experiment. Conditioned medium was collected, centrifuged at 250g for 15 min to remove cellular debris, decanted into clean eppendorffs. 200µl of conditioned media from each biological samples were then analysed on IMAC-Cu^{++} ProteinChip arrays as discussed in section 2.10.5, 2.10.10 and 2.10.11.

2.15 Statistical Analysis

Significance of data presented in section 3.0 was determined using 2 tailed T-tests with 2 unpaired samples with unequal varience.

T-tests were performed on invasion, siRNA and in treatment with MMP inhibitors assays in order to determine if data was significant.

A t-test > 0.05 was deemed not significant

A t-test > 0.05 was deemed significant

Section 3.0

Results

3.1 Proteomic analysis of cell lysates from HMEC, MCF-10A, MCF-7 and BT20

The transformation of a normal cell into a cancer cell and the process of tumour cell invasion are complicated multistage procedures that reflect well coordinated alterations in the expression of many genes and proteins within cells. The understanding of proteomic alterations may be helpful to a better understanding of the molecular as well as cellular mechanisms involved in the process transformation of cancerous and invasive phenotypes. Therefore, proteomics study of cell lysates from normal mammary epithelial cells (HMEC), non-tumorigenic immortalized epithelial (MCF-10A) and breast carcinoma cell lines (MCF-7 and BT20) were carried out (Table 3.1).

Table 3.1.1: Description of the cells used in this investigation in respect of cell type and invasive phenotypes.

Cells	**Cell Type**	**Type**	**Invasion Status**
HMEC	Basal	Normal-like	Invasive
MCF-10A	Basal	Normal	Non-invasive
MCF-7	Luminal	Cancerous	Non-invasive
BT20	Basal	Cancerous	Invasive

3.1.1 Invasion assay of HMEC, MCF-10A, MCF-7 and BT20

The invasive capabilities of HMEC, MCF 10A, MCF-7 and BT20 were measured using pre-coated matrigel invasion chambers over the 48hrs of culture as described in section 2.6 to ensure that the invasion status of cells reflects the phenotypes as described in the literature. The graph was plotted on the basis of total number of cells that crossed the matrigel membrane (Figure 3.1.1). The results indicated that the MCF-10A and MCF-7 cell lines are non-invasive, whereas HMEC is low invasive and BT20 is highly invasive in this group (Figure 3.1.1B). However, why HMEC cells are invasive is not clear.

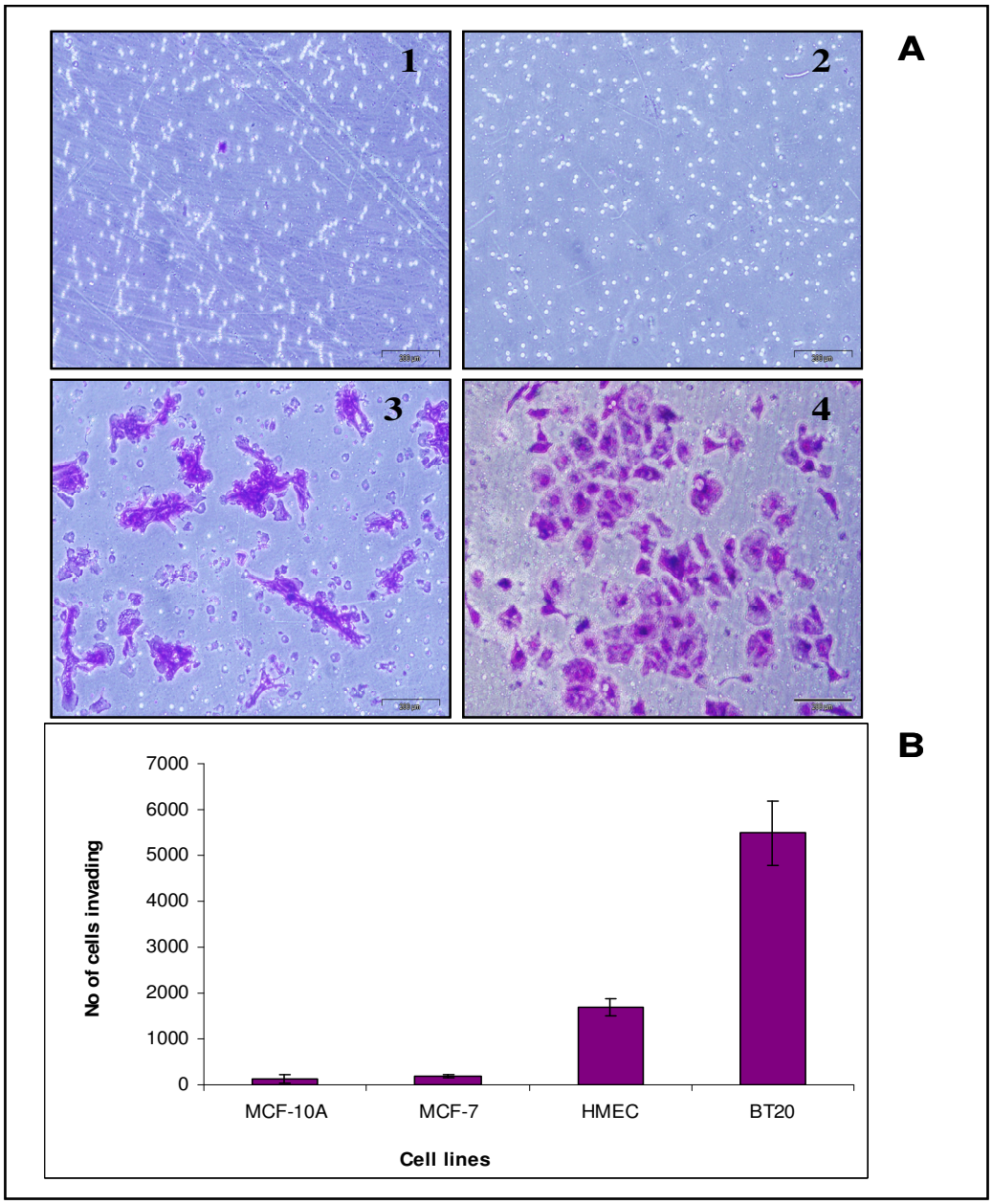


Figure 3.1.1 Investigation of invasion capabilities of cells from breast origin. **(A)** Images from invasion assay- (1) MCF-10A, (2) MCF-7, (3) HMEC, (4) BT20. **(B)** Graphical representation of the comparative levels of invasion in the cells tested. Magnification= 200x, Scale bar=200µm. Error bars represent the standard deviation calculated from data obtained from three biological samples.

3.1.2 Analysis of differential expression of proteins using 2D-DIGE

Investigations were carried out to establish the differential expression of cellular proteins between normal, cancerous, non-invasive and invasive breast cell lines as described in section 2.7.3. For this, four biological replicate samples from normal mammary epithelial cells (HMEC), non-tumorigenic immortalized mammary epithelial cells (MCF 10A), non-invasive breast cancer cells (MCF7) and invasive breast cancer cells (BT20) were prepared separately. Samples from each cell line were differentially labelled with 2D-DIGE dyes (i.e. Cy3 and Cy5) as outlined in Table 3.1.2. A pooled internal control containing a mixture of all biological replicate samples in the experiment was labelled with Cy2 dye.

2D-DIGE protein expression maps (PEMs) for normal mammary epithelial cells (HMEC), breast epithelial cells (MCF 10A), non-invasive breast cancer cells (MCF7) and invasive breast cancer cells (BT20) were created. The PEMs were then analysed using the Biological Variation Analysis (BVA) module of DeCyder 6.5 software as described in section 2.7.3.6. This was aided by the use of a pooled internal standard which facilitated spot matching and relative spot quantitation while minimizing gel-to-gel variation. Spots originating from dust particles and gel impurities in each gel image were checked and removed manually to improve the quantitation and matching process. Landmarks, which aid the gel-to-gel spot matching process, were also defined in the gel to increase the accuracy of the matching algorithm. Manual checks were carried out to allow for cases where spots were detected more than once (due to spot splits by the spot detection algorithms). These spots were then merged back to improve spot matching. After spot matching and filtering a total of 883 protein spots were matched across all of the gels in the experiment (i.e, a total of 21 gels, 1 gel of Cy2, Cy3 and Cy5 labelled samples was taken out from the analysis as the quality of this gel was not good).

Table 3.1.2. 2D-DIGE experimental design used for the analysis of differential protein expression in normal mammary epithelial cells (HMEC), breast epithelial cells (MCF 10A), non-invasive breast cancer cells (MCF7) and invasive breast cancer cells (BT20).

Gel number	**Cy2**	**Cy3**	**Cy5**
1	Pooled internal standard	HMEC Biological 1	MCF-10A Biological 1
2	Pooled internal standard	HMEC Biological 2	MCF-10A Biological 2
3	Pooled internal standard	HMEC Biological 3	MCF-10A Biological 3
4	Pooled internal standard	HMEC Biological 4	MCF-10A Biological 4
5	Pooled internal standard	MCF-7 Biological 1	BT20 Biological 1
6	Pooled internal standard	MCF-7 Biological 2	BT20 Biological 2
7	Pooled internal standard	MCF-7 Biological 3	BT20 Biological 3
8	Pooled internal standard	MCF-7 Biological 4	BT20 Biological 4

3.1.3 Creation of differentially expressed protein lists related to cancer and invasion

The objective of this investigation was to identify the proteins that are being up-regulated or down-regulated with the cancerous and invasion status of the cell lines. For this, PEMs of HMEC, MCF-10A, MCF-7 and BT20 were analysed in the following three comparison groups. Spots that were ±1.5 fold up or down-regulated with p-value ≤ 0.05 were considered significantly differentially expressed (DE) in this investigation (section 2.7.3.6). The software calculates the degree of difference in the standardized protein abundance between 2 spots from different groups and expresses these differences as an average ratio. The values generated by the software are displayed in the range of -∞ to -1 for a decrease in expression and +1 to +∞ for an increase in expression. For simplicity, the '**average ratio'** has been termed as '**fold change**' in this thesis. Hence, for example, a two-fold increase and decrease is represented by 2 and -2, respectively (not 2 and 0.5).

3.1.3.1 Cancerous (MCF-7+ BT20) vs. Normal (HMEC+ BT20)

To see which proteins are being differentially expressed between the cancer and normal cells, the PEMs of the cancerous cell lines (MCF-7+BT20) were compared with the PEMs of the normal cells (HMEC+MCF10A). The comparison revealed altered expression of 89 proteins; with 47 proteins up-regulated and 42 proteins down-regulated in the cancer cell lines (MCF-7+BT20) compared to the normal cells (HMEC+MCF10A).

3.1.3.2 Invasive (HMEC+ BT20) vs. Non-invasive (MCF-10A + MCF-7)

There is evidence in the literature that normal and malignant invasion use similar molecular mechanisms and the only difference between them is that malignant invasion can persist (Diamandis 2004, Baggerly *et al.* 2004). Therefore, grouping of cell lines by their invasion status, i.e. normal+cancer ((N+C) invasive (HMEC+ BT20) and normal+cancer ((N+C) non-invasive (MCF-10A + MCF-7), and comparing differential expression of proteins between the invasive and non-invasive cells could reveal potential targets that may have an important role in normal +cancer -related invasion. 31 proteins were found differentially expressed in this comparison with 11 proteins up-

regulated and 20 proteins down-regulated in the non-invasive cells. We have used the term **'normal + cancer-related invasion**' to describe this comparison in this thesis.

3.1.3.3 Cancerous invasive (BT20) vs. Normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7)

In another aspect to investigate alterations in the invasion-related protein expression, the proteins that are up-regulated or down-regulated only in the cancerous invasive cell line (BT20) compared to the normal invasive, normal non-invasive, cancerous non-invasive cells (HMEC+MCF-10A+MCF-7) could help to identify proteins that may have a potential role specifically in the cancer invasion. Therefore, the PEMs of BT20 were compared with the PEMs of HMEC+MCF-10A+MCF-7. This comparison also possibly eliminates protein changes that would be related to cell type rather than specifically to invasion as BT20, HMEC and MCF-10A are basal types, while MCF-7 is luminal type. In this comparison, 92 proteins were found differentially expressed, with 24 up-regulated and 86 down-regulated in BT20 cells. We used the term **'Cancer-related invasion'** to describe this comparison in this thesis.

A number of differentially expressed protein spots were observed in these three groups (Table 3.1.3).

Table 3.1.3. List of the total number of differentially expressed proteins in the comparison of the cancerous (MCF-7+BT20) vs. Normal (HMEC+MCF-10A), invasive (HMEC+BT20) vs. non-invasive (MCF-10A+MCF-7) and cancerous invasive (BT20) vs. normal invasive + normal non-invasive + cancerous non-invasive (HMEC+MCF-10A+MCF-7), and which pass the fold change ≥ 1.5 fold up/down regulated with t-test score of ≤ 0.05.

No.	**Comparison Groups**	**No. of differentially expressed proteins**	**Up-regulated**	**Down-regulated**
1	Cancerous (MCF-7+ BT20) vs. Normal (HMEC+ BT20)	96	41	54
2	Invasive (HMEC+ BT20) vs. Non-invasive (MCF-10A + MCF-7) **(Normal+ cancer-related invasion)**	31	20	11
3	Cancerous invasive (BT20) vs. Normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7) **(Cancer-related invasion)**	92	24	68

3.1.4 Identification of differentially expressed proteins using mass-spectrometry

For protein identification, preparative colloidal coomassie stained gels were used. One hundred and ninety three proteins spots were picked for identification using the Matrix Assisted Laser Desorption/ionization Time-of-Flight (MALDI ToF MS) mass-spectrometry (section 2.7.3.10). The same spots were also picked from different cell lines to confirm the identifications from these spots. A few identifications were also confirmed from repeat preparative gels. All identified proteins by MALDI ToF MS had an expectation value of 0.01 or better (a value of 0 denotes 100% confidence in the database match and a value of 0.01 denotes a 1% or less chance that the identification is random). Expectation value for proteins was determined by Ettan MALDI-ToF Pro evaluation software using the ProFound database search engine for peptide mass fingerprints. The proteins that were $\geq \pm 3$ fold up- or down-regulated were also analysed using Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) for identification or to confirm the identification obtained from MALDI ToF MS (section 2.7.3.12). The criteria for positive identification of peptides from LC-MS/MS were XCorr N1.5 for singly charged ions, XCorr N2.0 for doubly charged ions, and XCorr N2.5 for triply charged ions peptides containing a p-value ≤ 0.01. LC-MS/MS is a very sensitive technique and therefore in some cases more than one protein at the same molecular weight and isoelectric point (pI) were identified. The location of all identified proteins has been shown on a representative 2-D DIGE gel (Figure 3.1.2). Each protein has been denoted by its master number ID, which was generated by Decyder 6.5 BVA module during the analysis of differentially expressed spots (proteins).

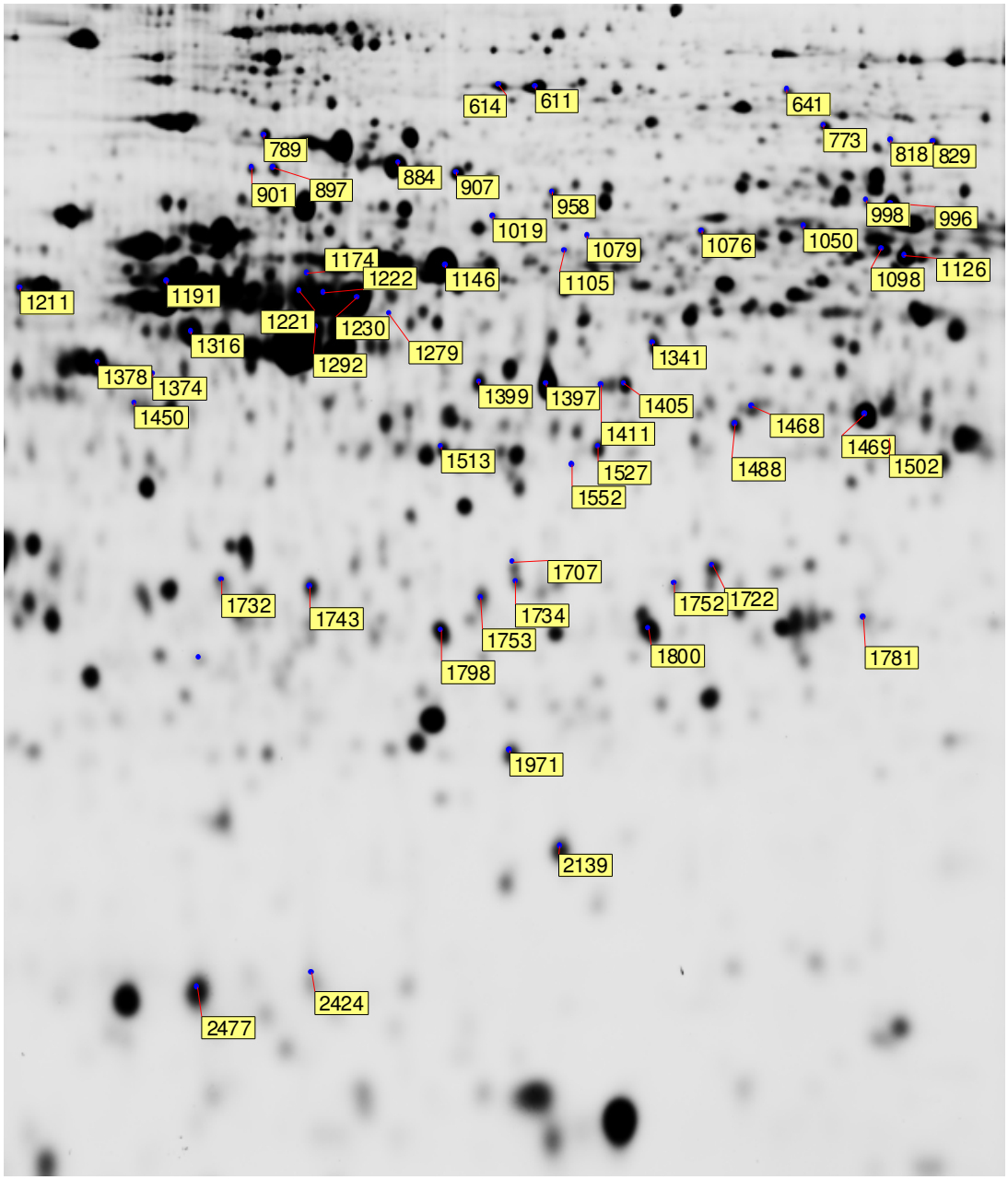


Figure 3.1.2 Representative 2D-DIGE gel image of Cy2-labelled pool of HMEC, MCF-10A, MCF-7 and BT20 cell lysate samples showing differentially expressed protein spots that have been successfully identified by MALDI-ToF MS and/or LC-MS/MS. Identified spots are represented on the gel using the Master Number ID generated by the Decyder software as a reference.

3.1.5 List of identified proteins differentially regulated in the cancerous vs. normal and invasive-related comparisons.

As described in section 3.1.3, PEMs from HMEC, MCF-10A, MCF-7 and HMEC were compared in three comparison groups, cancer cell lines (MCF-7+BT20) vs. normal cells (MCF-10A+ HMEC), invasive cells (HMEC+BT20) vs. non-invasive cells (MCF-10A+MCF-7) and cancer invasive cell line (BT20) vs. normal non-invasive, normal invasive and cancer non-invasive cell lines (MCF-10A+HMEC+MCF-7). A number of differentially expressed proteins were identified in each comparison group.

3.1.5.1 Cancerous (MCF-7+BT20) vs. normal (MCF-10A+ HMEC)

A total of 44 proteins were identified in the comparison of cancer cell lines (MCF-7+BT20) vs. normal cells (MCF-10A+HMEC) comparison group (Table 3.1.4.) as described in section 2.7.3. Among these proteins, 27 were up-regulated and 17 were down-regulated in the cancer cell lines compared to the normal cells. The cell lines are compared from cancerous to normal. The proteins that are up-regulated are increased in their expression in the cancerous cell lines compared to normal cells and are represented as positive fold changes, whereas the proteins that are down-regulated are expressed at lower levels in the cancerous cell lines compared to the normal cell lines and have been denoted by negative fold change. These alterations in protein expression, i.e. up or down regulation, of these proteins could potentially be associated with breast cancer.

A number of proteins that are already well known for their involvement in cancer were identified to be differentially expressed in the comparison, for example, Serpin B5, annexin A1 and KRT19 (Table 3.1.4.).

- Serpin B5 (SERPINB5), also known as Maspin, is a serine protease inhibitor and is involved in proteolysis in cells (Liotta and Stetler-Stevenson 1991). SERPINB5 was showed the highest differentially expression in this comparison and was 98-fold down-regulated in the cancer cell lines compared to the normal cells.
- Annexin A1 (ANXA1), also known as p35, is a cytoskeletal protein. Apart from cell motility, ANXA1 is a mediator of apoptosis and an inhibitor of cell

proliferation (Sheng *et al.* 1996). ANXA1 was found to be down-regulated (3.8-fold and 26.8-fold) in the cancerous cell lines compared to the normal cells.

- Keratin 19 (KRT19) was observed to be 37.8 fold up-regulated in the cancerous cell lines compared to the normal cells. KRT19 is an intermediate filament protein and is involved in maintenance of structural integrity of cells (Gerke and Moss 2002).

A number of proteins including SERPINB5, ANXA1 and transaldolase 1 (TALDO1) were identified at more than one location on the 2D-gel possibly indicating some form of post-translational modifications (PTMs) (Table 3.1.4.). For example, ANXA1 was down-regulated in both spots (3.8-fold and 26.8-fold) and SERPINB5 was down-regulated in both spots (98.6-fold and 20.6-fold) in the cancerous cell lines compared to the normal cells. TALDO1 was up-regulated in both spots (2.2-fold and 2.7-fold) in the cancerous cell lines compared to the normal cells. Investigation of potential PTMs in these proteins could potentially reveal a mechanistic view of breast cancer.

This comparison also revealed a number of potentially novel proteins, including EIF4A3, UQCRC1, DCI, SOD1 and SERPINB1 that have not been investigated extensively to date for their involvement in breast cancer and could have a significant role in regulation of the cancerous phenotype (Table 3.1.5.). Therefore, further study to establish the clinical relevance of these 5 proteins (EIF4A3, UQCRC1, DCI, SOD1 and SERPINB1) in breast tissue could be very helpful to understand the mechanisms of tumourigenesis in breast cells.

- Ubiquinol-cytochrome-c reductase complex core protein 1(UQCRC1) is a nuclear-encoded protein and is localized to the inner mitochondrial membrane. UQCRC1 was found to be 8.1-fold up-regulated in the cancer cell lines compared to the normal cells.

- Translation initiation factor 4A isoform 3 (EIF4A3) is an RNA helicase which participates in the unwinding of the 5' secondary structure in the mRNA and facilitates binding of the small ribosomal subunit during protein translation

(Tamai *et al.* 2000). The expression of EIF4A3 was 2.2-fold up-regulated in the cancer cell lines compared to the normal cells.

- 3,2-trans-enoyl-CoA isomerase (DCI) is involved in the biochemical reactions of lipids and fatty acid and was 3.1-fold up-regulated in the cancer cell lines compared to the normal cells.

- Chain J, Superoxide Dismutase Mutant With Lys 136 Replaced By Glu, Cys 6 Replaced By Ala And Cys 111 Replaced By Ser (SOD1) is one of the main reactive oxygen species in the cell and serves a key antioxidant role (Li *et al.* 1999). The SOD1 protein was 2.3-fold up-regulated in the cancer cell lines compared to the normal cells.

- Leukocyte elastase inhibitor (SERPINB1) displays tumour suppressor activity in cancer (Noor *et al.* 2002) and was found to be 2.9-fold down-regulated in the cancer cell lines compared to the normal in this study.

Table 3.1.4**. Cancer vs. normal list**: Proteins identified from the comparison of cancerous (MCF-7+BT20) vs. normal (HMEC+MCF-10A) groups using MALDI ToF MS and/or LC-MS/MS. 2D-DIGE number refers to location of the identified protein on the gel shown in the Figure 3.1.2. '+' represents up-regulation and '-' represent down-regulation of protein in terms of fold change in the given comparison. (Supplementary data containing additional information of proteins identified: Appendix 4).

2D-DIGE No	Gene Symbol	Protein Name	Cancerous vs. Normal (Fold Change)	Biological function
611	GSN	Gelsolin precursor	-2.21	Cell structure
641	EIF4A3	Translation initiation factor 4A isoform 3	2.24	Protein translation
773	LMNA	Lamin-A/C	-4.54	Cell structure
789	LMNB1	Lamin B1	2.51	Cell structure
818	G6PD	Glucose-6-Phosphate Dehydrogenase (Deletion Variant) Complexed With Glucose-6-Phosphate	2.17	Monosaccharide Metabolism
829	LMNA	Lamin A protein	2.13	Cell structure
884	HSPA	Chain A, Heat-Shock 70kd Protein 42kd Atpase N-Terminal Domain	2.03	Carbohydrate and amino acid metabolism
958	ME3	Chain C, Crystal Structure Of A Human Malic Enzyme	2.22	Cell adhesion
996	STIP1	STIP1 protein	1.86	Stress response
998	CBS	Cystathionine beta-synthase	6.97	Amino acid biosynthesis
1076	TXNRD1	Thioredoxin reductase 1, cytoplasmic precursor	3.31	Electron transport
1079	ARCN1	Coatomer subunit delta	-2.59	Constitutive exocytosis
1105	DYNC1LI2	Cytoplasmic dynein 1 light intermediate chain 2	-2.28	Cell cycle and Cell structure
1146	KRT8	Keratin, type II cytoskeletal 8	5.52	Cell structure
1174	ACTC1	Cardiac muscle alpha actin proprotein	3.49	Cytokinesis and cell structure

1191	VIM	Vimentin	-32.09	Cell structure
1211	gil31874087	Hypothetical protein	-5.98	Unclassified
1221	KRT18	Keratin 18	3.44	Cell structure
1222	ACTB	ACTB protein	3.44	Actin and actin related protein
	HNRPF	Heterogeneous nuclear ribonucleoprotein F		mRNA splicing
1230	UQCRC1	Ubiquinol-cytochrome-c reductase complex core protein 1, mitochondrial precursor	8.18	Electron transport
1279	NDRG1	N-myc downstream-regulated gene 1 protein	-2.82	Cell proliferation and differentiation
1292	NUDC	Nuclear migration protein NudC	2.2	Cell proliferation and differentiation
1316	KRT19	Keratin, type I cytoskeletal 19	37.85	Cell structure
1341	SERPINB1	Leukocyte elastase inhibitor (LEI)	-2.94	Proteolysis
1374	BTRC	WD-40 repeat protein	2.28	Proteolysis, Intracellular signaling cascade
1397	SERPINB5	Serpin B5 precursor	-98.67	Proteolysis
1399	SERPINB5	Serpin B5 precursor	-20.14	Proteolysis
1405	CAPG	Macrophage-capping protein	-11.98	Cell proliferation and differentiation
1411	SERPINB5	Serpin B5 precursor	-7.28	Proteolysis
1468	ANXA1	Annexin I	-3.81	Cell motility
1469	ANXA1	Annexin I	-26.8	Cell motility
1488	TALDO1	Transaldolase	2.26	Pentose-phosphate shunt

1513	ANXA3	Annexin A3	-7.34	Lipid and fatty acid metabolism
1527	TALDO1	Transaldolase	2.77	Pentose-phosphate shunt
1552	QPRT	Quinolinate phosphoribosyl transferase	3.66	NAD pyrophosphorylase activity
1707	PSME1	Proteasome activator subunit 1 isoform 2	2.86	Proteolysis
1722	DCI	3,2-trans-enoyl-CoA isomerase, mitochondrial precursor	3.18	metabolism
1734	PSME1	Proteasome activator subunit 1 isoform 2	2.79	Proteolysis
1753	CTSD	Cathepsin D At Ph 7.5	-2.07	Proteolysis
1800	HSPB1	Heat-shock protein beta-1	2.03	Stress response
1971	SOD1	Chain J, Superoxide Dismutase Mutant With Lys 136 Replaced By Glu, Cys 6 Replaced By Ala And Cys 111 Replaced By Ser	2.34	Immunity and defense
2139	NME1	Nm23 protein	3.02	Pyrimidine metabolism
2477	LGALS1	Galectin-1	-2.94	Cell adhesion

Table 3.1.5. The list of cellular protein targets either not previously associated with breast cancer or poorly known, identified from the comparison of cancerous cell lines (MCF-7+BT20) vs. normal cells (HMEC+MCF-10A).

2D-DIGE No	**Gene Symbol**	**Protein Identification Name**	**Cancerous vs. Normal Fold change**
641	EIF4A3	Translation initiation factor 4A subunit 3	2.24
1230	UQCRC1	Ubiquinol-cytochrome-c reductase complex core protein 1, mitochondrial precursor	8.18
1341	SERPINB1	Leukocyte elastase inhibitor (LEI)	-2.94
1722	DCI	3,2-trans-enoyl-CoA isomerase, mitochondrial precursor	3.18
1971	SOD1	Chain J, Superoxide Dismutase Mutant With Lys 136 Replaced By Glu, Cys 6 Replaced By Ala And Cys 111 Replaced By Ser	2.34

3.1.5.2 Invasive (HMEC+BT20) vs. non-invasive (MCF-10A+MCF-7) (normal+cancer invasion related)

Table 3.1.6. outlines the 15 identified proteins in the N+C invasive (HMEC+BT20) vs. N+C non-invasive (MCF-10A+MCF-7) comparison group. Among these proteins, 8 proteins were up-regulated and 7 proteins were down-regulated in the invasive cell lines compared to the non-invasive cells. The cell lines are compared from N+C invasive to N+C non-invasive therefore proteins that are up-regulated in the N+C invasive cell lines compared to the N+C non-invasive cells are represented as positive fold changes. The proteins that are down-regulated have negative fold changes as these proteins are expressed at lower levels in the N+C invasive cell lines compared to the N+C non-invasive cells.

A number of proteins, that are already known to be involved in the invasion of cancer cells were identified in this investigation (Table 3.1.6.). These include-

- Keratin 8 (KRT8) and 18 (KRT18) both identified at one spot on the 2D gel were found to be 2.7-fold down-regulated in the N+C invasive cell lines compared to the N+C non-invasive cells KRT8 and KRT18, members of the intermediate filament family of proteins, have been observed co-expressed in many types of cancer and are associated with metastatic spread in breast cancer (Remold-O'Donnell 1985).

- Nm23 protein (NME1) expression has been shown to be correlated with invasion and its up-regulation is suggested to interfere with PKC signalling pathways (Schaller *et al.* 1999). NME1 was found 2.4-fold up-regulated in the N+C non-invasive cells compared to the N+C invasive cells, indicating it may have an invasion-suppressing property.

This comparison list also revealed a number of proteins that have not been investigated extensively to date for their involvement in normal+cancer related invasion including AKR1A1, RAD23B, CLIC1 and PSME1 (Table 3.1.6.).

- RAD23B, a protein involved in the nucleotide excision repair (NER) (Ouatas *et al.* 2003), was 2.0-fold up-regulated in the N+C non-invasive cells compared to the N+C invasive cells.

- Chloride intracellular channel 1 (CLIC1) is a member of the p64 family; the protein localizes principally to the cell nucleus and exhibits both nuclear and plasma membrane chloride ion channel activity (Watkins *et al.* 1993). CLIC1 was found 4.2-fold up-regulated in the N+C non-invasive cells compared to N+C invasive cells.

- Proteasome activator subunit 1 isoform 2 (PSME1) alpha subunit of 11 S regulator of proteasome (Chen *et al.* 2007) and was 3-fold up-regulated in the N+C non-invasive cells compared to N+C invasive cells.

Table 3.1.6. **Invasive vs. Non-invasive (Normal + cancer-related invasion)**: Proteins identified from the comparison of invasive (HMEC+ BT20) vs. non-invasive (MCF-10A + MCF-7) comparison group using MALDI ToF MS and/or LC-MS/MS. 2D-DIGE number refers to the location of the identified protein on the gel shown in the Figure 3.1.2. '+' represents up-regulation and '-' represent down-regulation of protein in terms of fold change in the given comparison. (Supplementary data containing additional information of proteins identified: Appendix 4).

2D-DIGE No	Gene Symbol	Protein Identification	Invasive vs. non-invasive (fold change)	Molecular function
907	KT18	Keratin, type I cytoskeletal 18	-2.71	
	KT8	Keratin, type II cytoskeletal 8		Cell structure
1019	PDIA3	Protein disulfide-isomeraseA3 precursor	4.03	Protein disulfide isomerase reaction
1050	G6PD	Glucose-6-phosphate 1-dehydrogenase (G6PD)	3.84	Monosaccharide Metabolism
	CCT-beta	T-complex protein 1 subunit beta		Protein folding
1098	3-PGDH	3-phosphoglycerate dehydrogenase	4.11	Amino acid biosynthesis
1105	DYNC1LI2	Cytoplasmic dynein 1 light intermediate chain 2	3.48	Cell cycle and Cell structure
1378	RAD23B	RAD23B protein	-2.07	DNA repair

1450	TSSC1	Tumor-suppressing subchromosomal transferable fragment candidate gene 1 protein	-3.05	Protein binding
1502	AKR1A1	Chain A, Apo R268a Human Aldose Reductase	5.27	Oxidoreductase activity
1732	PSME1	proteasome activator subunit 1 isoform 2	-3.07	Proteolysis
1743	CLIC1	Chloride intracellular channel protein 1	-4.24	Anion transport
1781	TPI1	Chain B, Triosephosphate Isomerase Of New Crystal Form	2.23	Glycolysis
1798	Hsp27	Heat shock protein 27	-2.69	Stress response
1800	HSPB1	Heat-shock protein beta-1	-2.5	Proteolysis
2139	NME1	Nm23 protein	-2.47	Pyrimidine metabolism
2424	CLP	Chain A, Three Crystal Structures Of Human Coactosin-Like Protein	3.36	Cell adhesion

3.1.5.3 Cancerous invasive (BT20) vs. Normal invasive+normal non-invasive+cancerous non-invasive (MCF-10A+HMEC+MCF-7) (Cancer-related invasion)

Table 3.1.7. outlines the 25 identified proteins in the **Group 1** (cancer invasion (BT20)) vs. **Group 2** (Normal invasive+normal non-invasive+cancerous non-invasive (HMEC+MCF-10A+MCF-7)) comparison. Among these proteins, 16 proteins were up-regulated and 9 proteins were down-regulated in group 1 compared group 2.

A number of proteins identified in this comparison have already been shown to be involved in the invasion of cancer cells. Therefore our results indicate that the proteins which are up or down-regulated in the cancer invasive cells (BT20) could possibly have potential roles in breast cancer invasion.

- Gelsolin is a calcium-binding protein which binds to and regulates actin filaments (Zhang *et al.* 2007). Gelsolin was observed to be 4.3-fold down-regulated in group 1 compared to group 2.

- Heterogeneous nuclear ribonucleoprotein (HNRPK) has been implicated in chromatin remodelling, transcription and translational process and can also interact with kinases (Kumar *et al.* 2004). HNRPK was 9.48-fold down-regulated in group 1 compared to the group 2.

This comparison also revealed a number of proteins, including TXNRD1, ERp29, 3-PGDH, CLP, QPRT and TALDO1 that have not been investigated extensively to date for their involvement in cancer-related invasion (Table 3.1.7.).

- Thioredoxin reductase 1 (TXNRD1) reduces thioredoxins as well as other substrates, and plays a role in selenium metabolism and protection against oxidative stress (Mandal *et al.* 2001). TXNRD1 was observed to be 4.6-fold up-regulated in group 1 compared to group 2.

- Endoplasmic reticulum protein 29 (ERp29), a molecular cheparone, was 10.4-fold down-regulated in group 1 compared to group 2.

- 3-phosphoglycerate dehydrogenase (3-PGDH) involved in amino acid biosynthesis, was 5.2-fold up-regulated in group 1 compared to group 2.

- Coactosin-Like protein (CLP), actin binding protein, was found 5.1-fold up-regulated in group 1 compared to group 2.

- Quinolinate phosphoribosyltransferase (QPRT) involved in *de novo* synthesis of nicotinamide adenine dinucleotide, was found 5.2-fold up-regulated in group 1 compared to group 2.

- Transaldolase 1, involved in the pentose-phosphate pathway, was found to be 2.7 and 4.1-fold up-regulated in group 1 compared to group 2. This protein was identified at two locations on the 2D gel.

Table 3.1.7. **Group 1 vs. Group 2 (Cancer-related invasion)**: Proteins identified from the **Group 1** (cancer invasive cell line (BT20)) **vs. Group 2** (Normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7)) using MALDI ToF MS and/or LC-MS/MS. 2D-DIGE number refers to location of the identified protein on the gel shown in the Figure 3.1.2. '+' represents up-regulation and '-' represent down-regulation of protein in terms of fold change in the given comparison. (Supplementary data containing additional information of proteins identified: Appendix 4).

2D-DIGE No	**Gene Symbol**	**Protein Identification**	**Group1/ Group 2 Fold change**	**Molecular function**
611	GSN	Gelsolin precursor	-4.35	Cell structure
897	HNRPK	Heterogeneous nuclear ribonucleoprotein K	1.57	mRNA splicing
	HSP60	60 kDa heat shock protein		Stress response
901	HNRPK	heterogeneous nuclear ribonucleoprotein K	9.48	mRNA splicing
	TUBA6	Tubulin alpha-1C chain		Cell structure
907	KT18	Keratin, type I cytoskeletal 18	3.01	Cell structure
	KT8	Keratin, type II cytoskeletal 8		Cell structure
998	CBS	Cystathionine beta-synthase	3.12	Amino acid biosynthesis
1019	PDIA3	Protein disulfide-isomeraseA3 precursor	3.38	Protein disulfide-isomerase reaction
1076	TXNRD1	Thioredoxin reductase 1, cytoplasmic precursor	4.6	Electron transport
1098	3-PGDH	3-phosphoglycerate dehydrogenase	5.2	Amino acid biosynthesis
1105	DYNC1LI2	Cytoplasmic dynein 1 light intermediate chain 2 (Dynein light intermediate chain 2, cytosolic)	-2.8	Cell cycle and Cell structure

1126	TBCE	Tubulin-specific chaperone E	2.88	Cell structure
1191	VIM	Vimentin	20.39	Cell structure
1279	NDRG1	N-myc downstream-regulated gene 1 protein	-3.49	Cell proliferation and differentiation
1405	CAPG	Chain A, Ca2+-Binding mimicry In The Crystal Structure Of The Eu3+-Bound Mutant Human Macrophage Capping Protein Cap G	-12.16	Cell proliferation and differentiation
1411	SERPINB5	Tumour Suppressing Serpin	-9.27	Proteolysis
1468	ANXA1	Annexin I	-3.3	Cell motility
1469	ANXA1	Annexin I	-20.73	Cell motility
1488	TALDO1	Transaldolase	2.7	Pentose-phosphate shunt
1502	AKR1A1	Chain A, Apo R268a Human Aldose Reductase	7.48	Oxidoreductase activity
1527	TALDO1	Transaldolase	4.12	Pentose-phosphate shunt
1552	QPRT	Qinolinate phosphoribosyl transferase	5.27	NAD pyrophosphorylase activity
1722	DCI	3,2-trans-enoyl-CoA isomerase, mitochondrial precursor	2.06	Metabolism
1752	ERP29	Endoplasmic reticulum protein ERp29 precursor	-10.45	Constitutive exocytosis
1781	TPI 1	Chain B, Human Triosephosphate Isomerase Of New Crystal Form	2.36	Glycolysis
1798	Hsp27	Heat shock protein 27	-2.72	Stress response
2424	CLP	Chain A, Three Crystal Structures Of Human Coactosin-Like Protein	5.1	Cell adhesion

3.1.6 Identification of invasion-related proteins by overlapping differentially expressed proteins from the normal cells (MCF-10A vs. HMEC) with the cancerous cell lines (MCF-7 vs. BT20)

The aim of this analysis was to identify proteins that are only differentially expressed in the invasive phenotypes or in the non-invasive phenotypes. Two lists of differentially expressed proteins between normal non-invasive vs. normal invasive cell lines (MCF-10A vs. HMEC) and cancerous non-invasive vs. cancer invasive cell lines (MCF-7 and BT20) were generated (supplementary data: Appendix 1). The two generated differentially expressed protein lists were overlapped to determine unique and common differentially expressed proteins between these lists. A Venn diagram outlining the distribution of proteins between the lists of differentially expressed proteins in a comparison of cancerous non-invasive (MCF-7) vs. invasive (BT20) cell lines and normal non-invasive (MCF-10A) vs. normal invasive (HMEC) cells is shown in figure 3.1.3.

The overlapping of these lists identified 4 unique proteins, including SERPINB5 and ANXA1, in the normal comparison group (MCF-10A vs. HMEC). SERPINB5 and ANXA1 were up-regulated in the normal-non-invasive cells (MCF-10A) in comparison to the normal-invasive cells (HMEC). This indicates that inhibition of SERPINB5 and ANAXA1 could be associated with the development of the invasive phenotype in HMEC cells.

27 proteins, including well known cancer and invasion-associated proteins (KRT8, KRT18 and NME1) as well as other proteins, which have not been linked with breast invasion (ERP29 and QPRT), were found unique in the cancer non-invasive vs. invasive (MCF-7 vs. BT20) comparison group. KRT8, KRT18 and QPRT were up-regulated whereas ERp29 was down-regulated in the cancerous invasive cells (BT20) in comparison to the cancerous non-invasive cells (MCF-7). Over expression of KRT8, KRT18 and QPRT, and inhibition of ERp29 in the cancerous-invasive cells (BT20) could contribute to the development of the invasive phenotype in BT20 cells.

A total of 10 proteins were observed common in both comparison groups indicating the proteins involved in normal and cancer invasion. Alterations in the expression of these proteins could contribute to the development of invasive phenotypes. Of these 10

proteins, 7 proteins showed similar expression patterns in both comparison groups, normal-non-invasive vs. normal-invasive and cancer-non-invasive vs. cancer-invasive, while 3 protein spots, TALDO1 and NDRG1 (NDRG1 identified at 2 spots), were differentially expressed with opposite expression patterns. For example, TALDO1 and NDRG1 were over expressed in the normal-noninvasive cells (MCF-10A) in comparison to normal-invasive cells (HMEC), while they were down-regulated in the cancerous-non-invasive cells (MCF-7) in comparison to the cancerous-invasive cells (BT20). This suggests that inhibition of TALDO1 and NDRG1 could be involved in the development of the invasive phenotype in normal cells whereas over expression could be associated with invasion in cancerous cells.

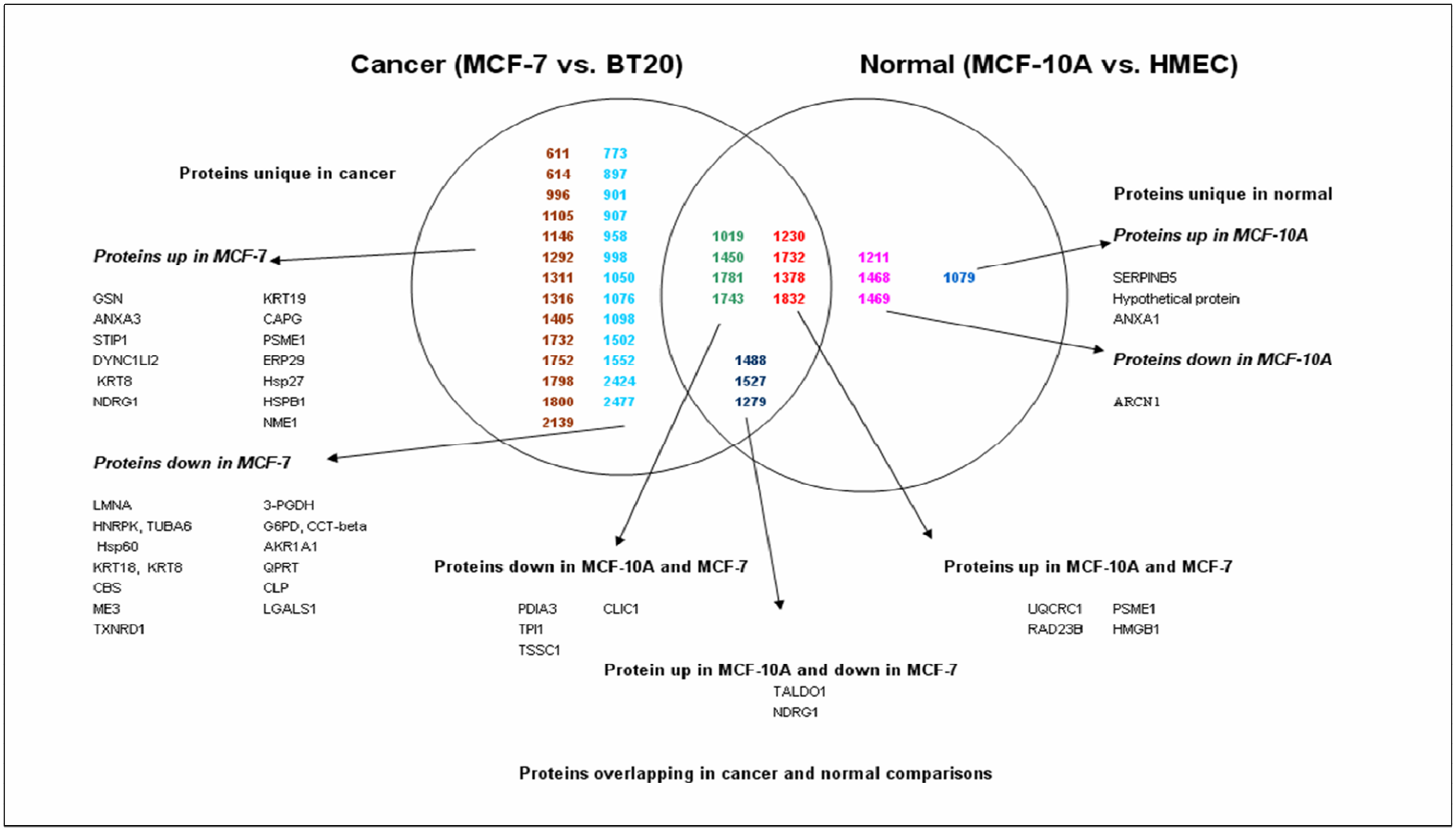


Figure 3.1.3. Venn diagram of differentially expressed overlapping proteins identified from the comparison of the normal non-invasive vs. invasive (MCF-10A vs. HMEC) cells, and the cancer non-invasive vs. invasive (MCF-7 vs. BT20). Proteins are represented by the 2D-DIGE number inside the circle and gene symbols outside the circle of protein.

3.1.7 Validation of proteomics data by Western blot analysis

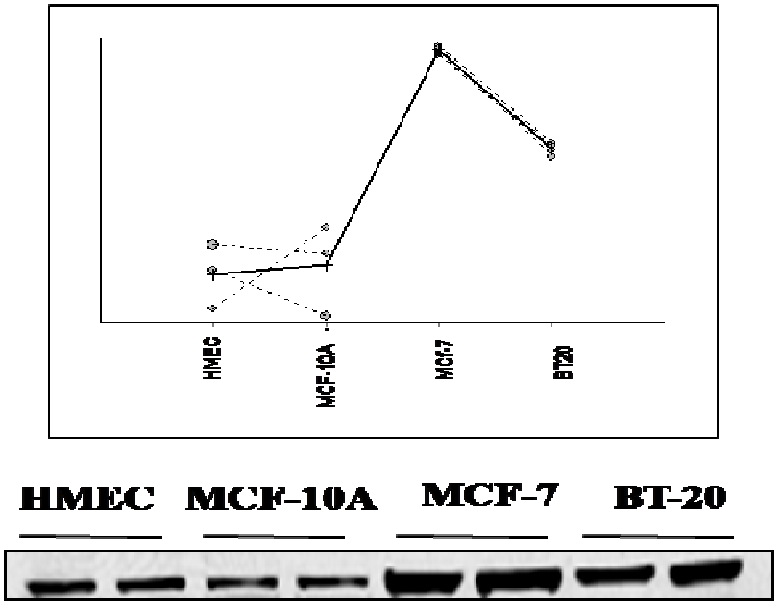
In order to validate the expression observed by 2D-DIGE analysis and identifications by MALDI-ToF and/or LC-MS/MS, Western blot analysis was carried out for selected target proteins (Keratin 8, Maspin (SERPINB5), vimentin, Nm23, transaldolase and STIP1) across the four cell lines (HMEC, MCF-10A, MCF-7 and BT20) as described in section 2.8.

Maspin (SERPINB5) was over expressed in the normal cells (HMEC+MCF-10A) compared to the cancerous cell lines (MCF-7+BT20), whereas transaldolase (TALDO1) was over expressed in the cancerous invasive cells (BT20) in comparison to normal non-invasive+normal invasive+cancerous-non-invasive cells (MCF-10A+HMEC+MCF-7) (Table 3.1.4. and 3.1.7.). This was in agreement with the published literature as SERPINB5 has been found down-regulated in breast cancer and TALDO1 was found increased in breast cancer tissues (Hirota *et al.* 2000b). Therefore, SERPINB5 and TALDO1 were selected as protein targets for Western blot analysis. Four other differentially expressed proteins, keratin 8, Nm23, vimentin and STIP1, were also considered for Western blotting since antibodies against them were available in the lab.

Biological duplicate samples from cell lysates were separated using 12% SDS gels and immunoblotted using appropriate antibodies (Section 2.8.2). Keratin 8, Maspin, vimentin, Nm23, transaldolase and STIP1 confirmed the trends observed in the proteomic analysis. Figures 3.1.4 to 3.1.6 show the expression pattern of these selected proteins observed using 2D-DIGE and Western blot analysis. Table 3.1.8 and 3.1.9 summarize the expression patterns observed in 2D-DIGE and western blot analysis for the targets. Two bands were detected by the antibody in Western blot analysis of Transaldolase-1 (Figure 3.1.7.1 A). The upper band confirmed the expression pattern observed at 2D-DIGE No. 1488 whereas and lower band followed the expression pattern observed at 2D-DIGE No. 1527 (Table 3.1.4).

Western blot analysis for GAPDH and BIP was also carried out as an internal loading control but both proteins were found differentially expressed across the four cell lines (Figure 3.1.7 A). Therefore commassie stained gels was used to demonstrate as best as possible equal loading between the samples (Figure 3.1.7 B).

Keratin 8



Maspin

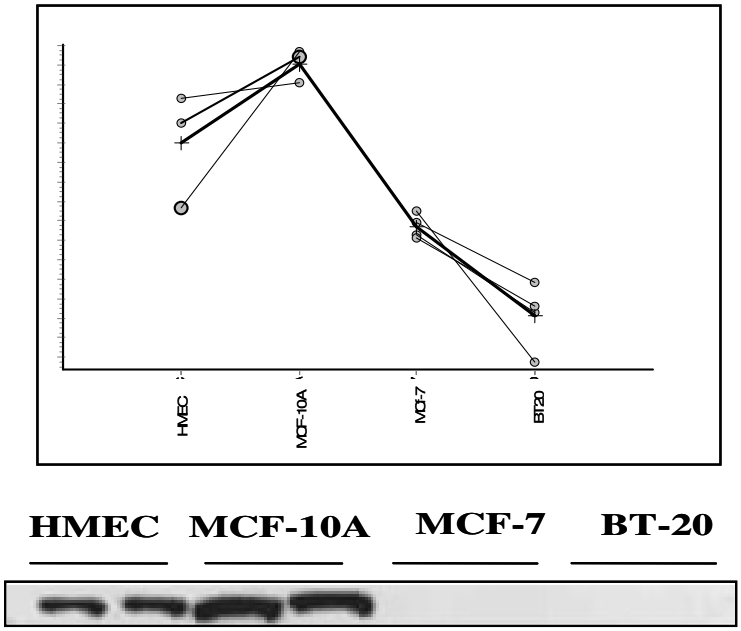
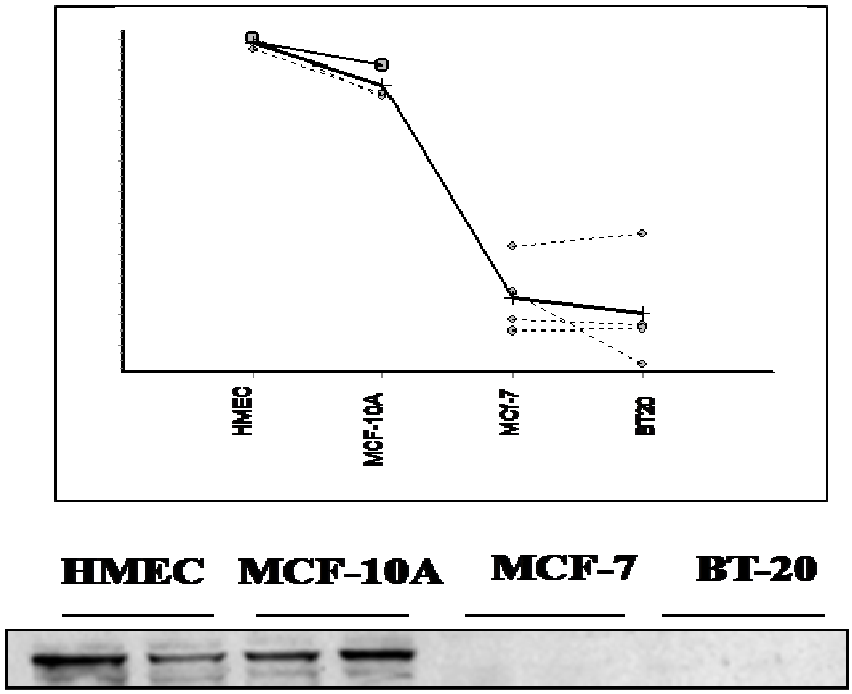


Figure 3.1.4 Standardized log abundance graph view (Decyder BVA module) and Western blot images of Keratin 8 and Maspin protein expression from HMEC, MCF-10A, MCF-7 and BT20. Biological duplicate samples were used in this investigation.

Vimentin



Nm23

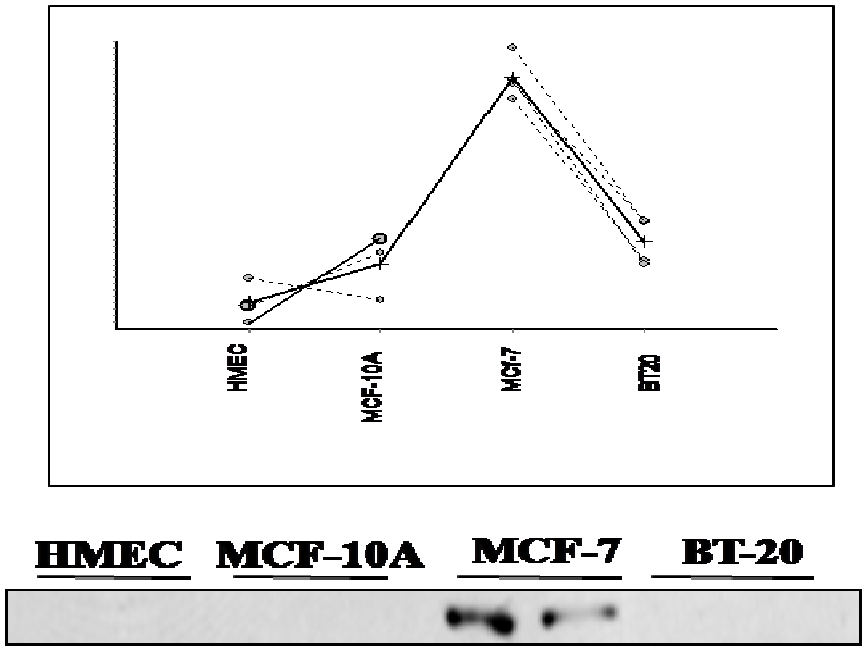
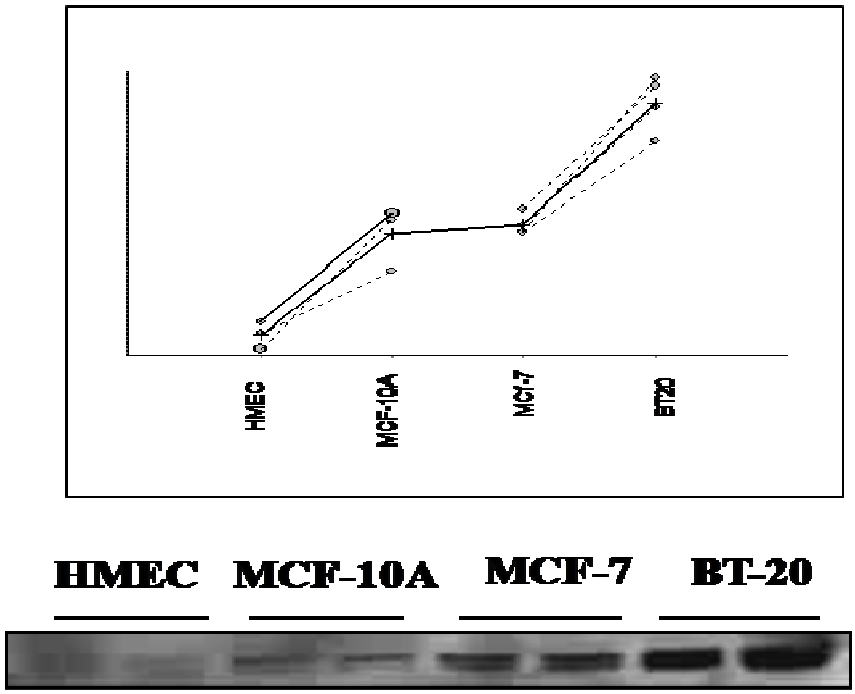


Figure 3.1.5 Standardized log abundance graph view (Decyder BVA module) and Western blot images of vimentin and Nm23 protein expression from HMEC, MCF-10A, MCF-7 and BT20. Biological duplicate samples were used in this investigation.

Transaldolase-1



STIP1

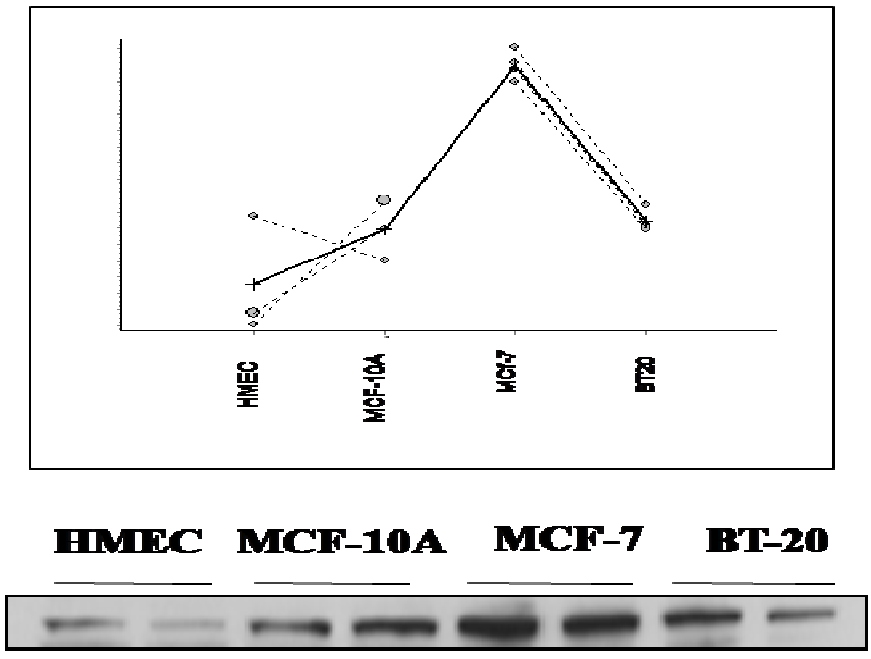


Figure 3.1.6 Standardized log abundance graph view (Decyder BVA module) and Western blot images of Transaldolase-1 and STIP-1 protein expression from HMEC, MCF-10A, MCF-7 and BT20. Biological duplicate samples were used in this investigation.

The differential expression of proteins observed in 2D-DIGE and results observed in western blot analysis has been summarised in Table 3.1.8 and 3.1.9.

Table 3.1.8. Expression pattern of keratin 8, Maspin and Vimentin observed in both 2D-DIGE and WB analysis.

Protein name	**2D-DIGE expression pattern (cancer vs. normal)**	**WB expression pattern (cancer vs. normal)**
Keratin 8	↑	↑
Maspin	↓	↓
Vimentin	↓	↓

Table 3.1.9. Expression patterns of Nm23, Transaldolase-1 and STIP-1 observed in 2D-DIGE and WB analysis. (↑) up-regulated, (↓) down-regulated, (NDE) not significantly differentially expressed.

Protein name	**Analysis**	**MCF-10A vs. MCF-7**	**MCF-7 vs. BT20**	**HMEC vs. BT20**	**MCF-10A vs. HMEC**
Nm23	2D-DIGE	↓	↑	NDE	NDE
	WB	↓	↑	NDE	NDE
Transaldolase-1	2D-DIGE	↓	↓	↓	NDE
	WB	↓	↓	↓	NDE
STIP-1	2D-DIGE	↓	↑	NDE	NDE
	WB	↓	↑	NDE	NDE

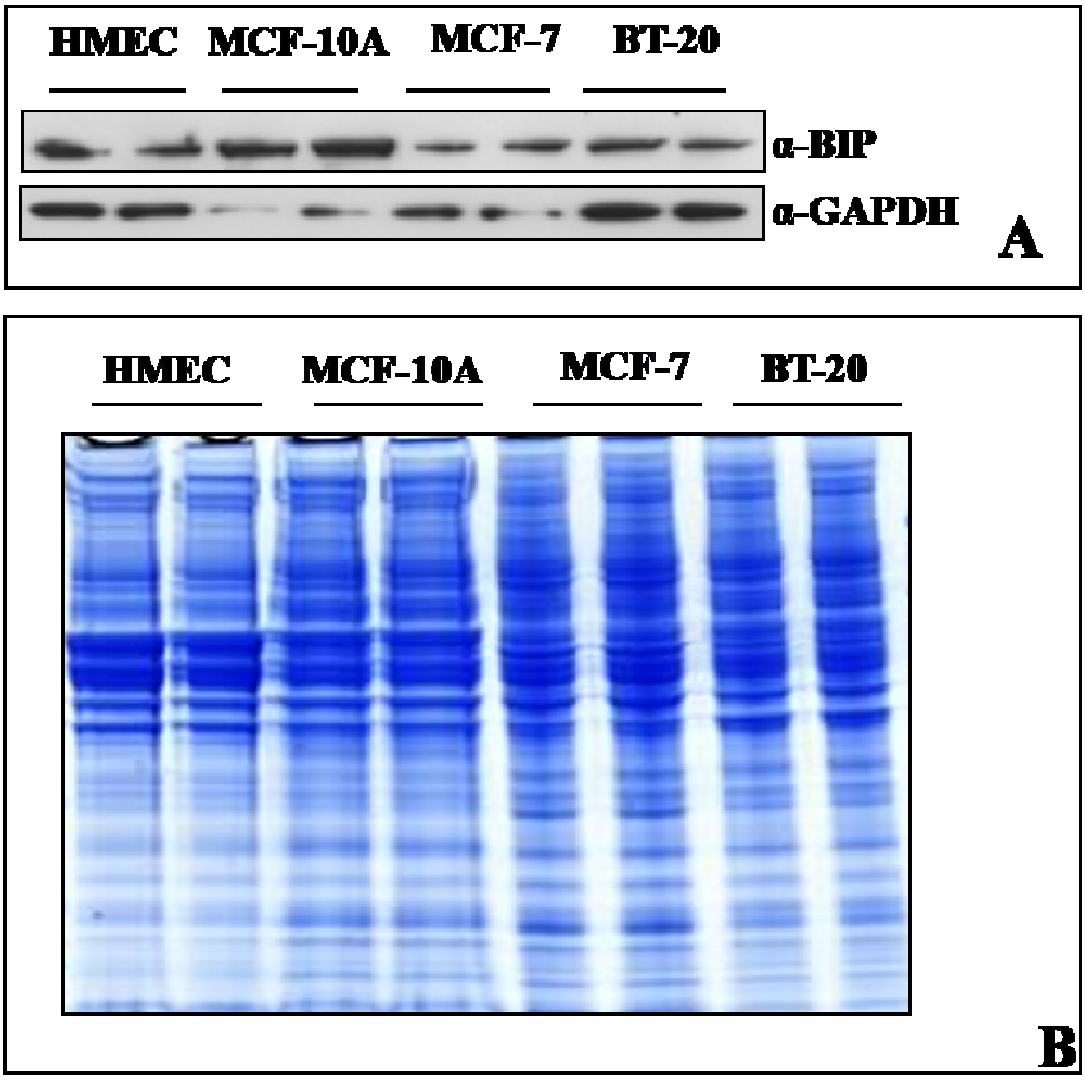


Figure 3.1.7 **A** represents Western blot images for the expression of BIP and GAPDH proteins in cell lysate sample from HMEC, MCF-10A, MCF-7 and BT20. Biological duplicate samples were used in this investigation. **B** represents coomassie stained gel of cell lysates samples from HMEC, MCF-10A, MCF-7 and BT20 to demonstrate as best as possible even loading between the samples.

3.1.8 Screening of potential invasion-specific protein targets for functional analysis

To investigate the role of proteins identified in the process of invasion, a few potential protein targets were chosen based on the following criteria for further screening for their possible involvement in the regulation of the invasive phenotypes in breast cells using Western blot analysis.

1. Proteins that have approximately similar expression patterns between normal and non-invasive cells (HMEC, MCF-10A and MCF-7) and are highly up-regulated or down-regulated in the cancerous invasive cell line (BT20) (Table 3.1.7). This criteria enables us to identify cancer invasion-related targets (Figure 3.1.8 A)
2. Proteins that have approximately similar expression patterns between the non-invasive cells (MCF-10A and MCF-7) and the invasive cells (HMEC and BT20) and are highly up-regulated or down-regulated in the invasive or non-invasive cell lines (Table 3.1.6). This analysis enables us to identify protein associated with invasion in both normal as well as cancer cells (Normal + Cancer- related invasion) (Figure 3.1.8 B).
3. All biological replicates are tightly clustered together statistically in their expression pattern from the analysis.
4. From the literature, we chose proteins which either have not been investigated previously in this context or for which only limited information is available regarding their potential role in cancer and/or invasion.

The following proteins have been found to pass the above selection criteria and therefore were selected for further western blot analysis in a panel of non-invasive and invasive breast cell lines in order to narrow down the protein list for functional validation using the siRNA knockdown technique. The selected protein targets were divided into two groups.

3.1.8.1 Cancer-related invasion (BT20 vs. HMEC+MCF-10A+MCF-7)

Six protein targets, thioredoxin reductase 1 (TXNRD1), 3-phosphoglycerate dehydrogenase (3-PGDH), endoplasmic reticulum protein 29 precursor (ERp29), transaldolase (TALDO1), coactosin-like protein (CLP) and quinolinate phosphoribosyltransferase (QPRT), were selected in this comparison group (Figure 3.1.8 A). TXNRD1, 3-PGDH, TALDO1, CLP and QPRT were observed to be up-regulated and ERp29 was found to be down-regulated in the cancerous invasive cell line (BT20) in comparison to the normal invasive+normal non-invasive+cancerous non-invasive cells (HMEC+MCF-10A+MCF-7). Investigation of these proteins in a panel of invasive and non-invasive breast cancer cell lines could further aid the selection of potential targets for cancer-related invasion in breast cancer.

3.1.8.2 Normal +Cancer- related invasion (HMEC+BT20 vs. MCF-10A+MCF-7)

Three proteins, proteasome activator subunit 1 isoform 2 (PSME1), nuclear chloride channel 1 (CLIC1) and RAD23B, were selected from this comparison group (HMEC+BT20 vs. MCF-10A+MCF-7) since these proteins were specifically down-regulated in the normal-invasive and cancer-invasive cells (HMEC+BT20) in comparison to the normal-non-invasive and cancer-non-invasive cells (MCF-10A+MCF-7) (Figure 3.1.8.B). Analysis of the expression of these protein targets in a panel of invasive and non-invasive breast cancer cell lines could help to improve the efficiency of potential target selection for normal + cancer- related invasion in breast cells.

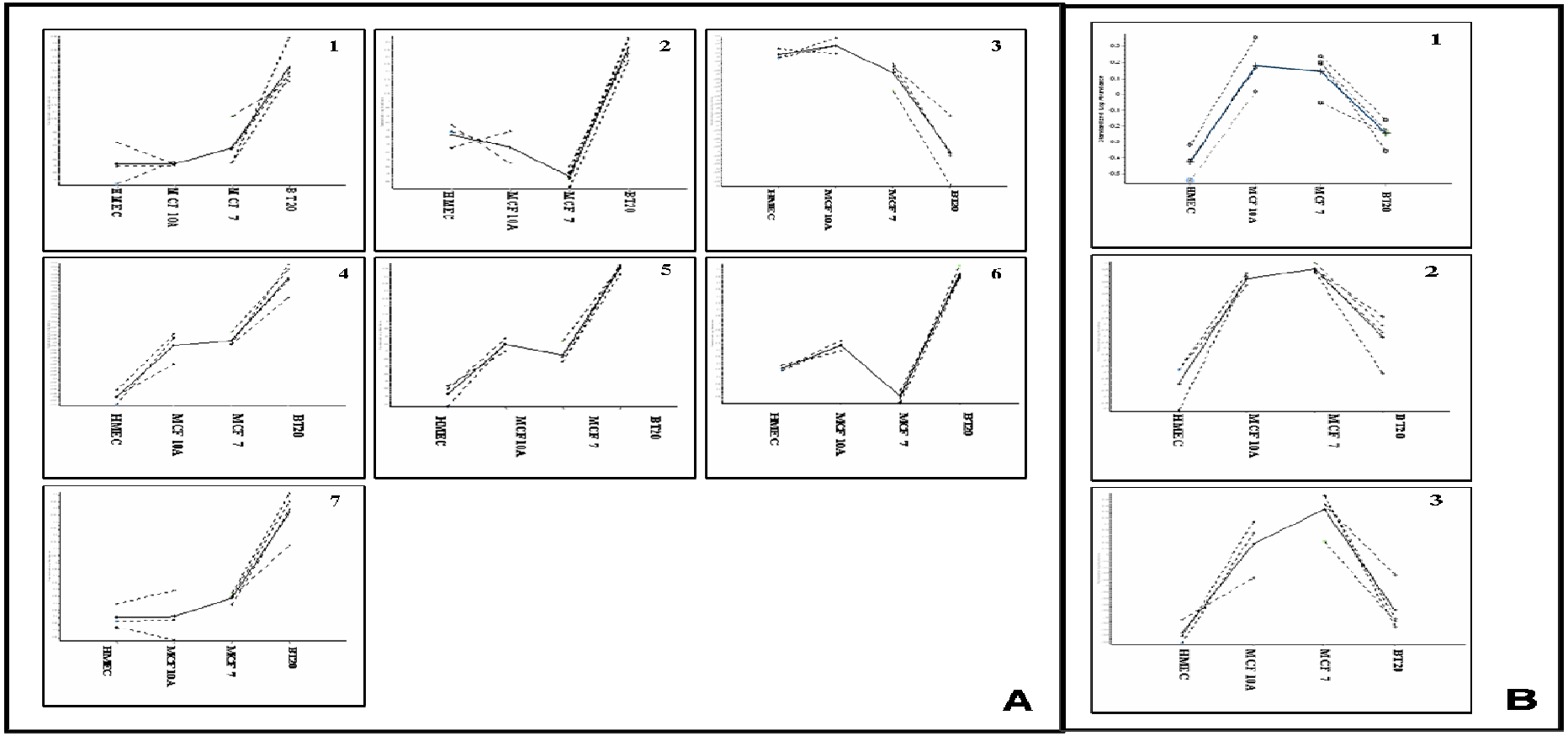


Figure 3.1.8. Standardised log abundance for each of the selected protein targets from 2D-DIGE analysis.(A) **Cancer- related invasion** (1), TXNRD1, (2) 3-PGDH, (3) ERp29, (4) TALDO1, (5) TALDO1 (6) CLP and (7) QPART. (B) **Normal + Cancer- related invasion** (1) PSME1 (2) CLIC1 and (3) RAD23B.

3.1.8.3 Invasion assay

A panel of breast cell lines including MCF-10A, MCF-7, T47D, UACC 812, HMEC, SKBR-3, MDA-MB 231, HCC 1937, BT20 and Hs578T were chosen on the basis of availability at the NICB culture collection. These cell lines covered a broad range of origin (basal, luminal and normal-like) and invasion status (low, medium and highly invasive) and therefore represent heterogenity of samples which often occurs in *in-vivo* models (Table 3.1.10). Thus it provided an appropriate model to identify real targets associated with invasion. The invasion assays, as described in section 2.6, were performed to confirm the invasion status of each cell line as outlined in the literature. Table 3.1.10. summarises the invasion status of the cell lines observed in the 48hrs invasion assay and as described in the literature. The invasive capabilities of normal mammary epithelial cells (HMEC), non-tumorigenic immortalized mammary epithelial (MCF-10A) and breast carcinoma cell lines (MCF-7, BT20, HCC1937, MDA-MB-231, Hs578T, T-47D, UACC-812 and SKBR-3) were measured using pre-coated matrigel invasion chambers over 48hrs of culture. The graph was plotted on the basis of the total number of cells that crossed the matrigel membrane (Figure 3.1.9 B). The results indicated that the MCF-10A, MCF-7, T-47D and UACC-812 cells were non-invasive, and HMEC, SKBR-3, BT20, HCC1937, MDA-MB-231 and Hs578T were invasive (Figure 3.1.9). HMEC and SKBR-3 were low invasive and HCC1937, MDA-MB-231 and BT20 were more invasive than HMEC and SKBR-3. Hs578T cells were found highly invasive in this assay.

Table 3.1.10. Summarised Invasion status of cell lines observed in 48hr invasion assay and described in the literature.

Cell line	**Cell Type**	**Phenotype**	**Invasion assay**	**Information from literature**
MCF-10A	Basal	Normal-like	Non-invasive	Non-invasive (Jung *et al.* 2007, Streuli 2002)
MCF-7	Luminal	Cancerous	Non-invasive	Non-invasive (Nagaraja *et al.* 2006)
T47D	Luminal	Cancerous	Non-invasive	Non-invasive (Nagaraja *et al.* 2006)
UACC-812	Luminal	Cancerous	Non-invasive	Non-invasive (Tong *et al.* 1999)
SKBR-3	Luminal	Cancerous	Low invasive	Low Invasive (Tong *et al.* 1999)
HMEC	Basal	Normal	Low invasive	Invasive (Feltes *et al.* 2002)
MDA-MB 231	Basal	Cancerous	Invasive	Invasive (Tong *et al.* 1999)
HCC-1937	Basal	Cancerous	Invasive	Invasive (Nagaraja *et al.* 2006)
BT20	Basal	Cancerous	Invasive	Invasive (Tong *et al.* 1999)
Hs578T	Basal	Cancerous	Invasive	Invasive (Tong *et al.* 1999)

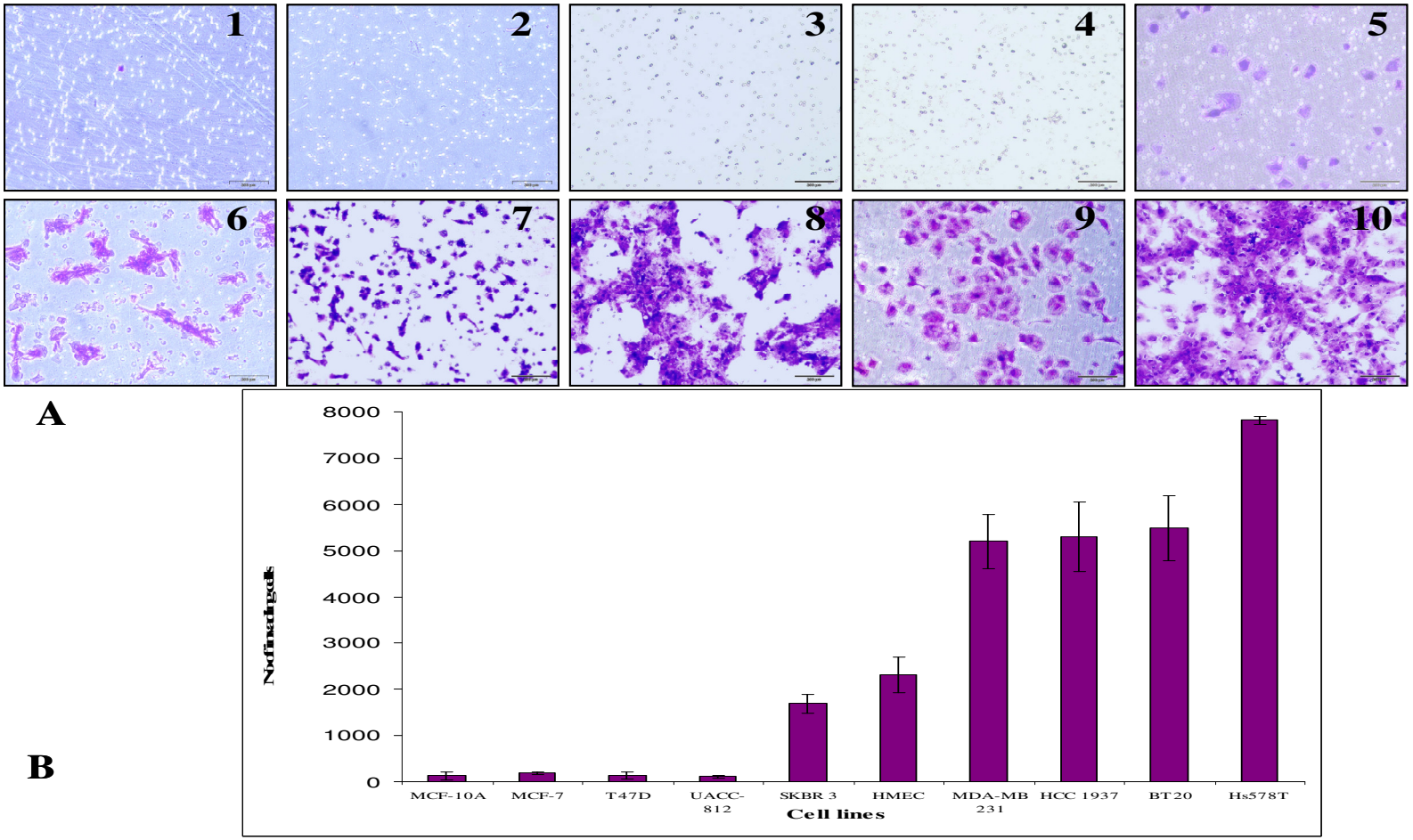


Figure 3.1.9. Investigation of the invasion capabilities of cells from breast origin. **(A)** Images from invasion assay- (1) MCF-10A, (2) MCF-7, (3) T47D, (4) UACC-812, (5) SKBR-3, (6) HMEC, (7) MDA-MB 231, (8) HCC-1937, (9) BT20 and (10) Hs578T. **(B)** Graphical representation of comparative levels of invasion in the cells tested. Magnification= 200x, Scale bar=200µm. Error bars represent standard deviation calculated from data obtained from three biological samples.

3.1.8.4 Western blot analysis

Western blot analysis was carried out to investigate the expression pattern for TXNRD1, 3-PGDH, ERp29, TALDO1, CLP, QPART, PSME1, CLIC1 and RAD23B targets in a panel of normal, non-invasive and invasive cell lines (MCF-10A, MCF-7, T47D, UACC-812, SKBR-3, HMEC, MDA-MB 231, HCC-1937, BT20 and Hs578T) (Figure 3.1.10 A).

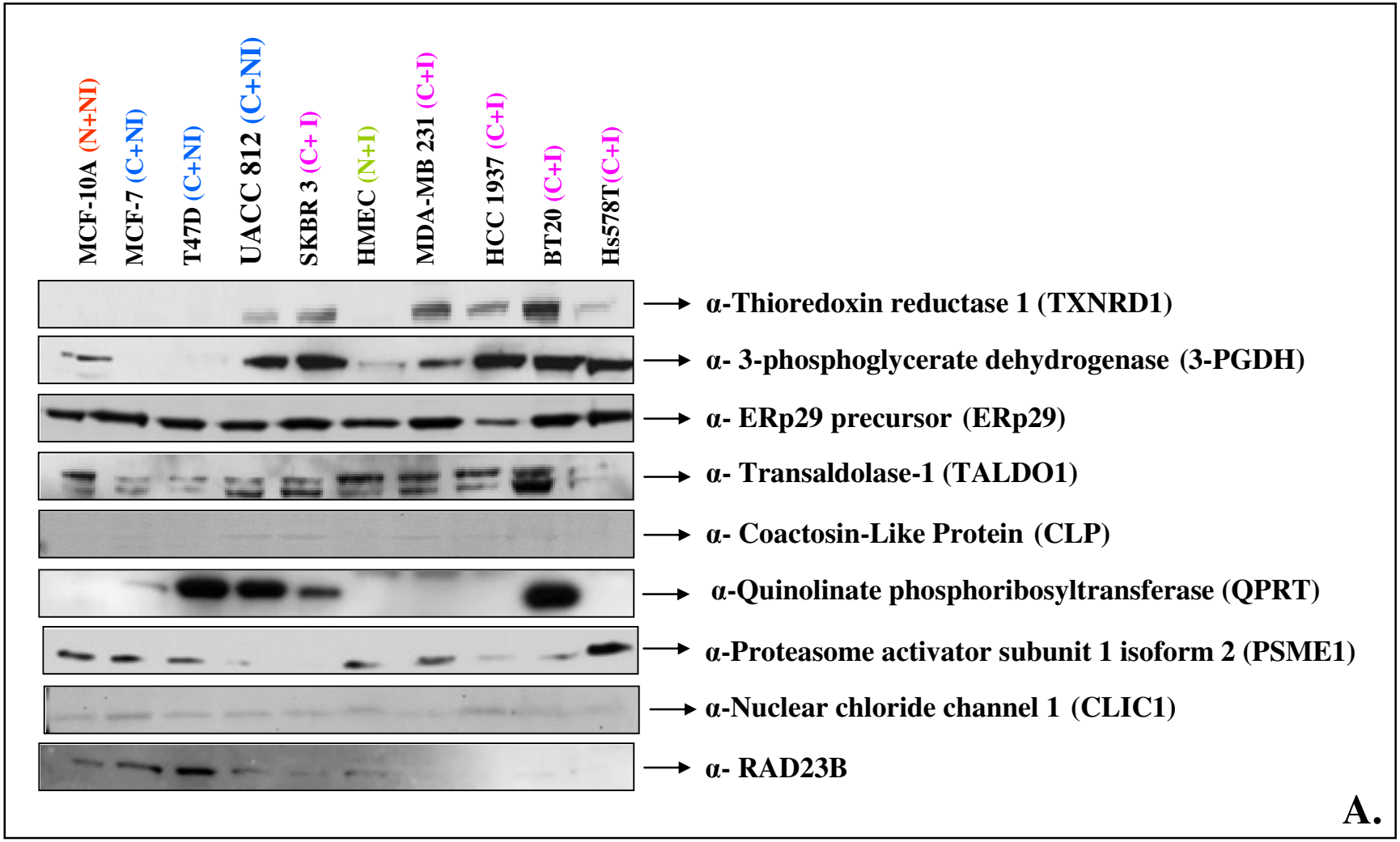
The western blot data of the nine target proteins across the four cell lines used in the 2D-DIGE analysis (HMEC, MCF-10A, MCF-7 and BT20) were matched with the results from the biological variant analysis (BVA module) of DeCyder (3.1.6 and 3.1.7). All of the proteins confirmed the trends observed in the proteomic analysis except ERp29 (Figure 3.1.10 A and Table 3.1.7). The expression pattern of ERp29 did not fully correspond to the trend of expression identified in the proteomic analysis of the cell lines. This discrepancy could possibly be due to the presence of other isoforms caused by post-translational modifications of the protein. Additionally this protein could also be present in other spots in the gel which we did not identify. Table 3.1.11 summarises the expression pattern of the nine proteins including TXNRD1, 3-PGDH, ERp29, TALDO1, CLP, QPART, PSME1, CLIC1 and RAD23B observed in 2D-DIGE of the 4 four cell lines (MCF-10A, HMEC, MCF-7 and BT20) and western blot analysis, again demonstrating the validation of the 2D-DIGE results.

Figure 3.1.10 A shows the images from the western blot analyses of the nine proteins, TXNRD1, 3 PGDH, ERp29, TALDO1, CLP, QPART, PSME1, CLIC1 and RAD23B from the ten cell lines (MCF-10A, MCF-7, T47D, UACC-812, SKBR-3, HMEC, MDA-MB 231, HCC-1937, BT20 and Hs578T). Coomassie stained gels was used to demonstrate as best as possible equal loading between the samples (Figure 3.1.10 B).

Table 3.1.11. Representation of the expression pattern of the nine proteins, including TXNRD1, 3-PGDH, ERp29, TALDO1, CLP, QPART, PSME1, CLIC1 and RAD23B, observed from 2D-DIGE analysis of the 4 four cell lines (MCF-10A, HMEC, MCF-7 and BT20) and WB analysis. (↑) up-regulated, (↓) down-regulated, (NDE) not significantly differentially expressed.

Protein name	**Analysis**	**MCF-10A vs. MCF-7**	**MCF-7 vs. BT20**	**MCF-10A+MCF-7 vs. HMEC+BT20**	**HMEC vs. BT20**	**MCF-10A vs. HMEC**
TXNRD1	2D-DIGE	NDE	↓	NDE	↓	NDE
	WB	NDE	↓	NDE	↓	NDE
3-PGDH	2D-DIGE	↑	↓	↓	↓	NDE
	WB	↑	↓	↓	↓	NDE
ERp29	2D-DIGE	↑	↑	NDE	↑	NDE
	WB	↓	NDE	NDE	NDE	NDE
TALDO1 (upper band)	2D-DIGE	↓	↓	NDE	↓	NDE
	WB	↑	↓	NDE	↓	NDE
TALDO1 (lower band)	2D-DIGE	NDE	↓	NDE	↓	↑
	WB	NDE	↓	NDE	↓	↑
CLP	2D-DIGE	NDE	↑	↑	NDE	↑
	WB	NDE	↑	↑	NDE	↑
QPART	2D-DIGE	NDE	↓	NDE	↓	NDE
	WB	NDE	↓	NDE	↓	NDE
PSME1	2D-DIGE	NDE	↑	↑	NDE	↑
	WB	NDE	↑	↑	NDE	↑

CLIC1	2D-DIGE	↑	↓	↓	↓	NDE
	WB	↑	↓	↓	↓	NDE
RAD23B	2D-DIGE	NDE	↑	↑	NDE	NDE
	WB	NDE	↑	↑	NDE	NDE


MCF-10A (N+NI)
MCF-7 (C+NI)
T47D (C+NI)
UACC 812 (C+NI)
SKBR 3 (C+ I)
HMEC (N+I)
MDA-MB 231 (C+I)
HCC 1937 (C+I)
BT20 (C+I)
Hs578T(C+I)
α-Thioredoxin reductase 1 (TXNRD1)
α- 3-phosphoglycerate dehydrogenase (3-PGDH)
α- ERp29 precursor (ERp29)
α- Transaldolase-1 (TALDO1)
α- Coactosin-Like Protein (CLP)
α-Quinolinate phosphoribosyltransferase (QPRT)
α-Proteasome activator subunit 1 isoform 2 (PSME1)
α-Nuclear chloride channel 1 (CLIC1)
α- RAD23B
A.

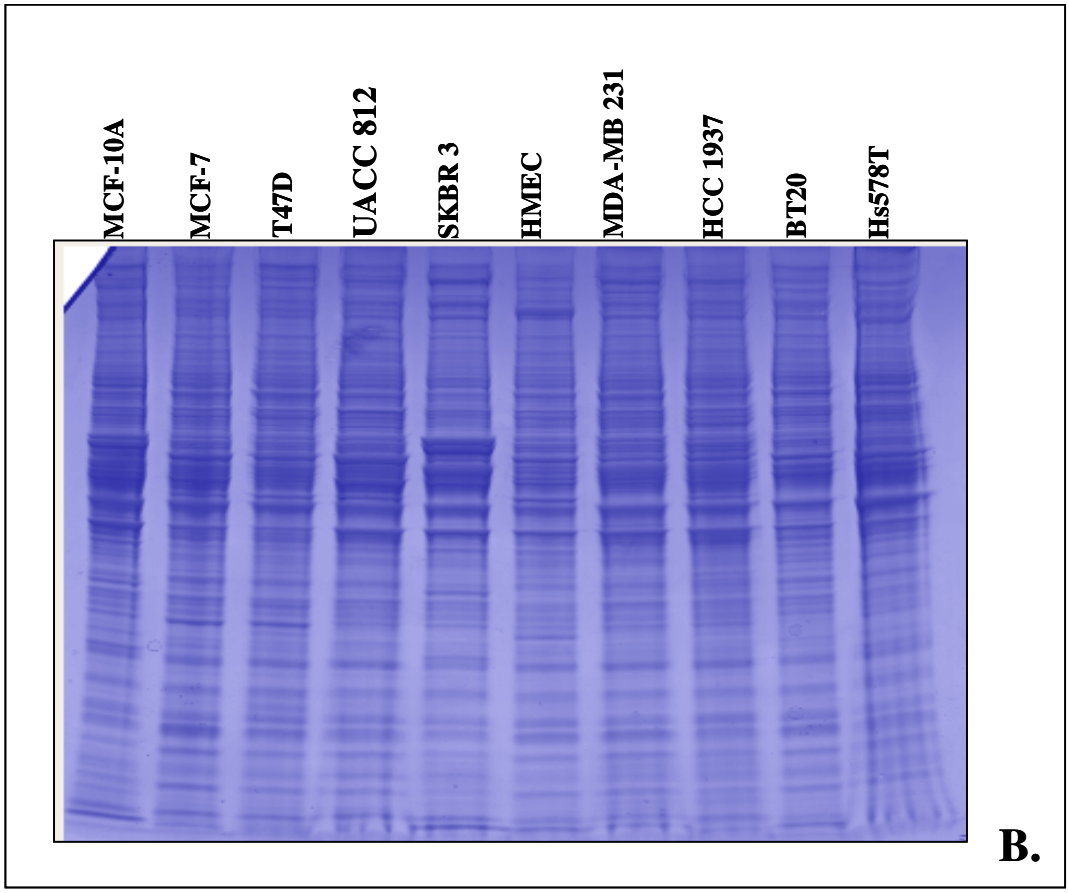


Figure 3.1.10. Analysis of the expression of the selected protein targets in a panel of breast cancer cell lines including non-invasive (MCF-10A, MCF-7, T47D and UACC 812), low invasive (HMEC and SKBR-3) and invasive (MDA-MB 231, HCC 1937, BT20 and Hs578T) using Western blot analysis. (**A)** represents Western blot images of PSME1, TXNRD1, CLIC1, 3-PGDH, ERp29, TALDO1, CLP, QAPRT and RAD23B in the cell lysate samples. Western blot images are representative of two biological samples**. (N+NI)** represents normal non-invasive, **(C+NI)** cancer non invasive, **(N+I)** normal invasive, **(C+I)** cancer invasive. (**B)** represents coomassie stained gel of cell lysate samples demonstrating as best as possible even loading between the samples.

After western blot analysis of 2 biological samples from each cell line, densitometry was carried out to calculate the intensity of the expressed protein. Average relative intensities were used to plot graphs from the two groups as mentioned below.

3.1.8.4.1 Cancer invasion- related targets

- TXNRD1 was found to be expressed in all cancerous invasive cell lines (SKBR-3, MDA-MB 231, HCC 1937, BT20 and Hs578T), whereas it was absent in the normal non-invasive and invasive cell lines (HMEC and MCF-10A) and in the cancerous non-invasive cell lines (MCF-7 and T47D), except UACC 812 (Figure 3.1.10A and 3.1.11).

- 3-PGDH was found to be expressed in all cancerous invasive cell lines (SKBR-3, MDA-MB 231, HCC 1937, BT20 and Hs578T) and was absent in the cancerous non-invasive cell lines (MCF-7 and T47D), except UACC 812 (Figure 3.1.10A and 3.1.11).. This protein was present at low levels in the normal non-invasive and invasive cell lines (HMEC and MCF-10A) compared to the cancer invasive cell lines.

- ERp29 was found to have similar levels of expression in the normal non-invasive (MCF-10A), normal invasive (HMEC), cancerous non-invasive (MCF-7, T47D and UACC 812) and cancerous invasive cell lines (SKBR-3, MDA-MB 231, HCC 1937, BT20 and Hs578T) (Figure 3.1.10A and 3.1.11A).
- TALDO1 showed a mixed expression pattern among normal, cancer non-invasive and cancer non-invasive cell lines. It was higly expressed in one cancerous non-invasive (UACC-812) and four cancerous invasive (SKBR-3, MDA-MB 231, HCC 1937 and BT20) cell lines. TALDO1 was expressed at low levels in one cancer invasive (Hs578T), two cancerous non-invasive cell lines (MCF-7 and T47D), normal invasive (HMEC), and normal non-invasive (MCF-10A) cell lines (Figure 3.1.10A and 3.1.11A).

- QPRT was found highly expressed in two cancerous non-invasive cell lines (T47D and UACC 812) and was expressed at low levels in one cancerous non-invasive cell line (MCF-7) (Figure 3.1.10A and 3.1.11A). This protein was absent in normal non-invasive and invasive cell lines (HMEC and MCF-10A). This protein was also found to be expressed in two cancer invasive cell line (SKBR-3 and BT20).

- Low levels of expression of CLP were observed in the normal non-invasive (MCF-10A), normal invasive (HMEC), and cancerous non-invasive cells (MCF-7 and T47D), except UACC 812 which has a slightly higher level of expression (Figure 3.1.10.A and 3.1.11A). Higher levels of CLP were found in the cancer invasive cell lines (MDA-MB 231, HCC 1937 and BT20) but not in Hs578T.

Out of the six proteins (TXNRD1, 3-PGDH, ERp29, TALDO1, CLP and QPRT) analysed in this comparison, only TXNRD1 and 3-PGDH were up-regulated in the cancerous invasive cell lines compared to the normal invasive+ normal non-invasive+ cancerous non-invasive cell lines, except UACC-812, as shown in figure 3.1.11B with the coloured lines. However, the other four proteins showed no real expression trends between the normal invasive+ normal non-invasive+ cancerous non-invasive vs. invasive cell lines. Therefore, ERp29, transaldose-1, QPRT and CLP were not observed to be associated with the invasion phenotype in breast cells in this investigation and were not selected for further siRNA studies. The proteins, TXNRD1 and 3-PGDH, were highly expressed in the invasive cells and therefore were selected for siRNA knockdown studies to investigate their potential involvement in the invasion process in breast cells (Figure 3.1.11B).

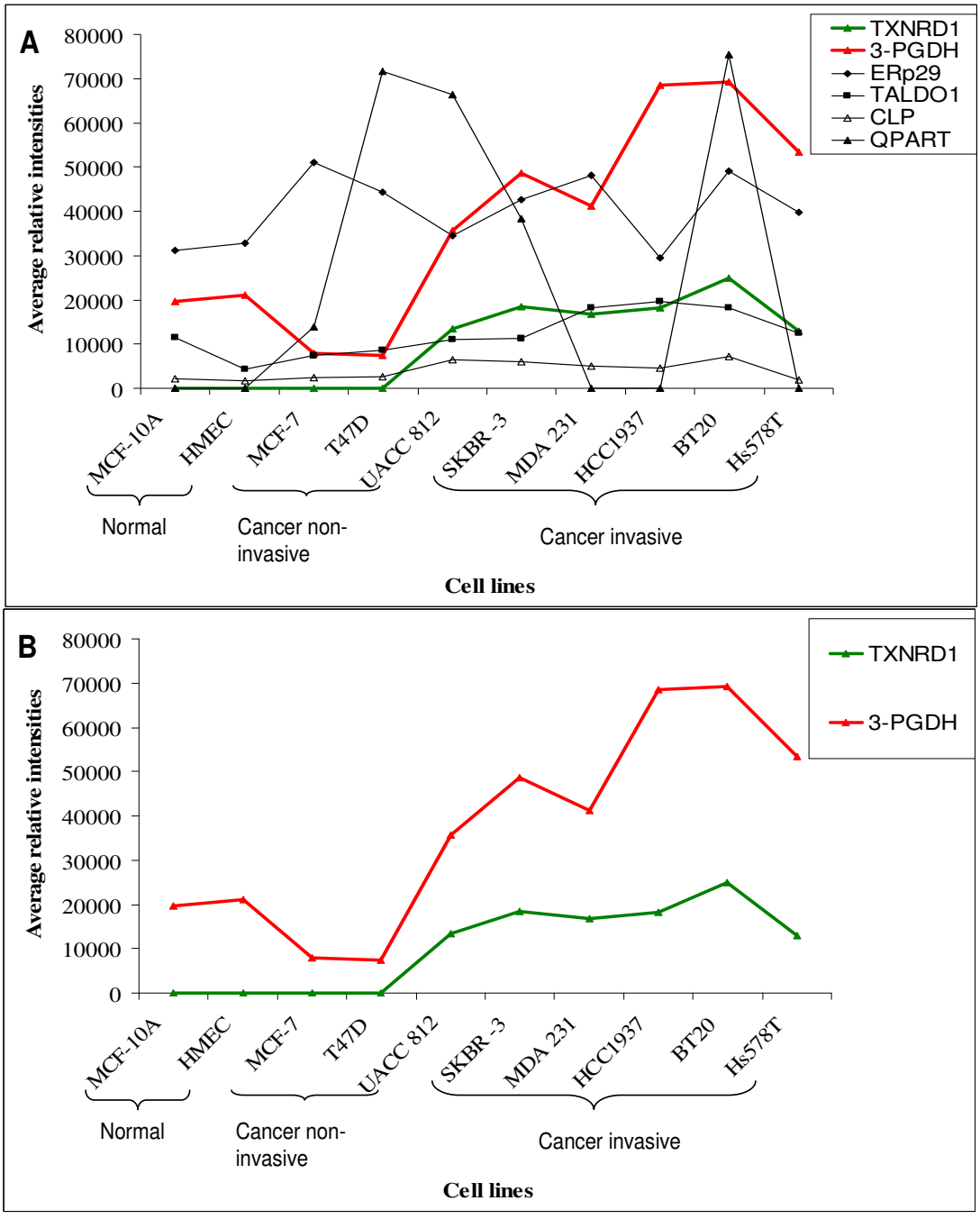


Figure 3.1.11. Graphical representation of average relative intensities calculated densitometry data from Western blotting analysis as shown in Figure 3.1.10 of selected cancer-invasion-related protein targets observed in a panel of cell lines. Average relative intensities represent the relative intensities obtained from 2 biological replicate samples. (A) TXNRD1, 3-PGDH, ERp29, TALDO1, TALDO1, CLP and QPART. (B) TXNRD1 and 3-PGDH

3.1.8.4.2 Normal + Cancer invasion-related targets

- PSME1 was found to be over expressed in three cell lines (MCF-10A, MCF-7 and T47D) but was expressed at low levels in one non-invasive cell line (UACC 812) (Figure 3.1.10A and 3.1.12A). In the invasive cells lines, it was also over expressed in 3 cell lines (HMEC, MDA-MB 231 and Hs578T), expressed at low levels in the remaining 2 invasive cell lines (HCC 1937 and BT20) and absent in the invasive cell line, SKBR-3.

- CLIC1 was found to have similar levels of expression in four non-invasive cell lines (MCF-10A, MCF-7, T47D and UACC 812) and two invasive cell lines (MDA-MB 231 and HCC 1937) whereas it was expressed at low levels in 3 invasive cell lines (HMEC, BT20 and Hs578T) (Figure 3.1.10A and 3.1.12A).

- RAD23B was found to be up-regulated in the normal + cancer non-invasive cell lines (MCF-10A, MCF-7, T47D and UACC 812) compared to the normal + cancer invasive cell lines (SKBR-3, HMEC, MDA-MB 231, HCC 1937, BT20 and Hs578T) (Figure 3.1.10A and 3.1.12A).

Of the three proteins (PSME1, CLIC1 and RAD23B), only RAD23B was highly expressed in the normal + cancerous non-invasive cell lines compared to normal + cancerous invasive cell lines as denoted by the coloured line in Figure 3.1.12. PSME1 and CLIC1 did not show this trend i.e. highly expressed in all non-invasive cell lines compared to the invasive cell lines (Figure 3.1.12A) and therefore were not selected for further functional studies. RAD23B was chosen for a further siRNA knockdown study to investigate potential involvement in the *in vitro* invasion process in normal and cancer cell lines (Figure 3.1.12B).

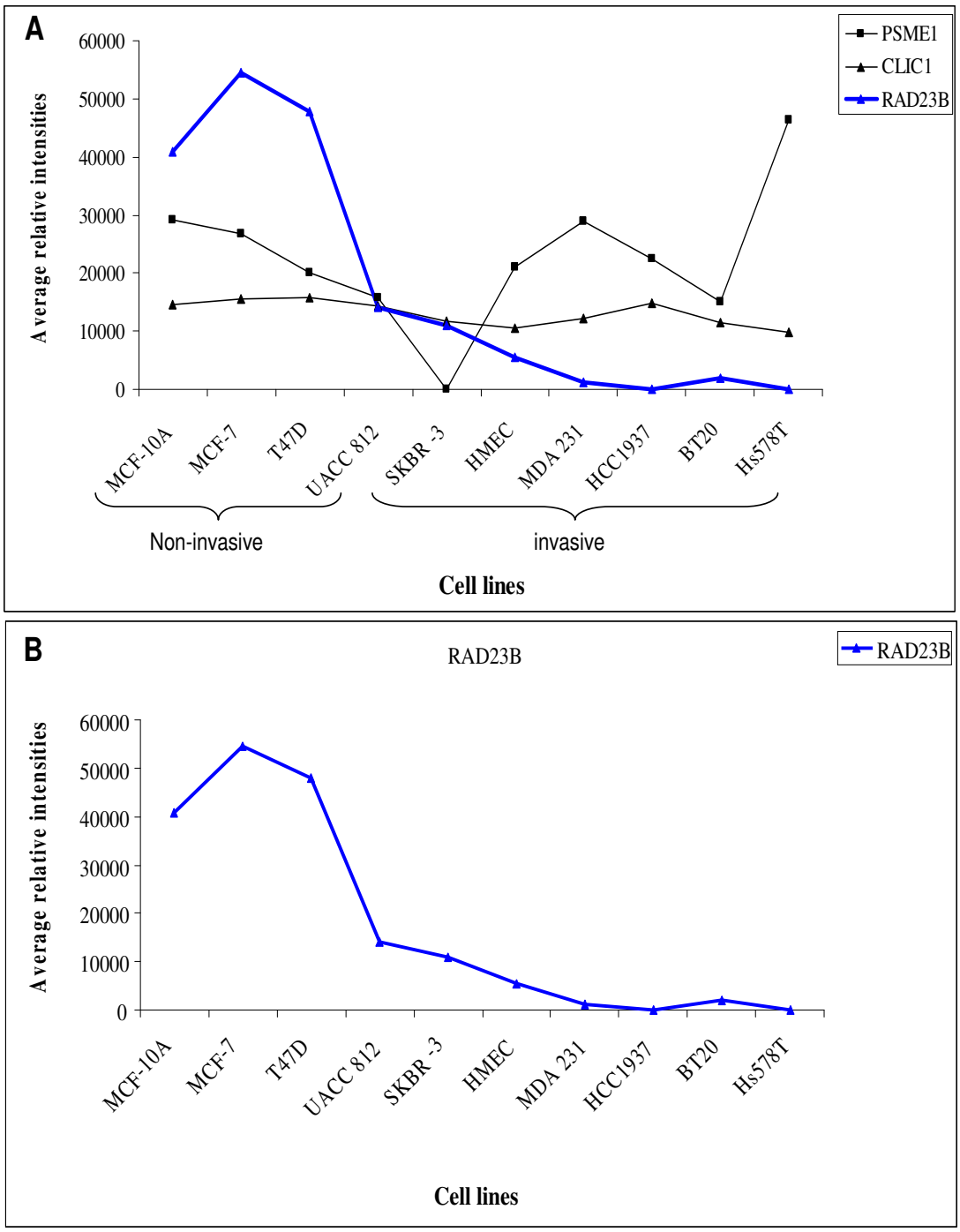


Figure 3.1.12**.** Graphical representation of average relative intensities calculated densitometry data from Western blotting analysis as shown in Figure 3.1.10 of selected normal+cancer invasion-related protein targets observed in a panel of cell lines. Average relative intensities represent the relative intensities obtained from 2 biological replicate samples. (A) PSME1, CLIC1 and RAD23B. (B) RAD23B

3.1.9 Optimization of transfection conditions of MCF-10A, MCF-7, SKBR-3 and BT20

TXNRD1 and 3-PGDH were up-regulated in the invasive cell lines and therefore knockdown of these targets was expected to reduce the level of invasion. RAD23B was up-regulated in the non-invasive cell lines and therefore knockdown of RAD23B was expected to induce the level of invasion. For TXNRD1 and 3-PGDH siRNA knockdown, two invasive cell lines (SKBR-3 and BT20) were chosen for optimization. For RAD23B siRNA knockdown, one normal non-invasive (MCF-10A) and one cancer non-invasive (MCF-7) cell line were chosen for optimization.

siRNAs can inhibit the expression of selected target proteins by hindering the transcription of specific genes. siRNA delivery through transfection can result in temporary changes in the cell, and in some cases cells may become resistant to conditions of delivery. To further ensure optimal target knockdown, siPORT™ *NeoFX*™, a lipid-based transfection reagent was used. The duration of gene silencing also varies greatly between cells, i.e. slow growing cells can show the effects of siRNA up to several weeks, whereas rapidly dividing cells can not show effects of siRNA for longer than 1 week (Tong *et al.* 1999). Therefore to achieve maximum effectiveness of exogenously introduced siRNA transfection optimization experiments were carried out as described in section 2.9.1.1.

Optimisation of siRNA transfection conditions, using kinesin siRNA and scrambled siRNA, was carried out for MCF-10A, MCF-7, SKBR-3 and BT20 in 96 well plates. Three different cell densities and siRNA/NeoFX reagent concentrations were used. Scrambled treated cultures were considered as control. The plates were assayed for changes in proliferation at 72 hours using the acid phosphatase assay as mentioned in section 2.9.1.1. Optimal conditions for transfection were determined as the combination of conditions which gave the greatest reduction in cell number after kinesin siRNA transfection and the least cell death in scrambled siRNA transfected cells. Figure 3.1.13. shows the proliferation assay results obtained for MCF-10A, MCF-7, SKBR-3 and BT20, and table 3.1.12 summarises the optimal conditions for siRNA transfection in 96 well plates.

Table 3.1.12. The optimal conditions for siRNA transfection in MCF-10A, MCF-7, SKBR-3 and BT20 in 96 well plate.

Cell lines	Seeding density/ well	Volume of NeoFX reagent(µl)
MCF 10A	1x 10^4	0.4
MCF-7	7.5x 10^3	0.6
SKBR-3	3x 10^3	0.4
BT20	5x 10^3	0.8

GAPDH, often used as an endogenous control, has been found to be an 'easy target' for siRNA, with efficient silencing observed in many different systems (www.Ambion.com). Optimisation of siRNA against GAPDH was also carried out in MCF-10A, MCF-7, SKBR-3 and BT20 cell lines to determine transfection knock down conditions at the protein level in 6 well plates using three different concentrations of NeoFX and cell density as mentioned in Table 3.1.13. Western blot analysis was carried out to establish the optimal conditions for siRNA transfection as described in section 2.8. Alpha tubulin was used as a loading control. Figure 3.1.14 shows the western blots of GAPDH siRNA transfected cells for MCF-10A, MCF-7, SKBR-3 and BT20. The same conditions of siRNA transfection in MDA-MB 231 were used as recommended by Ambion and also used by Timoshenko et al (2006). Table 3.1.13 shows the optimized conditions for the cell lines in 6 well plates.

Table 3.1.13. The optimal conditions for siRNA transfection in MCF-10A, MCF-7, SKBR-3 and BT20 in 6 well plate.

Cell lines	Seeding density/ well	Volume of NeoFX reagent(µl)
MCF 10A	5x 10^5	4
MCF-7	5x 10^5	4, 6, 8
SKBR-3	1x 10^5	3
BT20	5x 10^5	6
MDA-MB 231	2x 10^5	4

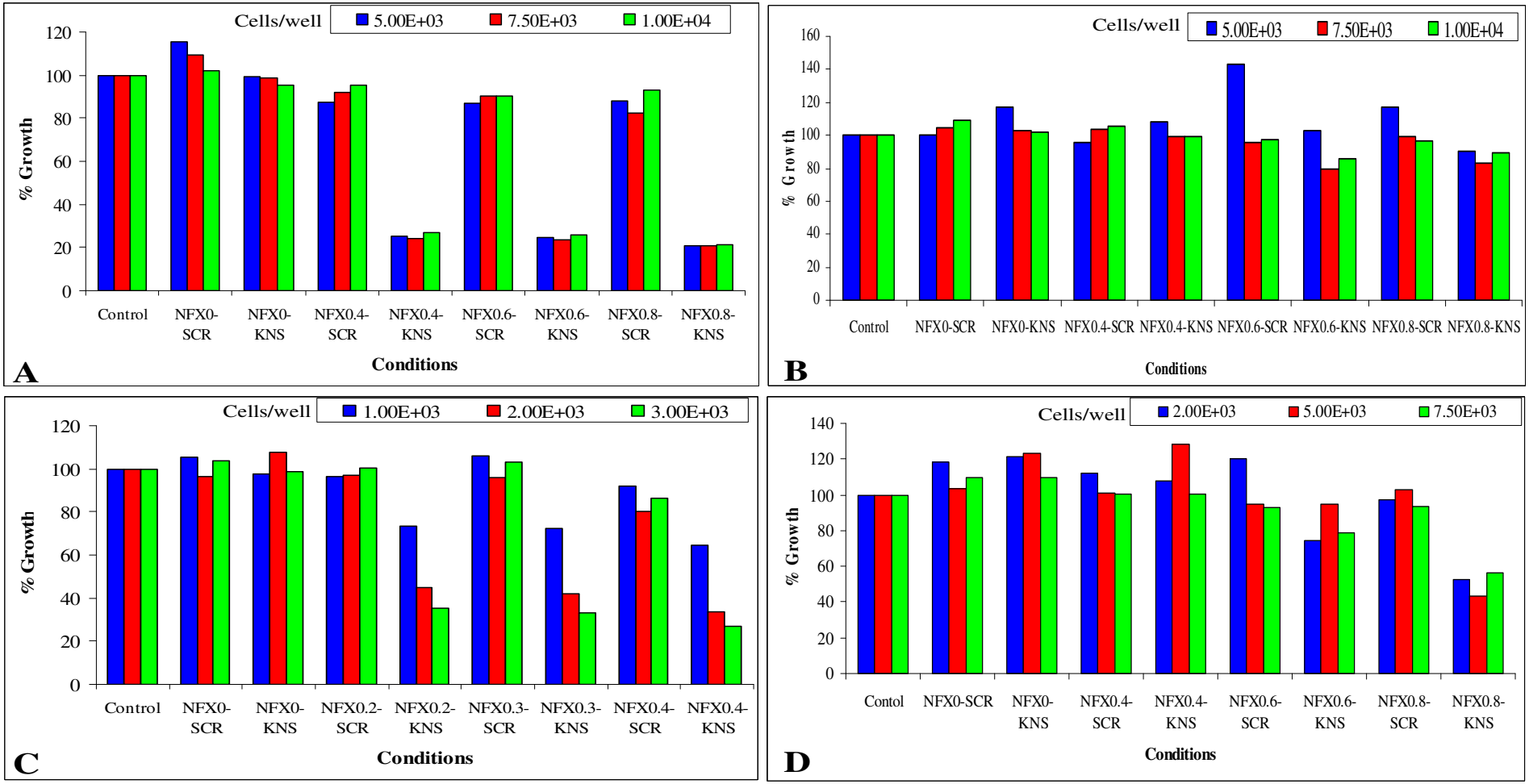


Figure 3.1.13. Proliferation assay of cell lines transfected with three different cell densities, three different concentrations of Kinesin siRNA and scrambled siRNA and three concentrations of NeoFX. (A) MCF-10A (B) MCF-7 (C) SKBR-3 (D) BT20. Control denotes untreated cultures.

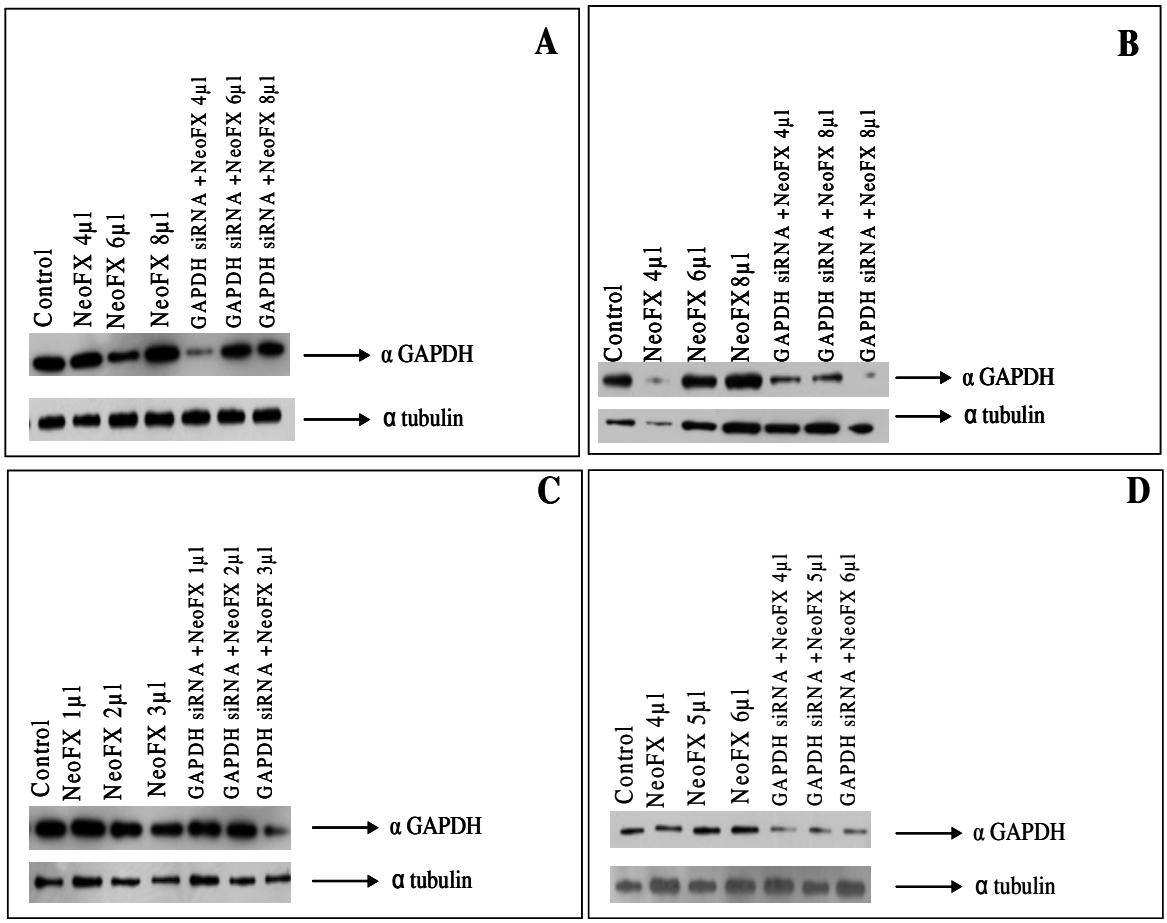


Figure 3.1.14. Western blot analysis of cell lines transfected with GAPDH siRNA with three concentrations of NeoFX, and optimal cell densities as described in Table 3.1.9.2 (A) MCF-10A (B) MCF-7 (C) SKBR-3 (D) BT20

3.1.10 Functional analysis of final targets

Of the 9 targets from section 3.1.8.4, 3 protein targets were chosen for further siRNA knockdown to investigate their functional role in invasion. The levels of silencing can vary between species, cells and tissues due to differences in the efficiency with which the siRNAs are taken up by target cells. Due to this problem the conditions for siRNA transfection in each cell line were optimised in 96- and 6-well plates using GAPDH and kinesin as positive controls, and scrambled siRNA as an siRNA transfection control. 2-3 siRNAs were chosen for each target for transfection into the cells as appropriate. For each set of siRNA transfections, untreated, NeoFX without siRNA and scrambled siRNA transfected culture were used as a control. Kinesin was also used as positive control to show efficient transfection as inhibition of kinesin reduces proliferation in culture.

3.1.10.1 Investigation into the role of TXNRD1 (cancer invasion related target) in invasion in breast cancer cells

TXNRD1 was identified as a protein potentially involved in invasion in our *in vitro* breast cancer cell line models. This protein was 4.6 fold up-regulated in the cancerous invasive cell line (BT20) compared to normal invasive (HMEC) + normal non-invasive (MCF-10A) + cancerous non-invasive (MCF-7) cell lines in 2D DIGE analysis (Table 3.1.7). Western blot analysis also showed that TXNRD1 was highly expressed in the invasive cell lines compared to the normal and non invasive cell lines. However, UACC-812 is a non-invasive cell line but is expressing TXNRD1 and is possibly an outlier in this comparison (Figure 3.1.12A and 3.1.13B). The invasive cell line (MDA-MB 231) and the low invasive cell line (SKBR-3) were used to investigate the effects of inhibition of the expression of TXNRD1 on cell proliferation and invasion using siRNA transfection. The endogenous levels of TXNRD1 were higher in the invasive cell line, BT20, compared to MDA MB 231, therefore MDA MB 231 was chosen for siRNA knockdown, as the effect of knock down may be more difficult to see in BT20 cells.

3.1.10.1.1 Effect of TXNRD1 siRNA transfection on MDA-MB 231 and SKBR-3

2 independent siRNA molecules were used to inhibit the expression of TXNRD1 in this investigation and the inhibition was confirmed for biological duplicate samples using Western blotting after 72hrs of transfection. 30nM of siRNA in SKBR-3 and 50nM siRNA in MDA-MB 231 were used. Figure 3.1.15A and B show Western blot analysis of siRNA transfection of TXNRD1 in MDA-MB 231 and SKBR-3 cells compared to untreated, NeoFX without siRNA, and siRNA scrambled transfected control cultures. Results indicate that the expression of TXNRD1 was reduced following siRNA transfection for both siRNA molecules in comparison to scrambled treated control cultures.

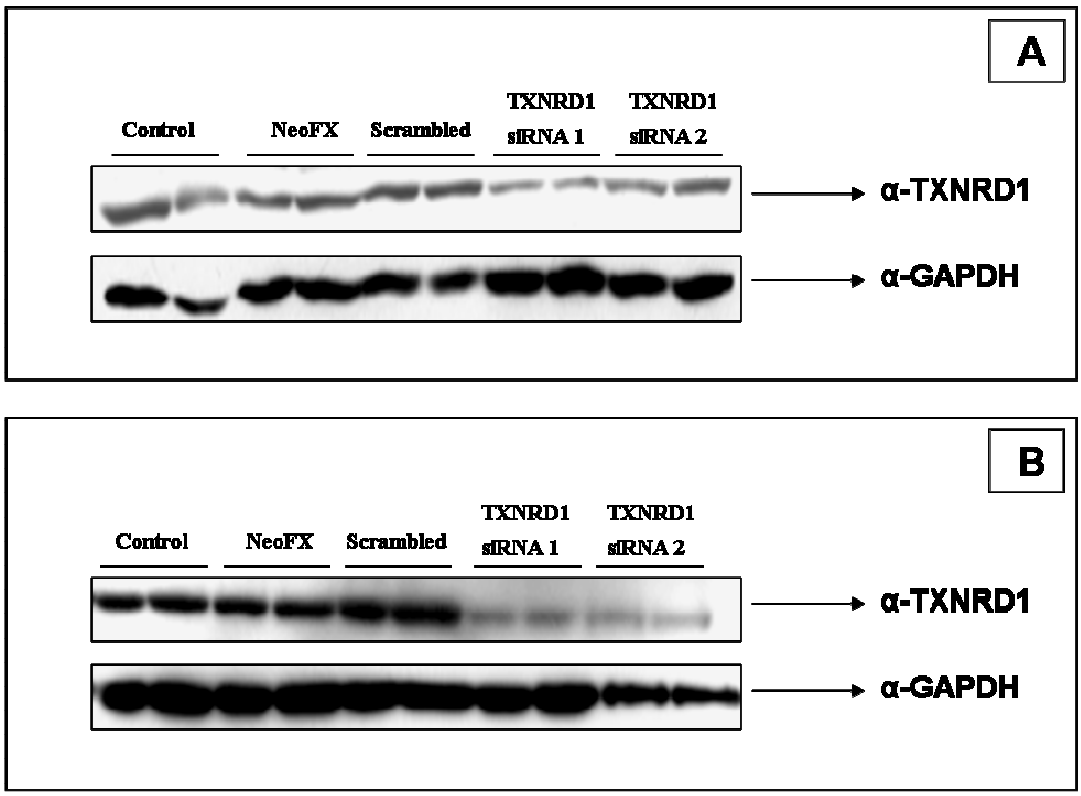


Figure 3.1.15. Western blot analysis of the inhibition of expression of TXNRD1 using siRNA knockdown in MDA-MB 231 (A) and SKBR-3 (B) cells. Two independent siRNA molecules of TXNRD1 were transfected into the cells. Protein was harvested 72 hrs post-transfection and used to determine the TXNRD1-siRNA specific decrease at the protein level using Western blot. Expression of GAPDH was used to demonstrate even loading between the samples in this investigation.

3.1.10.1.2 Investigation of the effects of inhibition of expression of TXNRD1 on the invasion status of cells

After 48hrs of siRNA transfection, invasion assays were performed to investigate the effects of the inhibition of expression of TXNRD1 in MDA-MB 231 and SKBR-3 cells using precoated matrigel invasion chambers for a further 48hrs as described in section 2.6. The total number of cells invading the membrane of the invasion chamber was reduced following siRNA transfection (Figure 3.1.16 and 3.1.17). The reduction in the invasion was 1.4-fold (p-value=0.00033) with siRNA TXNRD1 (1) and 1.3-fold (p-value=0.00035) with siRNA TXNRD1 (2) after transfection in MDA-MB 231 (Figure 3.1.16). Invasion was reduced by 3.1-fold (p-value=0.0015) with siRNA TXNRD1 (1) and 2.2-fold (p-value=0.00041) with siRNA TXNRD1 (2) after transfection in SKBR-3 (Figure 3.1.17).

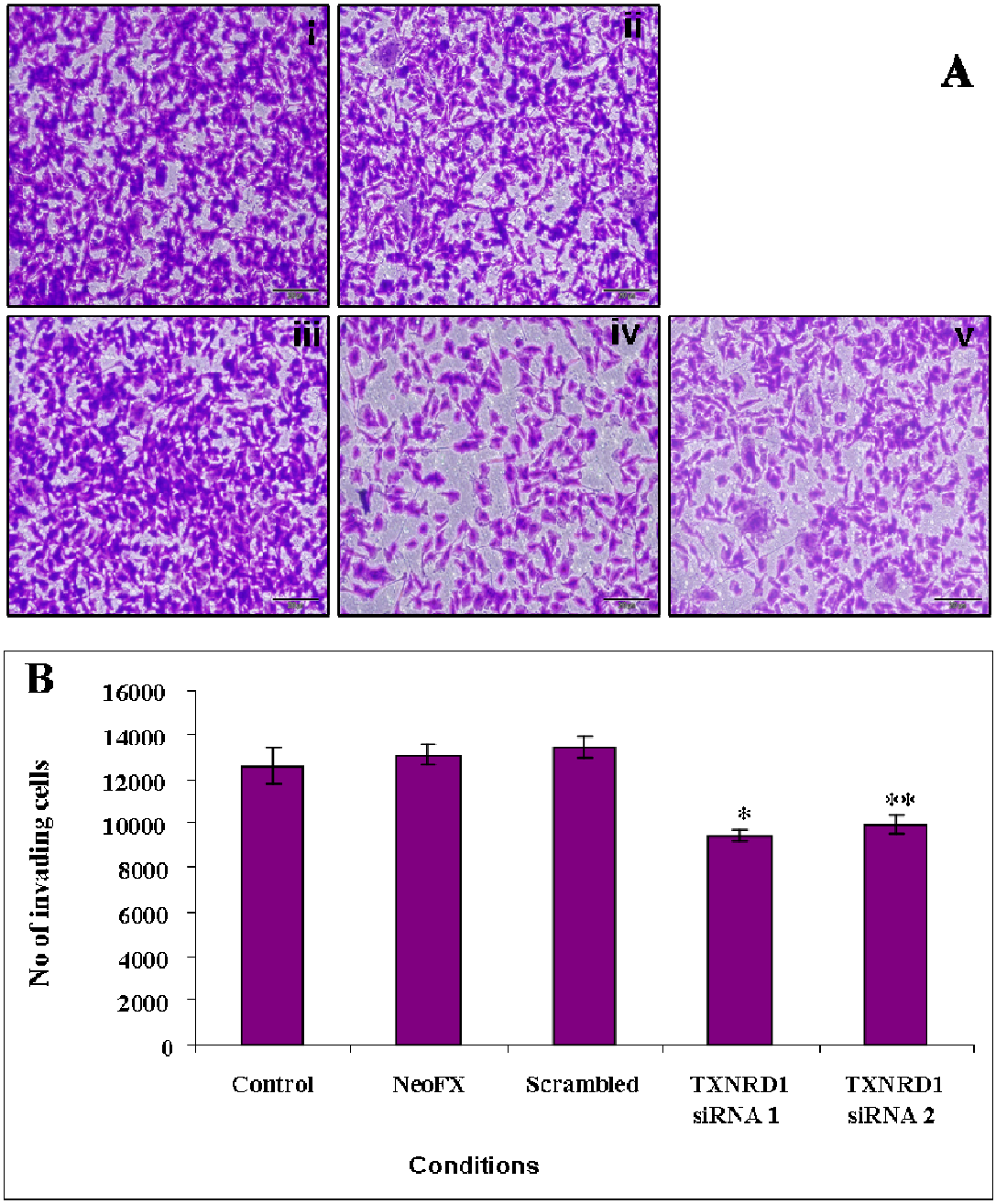


Figure 3.1.16. Investigation of the effects of inhibition of expression of TXNRD1 on the invasion status of MDA-MB 231 cells. **(A)** Images of invasion assays- (i) un-treated, (ii) NeoFX without siRNA, (iii) transfected with scrambled, (iv) transfected with TXNRD1 siRNA (1), and (v) transfected with TXNRD1 siRNA (2). Magnification= 200x, Scale bar=200µm. **(B)** Total number of cells invading through the membrane of invasion chambers after siRNA transfection. Error bars represent standard deviation calculated from data obtained from three biological samples. * P-value =0.0015 and ** P-value =0.00041.

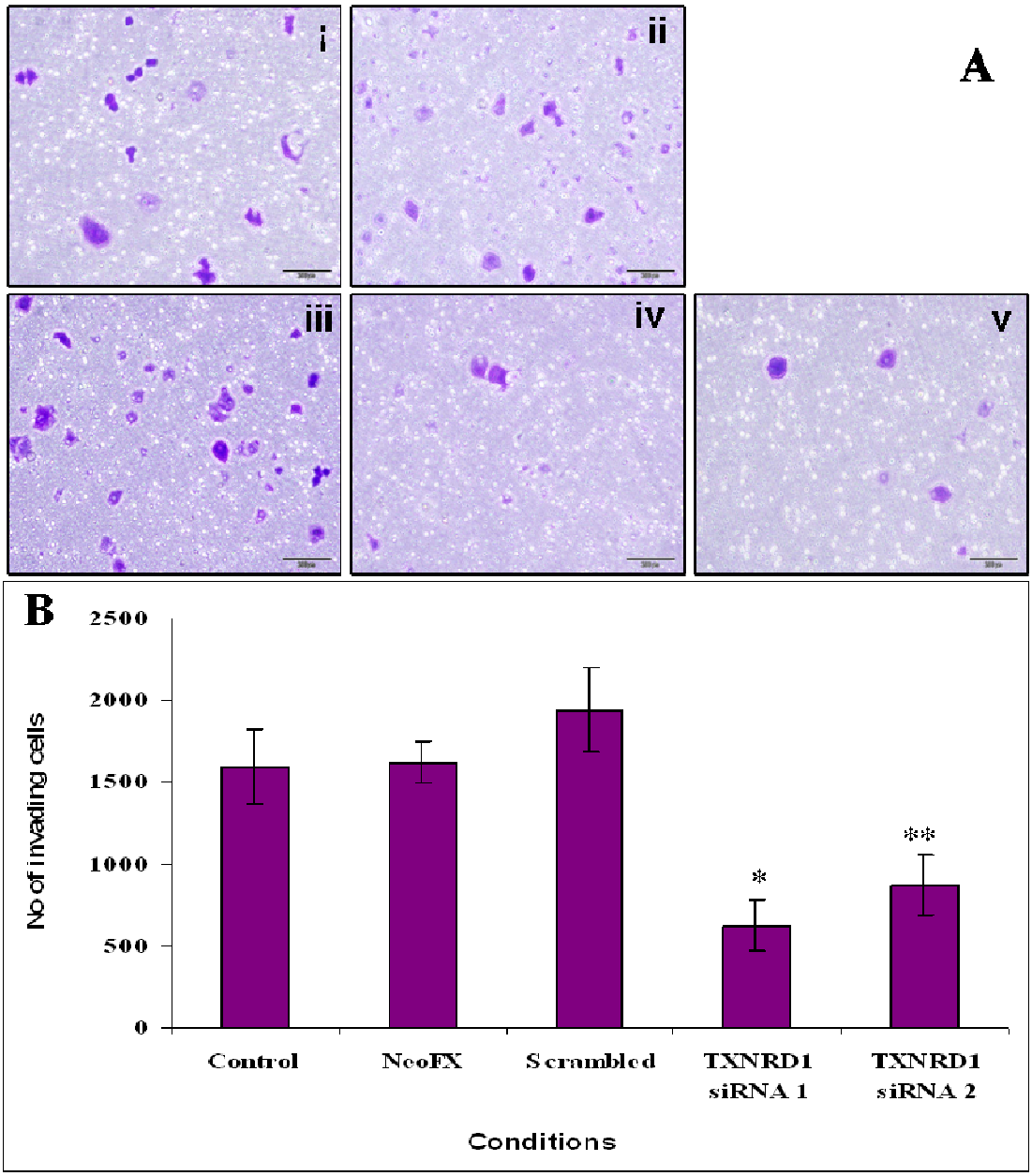
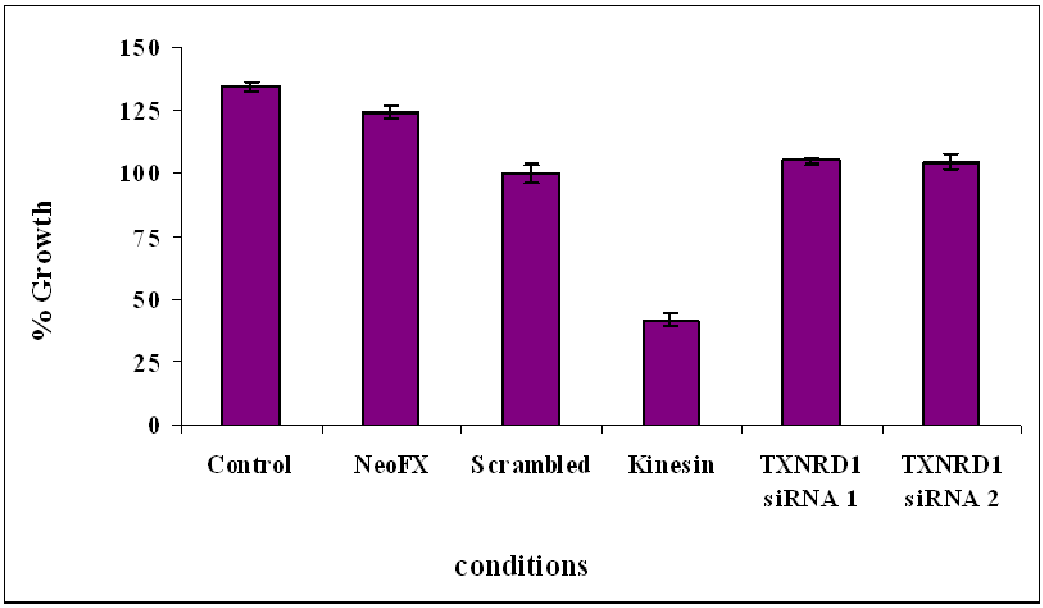


Figure 3.1.17. Investigation of the effects of inhibition of expression of TXNRD1 on invasion status of SKBR-3 cells. **(A)** Images of invasion assays- (i) un-treated, (ii) NeoFX without siRNA, (iii) transfected with scrambled, (iv) transfected with TXNRD1 siRNA (1), and (v) transfected with TXNRD1 siRNA (2). Magnification= 200x, Scale bar=200µm. **(B)** Total number of cells invading through the membrane of invasion chambers after siRNA transfection. Error bars represent standard deviation calculated from data obtained from three biological samples. * P-value =0.00033 and ** P-value =0.00035.

3.1.10.2 Effect of TXNRD1 siRNA on proliferation

Proliferation assays were carried out after 72hrs of transfection of TXNRD1 siRNAs into MDA-MB 231 and SKBR-3 cells to measure changes in growth. Figure 3.3.18 displays the percentage survival of transfected cells relative to untreated control (Section 2.9.2.2). Kinesin was used as a positive control to represent the efficiency of transfection. No significant difference in proliferation of TXNRD1-siRNAs treated cells compared to scrambled cells was observed which indicates that the loss of expression of TXNRD1 did not affect proliferation in MDA-MB 231 cells (Figure 3.3.18A). Similar results were observed in the case of TXNRD1-siRNA transfection in SKBR-3 cells (Figure 3.3.18B)



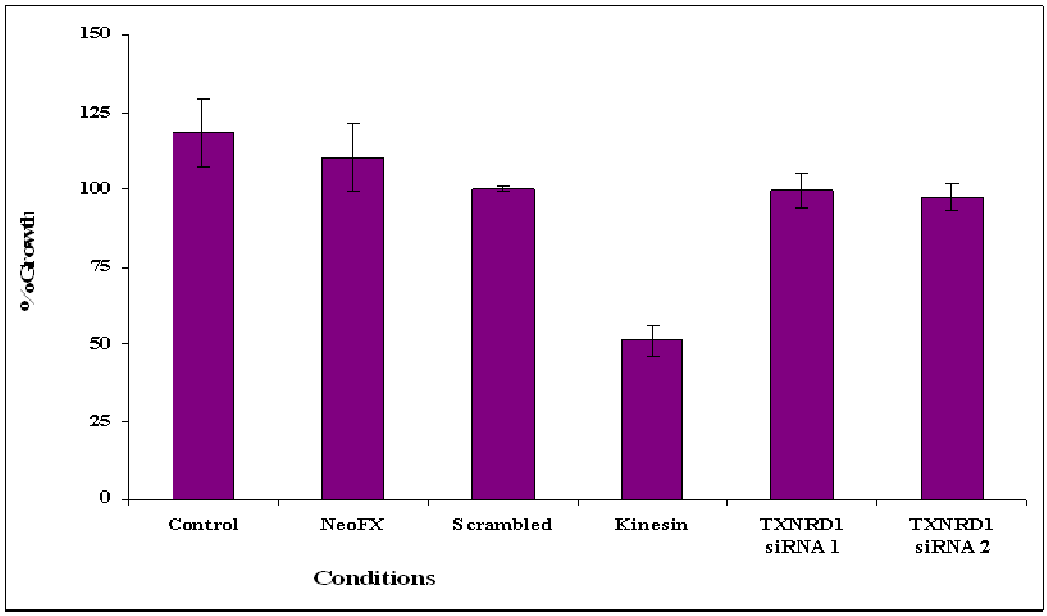


Figure 3.3.18**.** Percentage survival of MDA-MB 231 (A) and SKBR-3 (B) transfected with TXNRD1-siRNAs relative to untreated, NeoFX without siRNA, and Scrambled siRNA transfected control cultures. Error bars represent standard deviation calculated from data obtained from three biological replicate samples.

3.1.10.3 Investigation of the role of RAD23B (normal+ cancer invasion-related target) in invasion in breast cells

RAD23B was identified as a protein potentially involved in suppressing invasion in our *in vitro* breast cell line models. This protein was 2.07 fold up-regulated in non-invasive (MCF-10-A+ MCF-7) compared to invasive (HMEC+ BT20) cell lines from the 2D DIGE analysis (Table 3.1.6). Western blot analysis showed that RAD23B was highly expressed in the non-invasive cell lines (MCF-7, MCF-10 and T47D) compared to the invasive cell lines. However, UACC-812, which is a non-invasive cell line was found to express low levels of this protein and is a possible outlier in this experiment (Figure 3.1.10A and 3.1.11B). Low levels of expression of RAD23B were also found in HMEC, SKBR-3 and BT20 cells (Figure 3.1.10A). Therefore the non-invasive cell line (MCF-10A and MCF-7) were used to investigate the effects of inhibition of the expression of RAD23B on cell proliferation and invasion using siRNA transfection.

3.1.11.3.1 Effect of RAD23B siRNA transfection on MCF-7 and MCF-10A

3 independent siRNA molecules were used to inhibit the expression of RAD23B in this investigation and the inhibition was confirmed for biological duplicate samples using Western blotting after 72hrs of transfection. Figure 3.1.18A and 3.1.18B show Western blot analysis of siRNA transfection of RAD23B in MCF-7 and MCF-10A cells compared to un-treated, NeoFX without siRNA and siRNA scrambled transfected control cultures. The results indicate that the expression of RAD23B was significantly reduced following siRNA transfection for both siRNA molecules in comparison to scrambled treated control cultures.

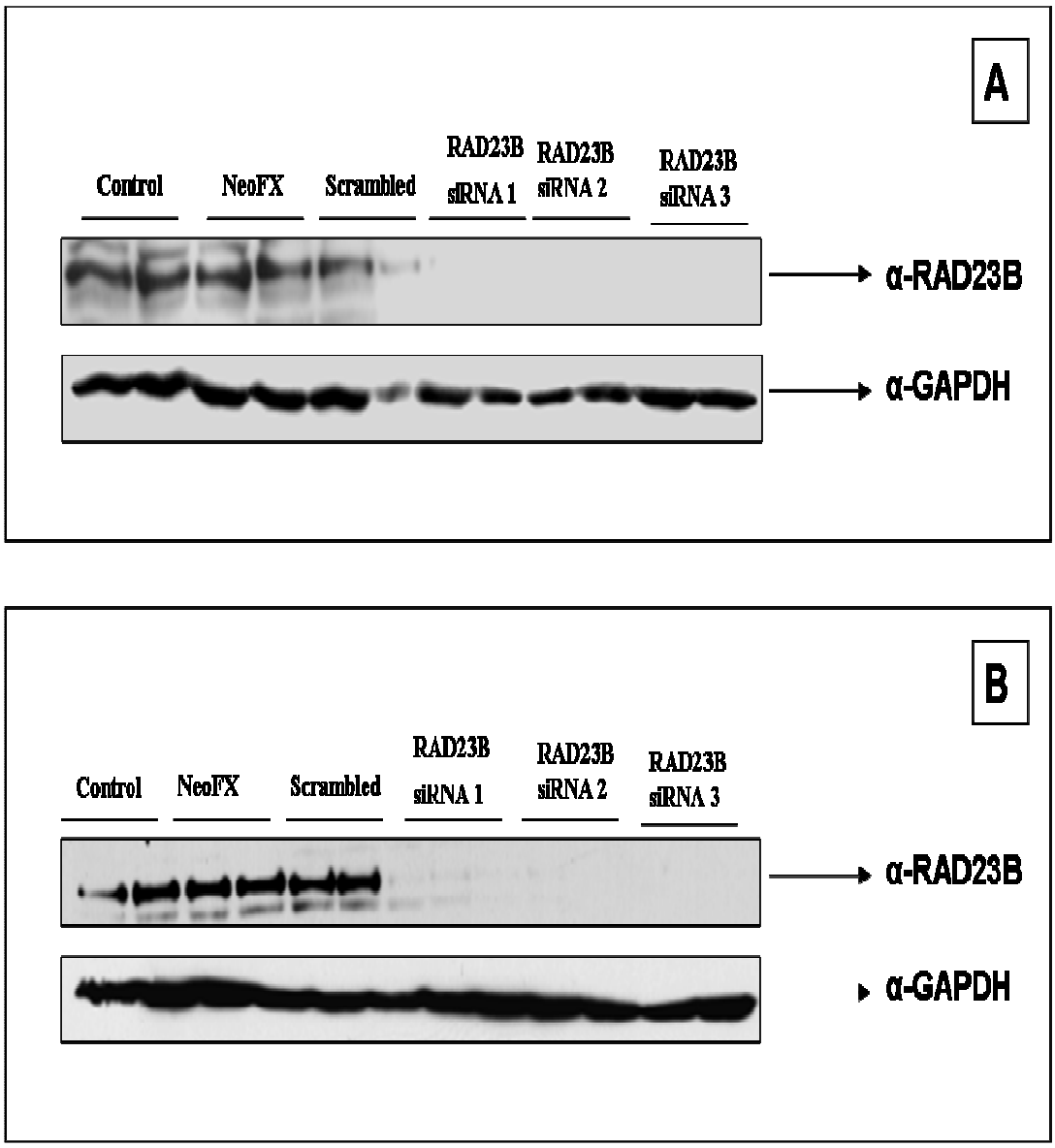


Figure 3.1.19. Western blot analysis of the inhibition of expression of RAD23B using siRNA knockdown in MCF-7 (A) and MCF-10A (B) cells. 3 independent siRNA molecules of RAD23B were transfected into the cells. Protein was harvested at 72 hrs for MCF-10A and 96 hrs for MCF-7 post-transfection and used to determine the RAD23-siRNA specific decrease at the protein level using Western blot. Expression of GAPDH was used to demonstrate even loading between the samples in this investigation.

3.1.10.3.2 Effect of RAD23B siRNA on invasion

After 48hrs of siRNA transfection, invasion assays were performed to investigate the effects of inhibition of expression of RAD23B in MCF-7 and MCF-10A cells using pre-coated matrigel invasion chambers for a further 72 and 48hrs respectively (section 2.9.2.1). The total incubation time for transfection was 96 hrs for MCF-7 and 72 hrs for MCF-10A. The choice of a longer incubation period (72hrs) for MCF-7 in invasion chambers was driven by the previous observations in this laboratory that it is difficult to induce invasion of MCF-7 cells. The total number of cells invading the membrane of the invasion chamber was increased following siRNA transfection (Figure 3.1.20 and 3.1.21). The increase in the invasion was 1.9-fold ($p=0.020$) with siRNA RAD23B (1), 2.6-fold ($p=0.00025$) with siRNA RAD23B (2) and 2.9-fold ($p=0.00018$) with siRNA RAD23B (3) after transfection in MCF-7 cells (Figure 3.1.20). Invasion was increased by 2.7-fold ($p=0.015$) with siRNA RAD23B (1), 3.5-fold ($p=0.0029$) with siRNA RAD23B (2) and 4.0-fold ($p=0.0025$) with siRNA RAD23B (3) following transfection in MCF-10A (Figure 3.1.21). A few cells in control (neoFX, scrambled and non-transfected cells) were observed in MCF-7 in invasion assays which could be an artefact of longer incubation (72hrs) of cells in invasion chambers.

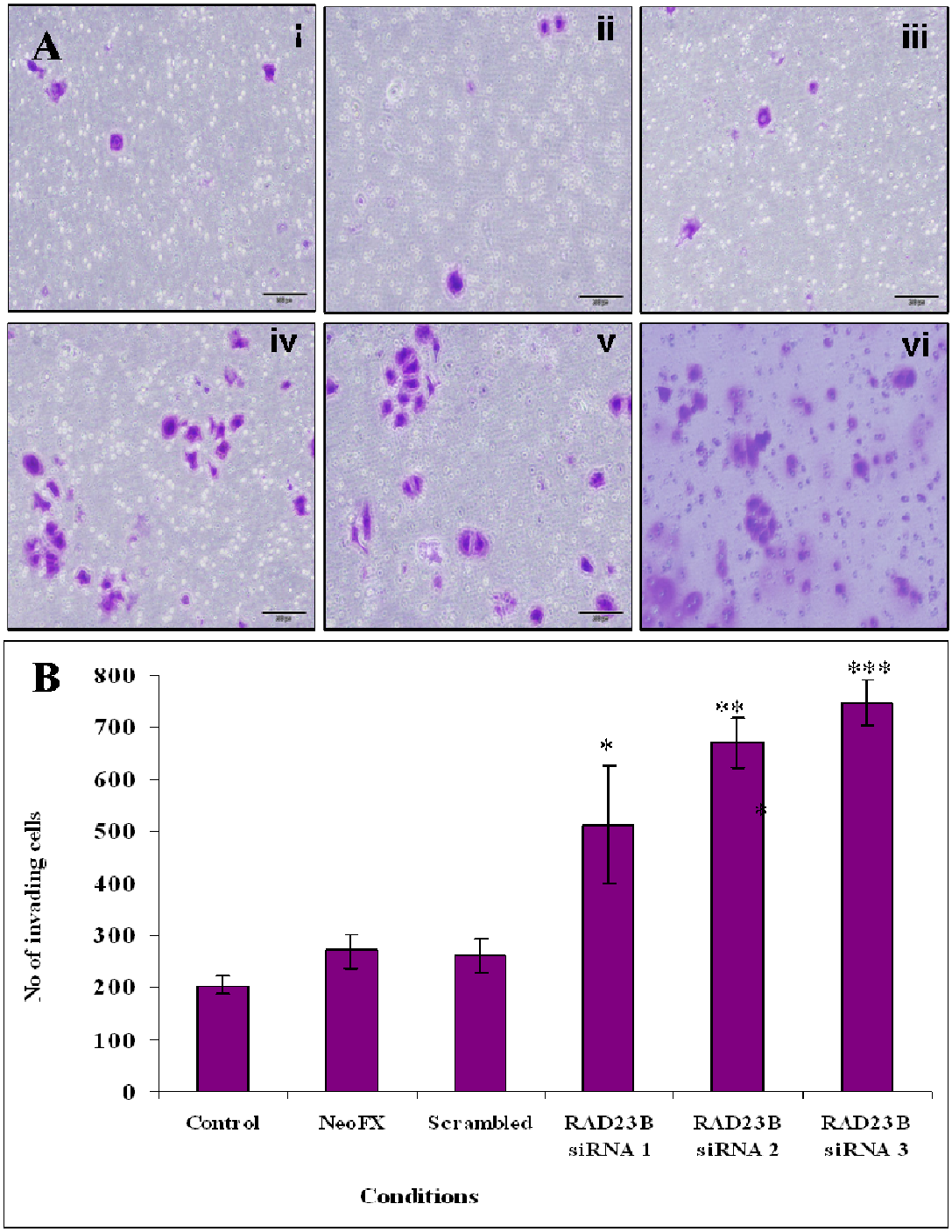


Figure 3.1.20. Investigation of effects of the inhibition of expression of RAD23B on the invasion status of MCF-7 cells. **(A)** Images of invasion assays- (i) un-treated, (ii) NeoFX without siRNA, (iii) transfected with scrambled, (iv) transfected with RAD23B siRNA (1), (v) transfected with RAD23B siRNA (2) and (vi) transfected with RAD23B siRNA (3). Magnification= 200x, Scale bar=200µm. **(B)** Total number of cells invading through the membrane of invasion chambers after siRNA transfection. Error bars represent standard deviation calculated from data obtained from three biological samples. * P-value =0.020, ** P-value =0.00025 and *** P-value =0.00018.

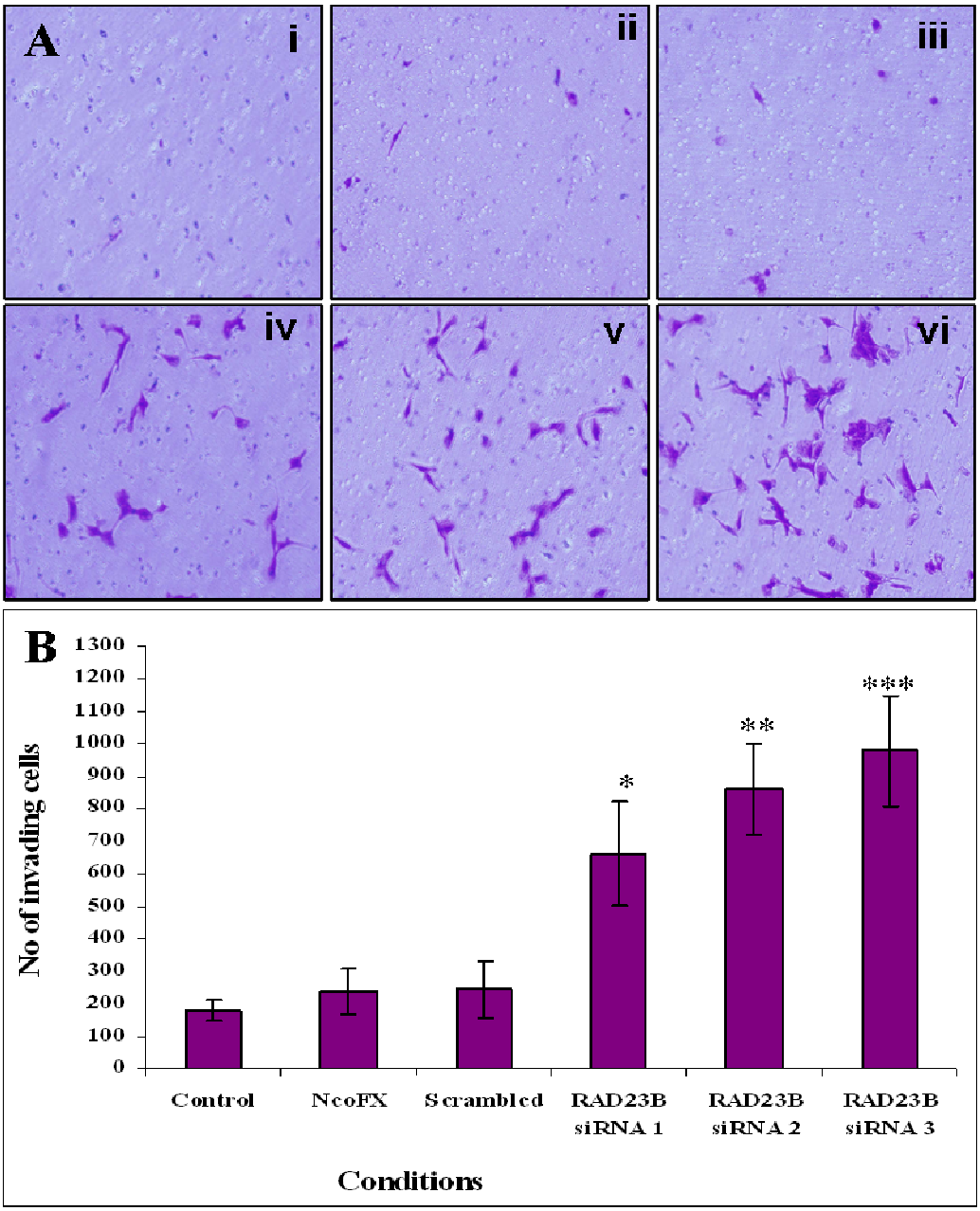
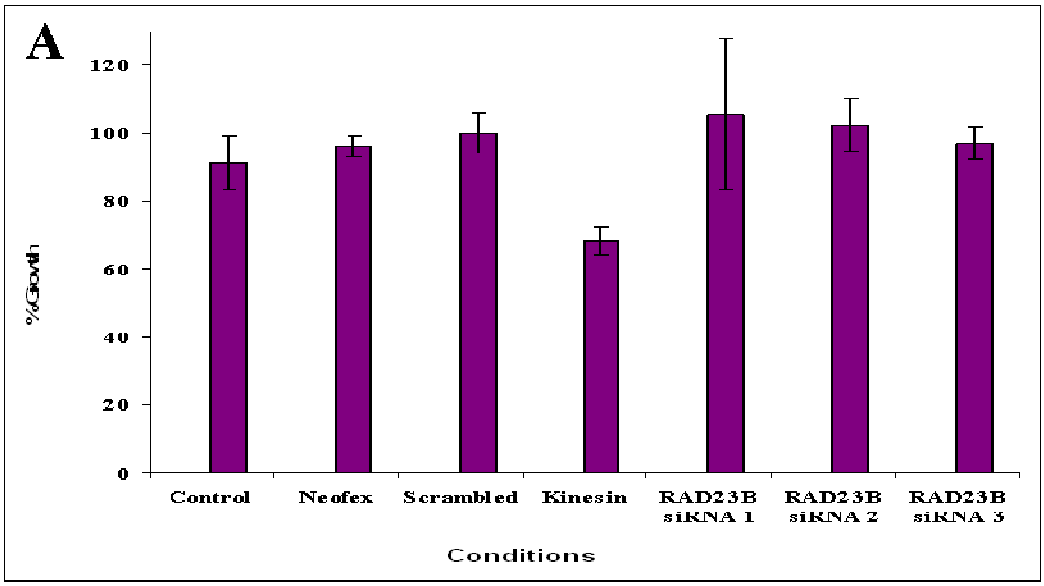


Figure 3.1.21. Investigation of effects of the inhibition of expression of RAD23B on the invasion status of MCF-10A cells. **(A)** Images of invasion assays- (i) un-treated, (ii) NeoFX without siRNA, (iii) transfected with scrambled, (iv) transfected with RAD23B siRNA (1), and (v) transfected with RAD23B siRNA (2), (vi) transfected with RAD23B siRNA (3). Magnification= 200x, Scale bar=200µm. **(B)** Total number of cells invading through the membrane of invasion chambers after siRNA transfection. Error bars represent standard deviation calculated from data obtained from three biological samples. * P-value =0.015, ** P-value =0.0029 and *** P-value =0..0025.

3.1.10.4 Effect of RAD23B siRNA on proliferation

Proliferation assays were carried out after 72hrs of transfection of RAD23B siRNAs into MCF-7 and MCF-10A cells to measure the changes in growth. Figure 3.1.22 displays the percentage survival of transfected cells relative to untreated control (section 2.9.2.1). Kinesin was used as a positive control to represent the efficiency of transfection. No significant difference in proliferation of RAD23B-siRNA treated cells compared to scrambled siRNA treated cells was observed which indicates that the loss of expression of RAD23B did not affect proliferation in MCF-7 cells (Figure 3.1.22A). Similar results were observed in the case of RAD23B-siRNA transfection in MCF-10A (Figure 3.1.22B).



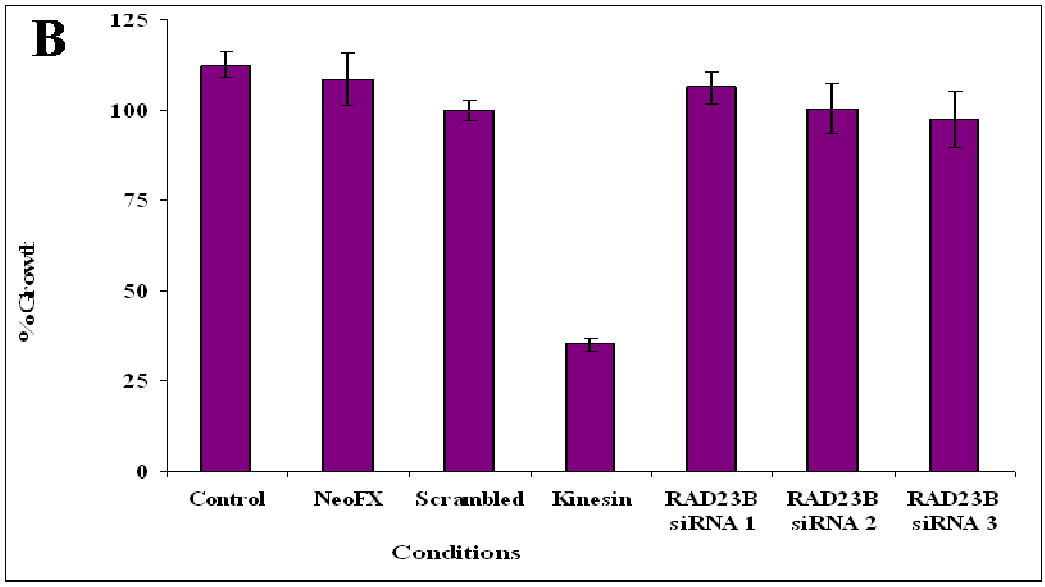


Figure 3.1.22. Percentage survival of MCF-7 (A) and MDA-10A (B) transfected with RAD23B-siRNAs relative to untreated, NeoFX without siRNA, and Scrambled siRNA transfected control cultures. Error bars represent standard deviation calculated from data obtained from three biological samples.

3.1.10.5 Investigation of the role of 3-PGDH (cancer invasion-related target) in breast cancer cell invasion

3-PGDH was identified as a protein potentially involved in invasion in the *in vitro* breast cancer cell lines used in this study. This protein was 4.6 fold up-regulated in the cancerous invasive cell line (BT20) compared to the normal invasive (HMEC)+ normal non-invasive (MCF-10A) + cancerous non-invasive (MCF-7) cell lines from 2D-DIGE analysis (Table 3.1.7). Western blot analysis showed 3-PGDH to be highly expressed in the invasive cell lines compared to the normal and non invasive cell lines, with the exception of UACC-812 cells (Figure 3.1.10A and 3.1.11A). Therefore, the invasive cell lines (MDA-MB 231 and SKBR3) were used to investigate the effects of inhibition of the expression of 3-PGDH on cell proliferation and invasion using siRNA transfection.

3.1.10.5.1 Effect of 3-PGDH siRNA transfection on MDA-MB 231 and SKBR-3

3 independent siRNA molecules (30nM) were used to inhibit the expression of 3-PGDH in this investigation and no inhibition was observed for biological duplicate samples using Western blotting after transfection. Figure 3.1.23 show western blot analysis of siRNA transfection of 3-PGDH in MDA-MB 231 and SKBR-3 cells compared to un-treated, NeoFX and scrambled siRNA transfected control cultures. The results indicate that the expression of 3-PGDH was not inhibited following siRNA transfection for all of the siRNA molecules in comparison to scrambled treated control cultures. siRNAs were then transfected into another invasive cell line (BT20) and the protein samples were analyzed using Western blotting. No difference in siRNA treated vs. control samples was observed. Therefore increased concentrations of siRNA molecules (50nM for SKBR-3 and 125nM for MDA-MB 231 cells) were transfected into the cell lines and protein samples were collected after 72hrs of transfection from SKBR-3 and MDA-MB 231 cells and were analysed using western blotting.

Higher concentrations of siRNA (100nM) have been recommended by Ambion (www.ambion.com) and a concentration of 125nM siRNA concentration on MDA-MB 231 has been used by Timoshenko et al, (2006). No knockdown in the expression of siRNA treated vs. control samples was observed. This indicates that the siRNA molecules are not able to inhibit the expression of 3-PGDH. Two reasons for this could be possible that 3-PGDH may have slow turn over in the cells and confirmation

of knockdown at the mRNA level using qRT-PCR might be useful. The siRNA molecules used in this investigation are ‘not-validated-siRNAs’ (no experimental proof for efficiency of siRNAs) and therefore inhibition of 3-PGDH using validated-siRNA from other providers could help to investigate the effects of 3-PGDH on invasion process in breast cancer.

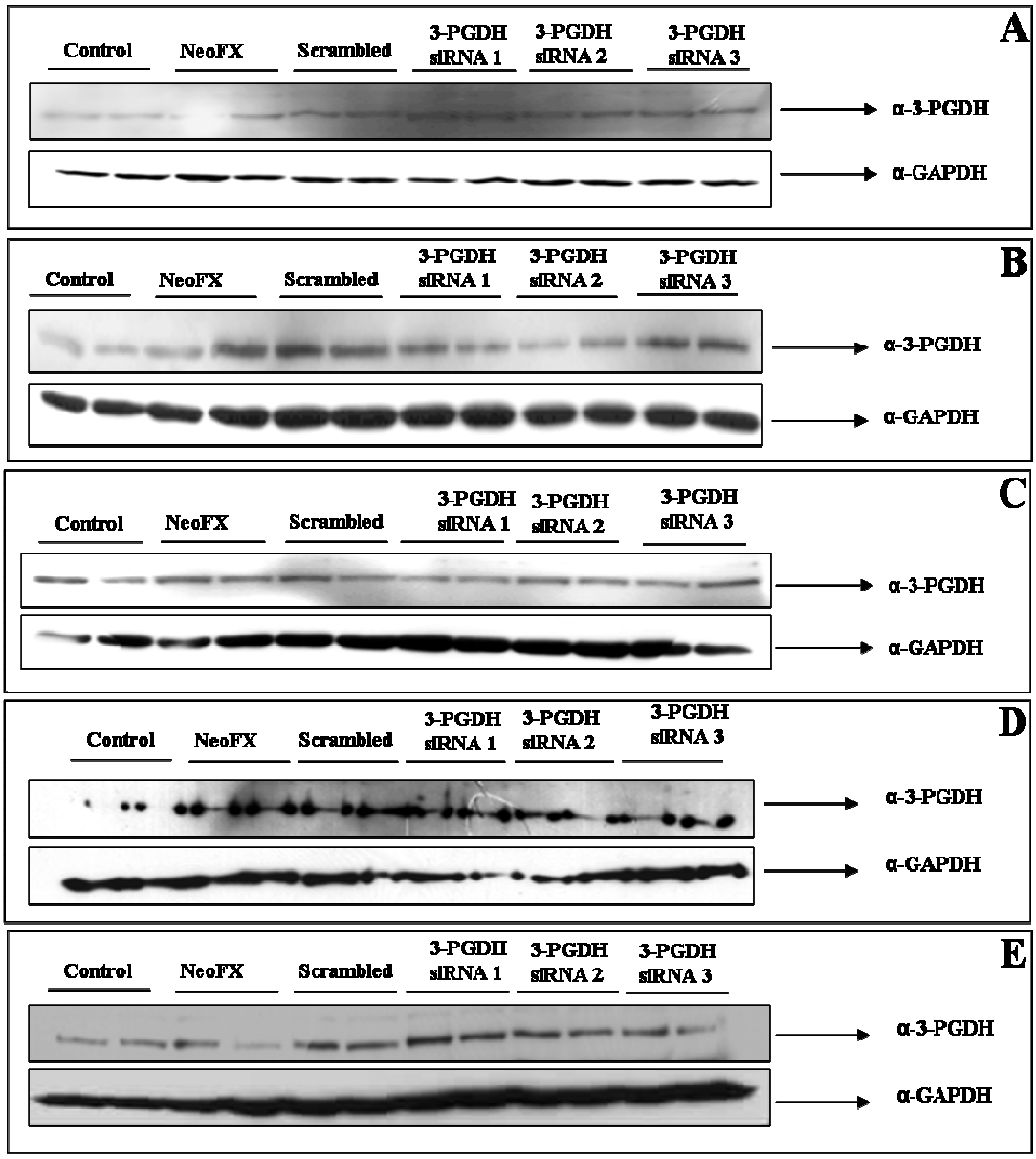


Figure 3.1.23. Western blot analysis of the inhibition of expression of 3-PGDH using siRNA knockdown technique in MDA-MB 231, SKBR-3 and BT20 cells. (A) transfected with 3-PGDH 30nM siRNAs in MDA-MB 231, (B) transfected with 3-PGDH 30nM siRNAs in SKBR-3, (C) transfected with 3-PGDH 30nM siRNAs in BT20, (D) transfected with 3-PGDH 50nM siRNAs in SKBR-3, (E) transfected with 3-PGDH 125nM siRNAs in MDA-MB 231. Expression of GAPDH was used to demonstrate even loading between the samples in this investigation.

3.1.10.6 Effect of 3-PGDH siRNA on cell proliferation

Proliferation assays were carried out after 72hrs of transfection of 3-PGDH siRNAs into MDA-MB 231, SKBR-3 and BT20 cell lines to measure changes in growth. Figure 3.1.24 displays the percentage survival of transfected cells relative to untreated control. Kinesin was used as a positive control to represent efficient transfection. The reduction in expression of 3-PGDH was not achieved following siRNA tranfection resulting insignificant difference in proliferation of siRNA treated cells compared to scrambled siRNA treated cells. However, kinesin treated culture showed a significant reduction in cell growth demonstrating efficient transfection (Figure 3.1.24).

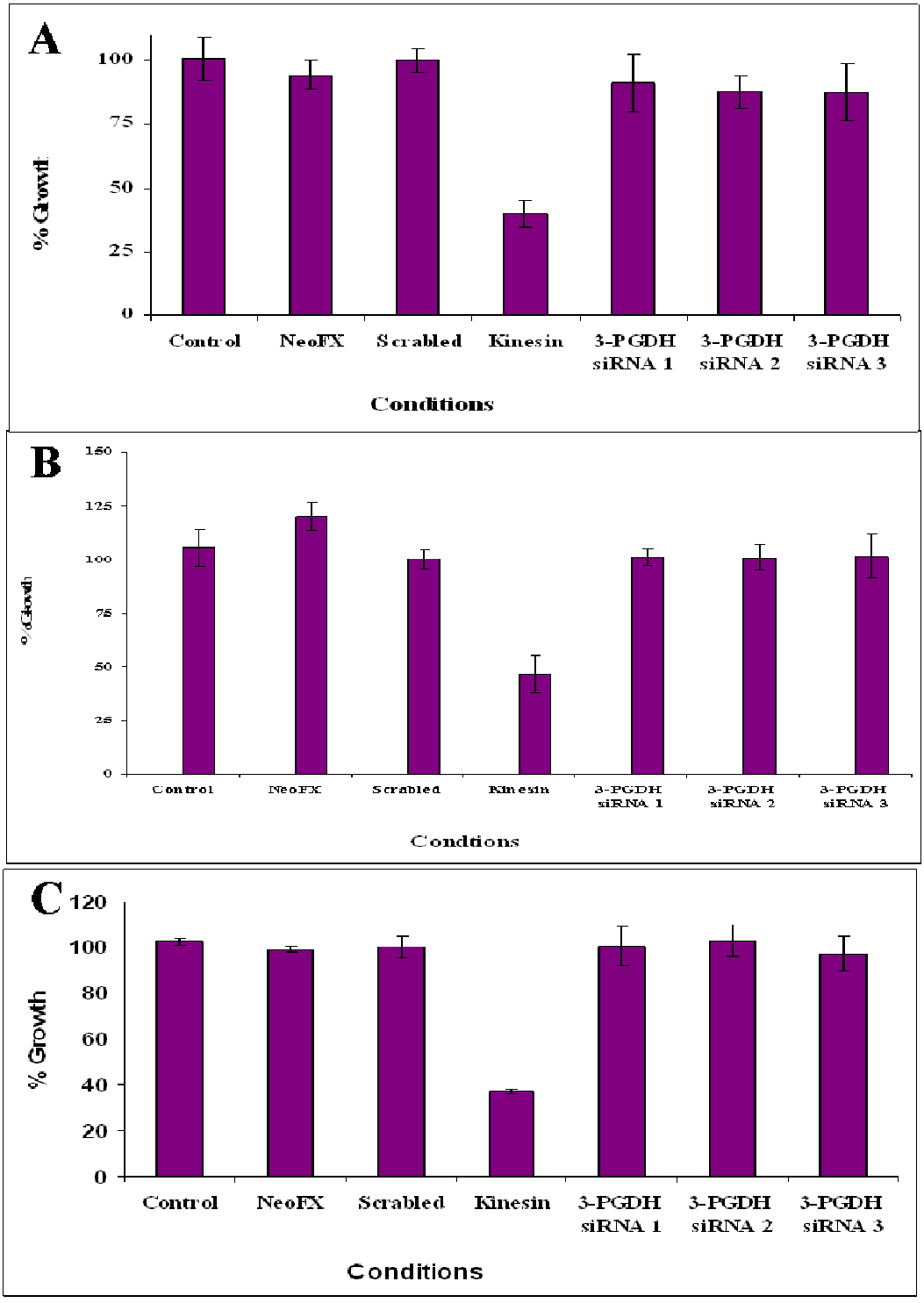


Figure 3.1.24. Percentage survival of MDA-MB 231 (A), SKBR-3 (B) and BT20 (C) transfected with 3-PGDH-siRNAs relative to untreated, NeoFX without siRNA, and Scrambled siRNA transfected control cultures. Error bars represent standard deviation calculated from data obtained from three biological samples.

3.2 Proteomic analysis of conditioned media from HMEC, MCF-10A, MCF-7 and BT20

The proteins released by cells or shedding proteins are a record of its physiological status and are commonly associated with diseases including cancer. Invasion occurs within the tumour-host microecology, where stroma and tumour cells exchange enzymes and cytokines that modify the local extracellular matrix, stimulate migration and promote proliferation. Therefore, either the tumour itself or its microenvironment could be sources for biomarkers that would ultimately be shed into the serum proteome and possibly be important for early detection, monitoring therapeutic efficacy or understanding the biology of cancer.

In vitro cell culture model systems, in which serum-free conditioned media are collected from cells, are being used to understand biological processes and functions (Ryther *et al.* 2005). Therefore, investigation of protein expression changes in breast cell lines representative of normal, non-invasive and invasive phenotypes might be helpful in understanding the biological mechanisms leading to breast cancer and early detection of breast cancer and invasion. For this, differential proteomics study of conditioned media from HMEC, MCF-10A, MCF-7 and BT20 were carried out.

3.2.1 Analysis of viability of cells in serum-free medium

MCF-10A, MCF-7 and BT20 cells were grown in serum free medium (SFM) to ensure that the conditioned media contained as few as possible other exogenous proteins (such as serum proteins). To minimise cell death and maximize the concentration of secreted protein in the conditioned media, cells were grown in complete media until 60% confluency and then media was replaced with serum-free media**.** The viability of MCF-10A, MCF-7 and BT20 cells were measured at the time of sample collection as described in sections 2.4.6 and 2.7.1.2. The 10% serum-supplemented cells were used as a control in this study. Figure 3.2.1 shows the viability of cells cultured in serum and serum-free medium at 24, 48 and 96 hrs of MCF-10A, MCF-7 and BT20. The results indicate that the viability of MCF-10A, MCF-7 and BT20 after 96 hrs of culture in serum supplemented medium was 99%, 97% and 99% respectively. The viability of cells grown in serum-free was 95%, 96% and 97% which is comparable to the control culture and therefore represents the healthy state of cell at time of conditioned medium harvesting. HMEC grows in specialised media and has a short life-time, therefore the same conditions as optimized using MCF-10A, MCF-7 and BT20 were applied for HMEC for further investigations.

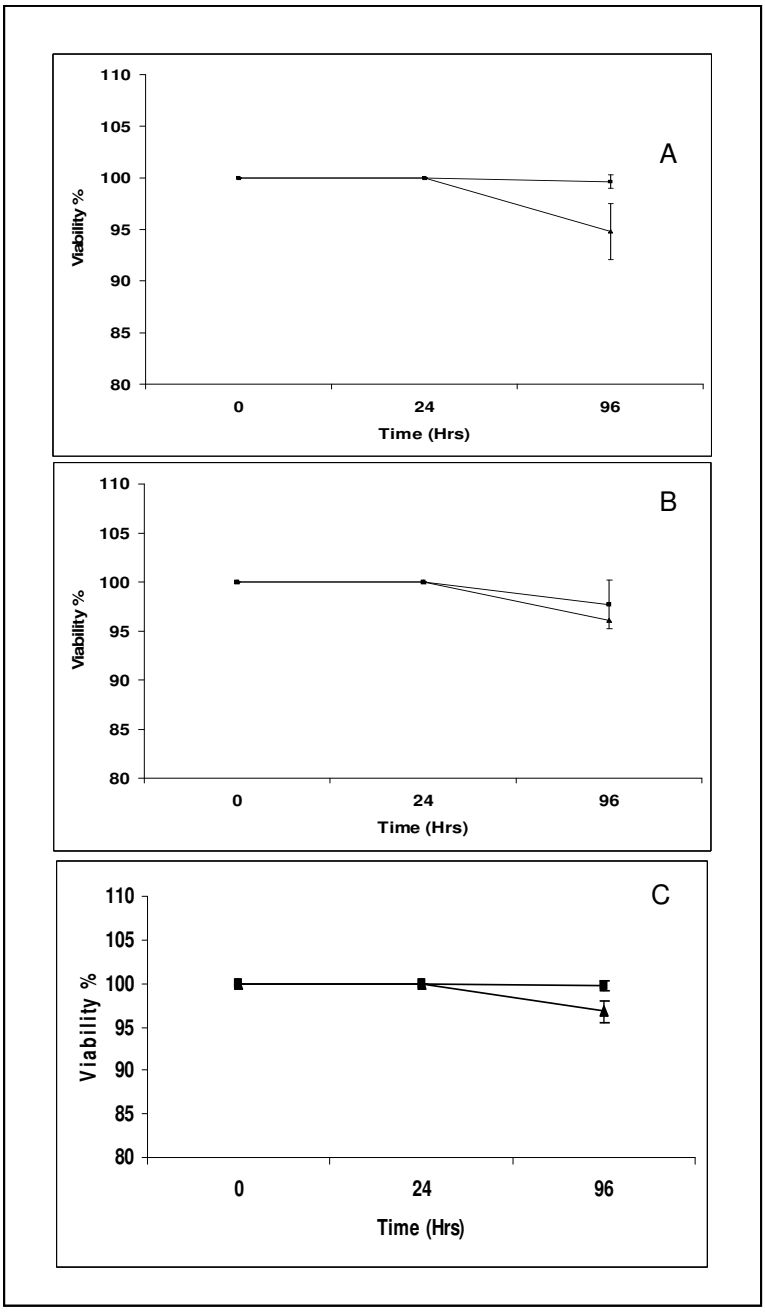


Figure 3.2.1: (A) Percentage viability of MCF10A, (B) Percentage viability of MCF-7, (C) Percentage viability of BT20 grown over 96 hours of culture. (■) represents the cells viability in serum supplemented medium; (▲) represents the cells viability in serum-free media. Error bars represents the standard deviation calculated from data obtained from the experiment (n=3)

3.2.2 Experimental outline

Investigations were carried out to establish differential expression of secreted proteins as described in section 3.1.3. For this, four biological samples from normal mammary epithelial cells (HMEC), non-tumorigenic immortalized mammary epithelial cells (MCF-10A) non-invasive breast cancer cells (MCF-7) and invasive breast cancer cells (BT20) were prepared separately. Each cell line sample was labelled with 2-D DIGE dyes (i.e. Cy3 and Cy5) as outlined in Table 3.1.2. A pooled internal control containing a mixture of all biological replicate samples in the experiment was labelled with Cy2 dye.

3.2.3 Analysis of differential expression of proteins using DeCyder

2D-DIGE protein expression maps (PEMs) for conditioned media from HMEC, MCF-10AMCF-7 and BT20 were created. Analysis was carried out as described in section 3.1.3. After spot matching and filtering a total of 305 protein spots were matched across all of the gels in the experiment, i.e. 24 gels in total.

The objective of this investigation was to identify the proteins that are up-regulated or down-regulated with the cancerous and invasion status of the cell lines. For this, analyses of the following three comparison group were performed from the 2D-DIGE PEMs. Spots that were ±1.5 fold up or down-regulated with p-value ≤ 0.05 were considered significantly differentially expressed (DE).

3.2.3.1 Cancerous (MCF-7+ BT20) vs. Normal (HMEC+ BT20)

To see which proteins are being differentially expressed between the cancer and normal cells, the PEMs for the cancerous cells (MCF-7+BT20) were compared with the PEMs for the normal cells (HMEC+MCF10A). The comparison revealed altered expression of 81 proteins, of which 56 proteins up-regulated and 23 proteins down-regulated in the cancerous cell lines compared to the normal cells.

3.2.3.2 Invasive (HMEC+BT20) vs. Non-invasive (MCF-10A+MCF-7)

Proteins that are up-regulated or down-regulated in the normal+cancer ((N+C) invasive cells (HMEC+BT20) compared to normal+cancer (N+C) non-invasive cells (MCF-10A + MCF-7) were investigated to identify invasion-specific targets. 33 proteins were found differentially expressed in this comparison, of which 4 proteins were up-regulated and 29 proteins were down-regulated in the invasive cells compared to non-invasive

cells. We have used **'normal + cancer related invasion**' term for this comparison in this thesis.

3.2.3.3 Cancerous invasive (BT20) vs. Normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7)

To identify protein alterations in the cancer invasive cell lines, the PEMs of the cancerous invasive cell line (BT20) were compared with the PEMs of the normal invasive + normal non-invasive + cancerous non-invasive cells (HMEC+MCF-10A+MCF-7). In this comparison, 52 proteins were found differentially expressed, of which 35 proteins were up-regulated and 17 proteins were down-regulated in BT20 compared to HMEC+MCF-10A+MCF-7. We used **'Cancer-related invasion'** term for this comparison in this thesis.

Table 3.2.1: List of total number of differentially expressed proteins in the comparison of cancerous (MCF-7+BT20) vs. Normal (HMEC+MCF-10A), Invasive (HMEC+BT20) vs. non-invasive (MCF-10A+MCF-7), and cancerous invasive (BT20) vs. normal invasive + normal non-invasive + cancerous non-invasive (HMEC+MCF-10A+MCF-7) and which pass the fold change ≥ 1.5 fold up/down regulated with t-test score of ≤ 0.05.

No.	**Comparison Groups**	**No. of differentially expressed proteins**	**Up-regulated**	**Down-regulated**
1	Cancerous (MCF-7+ BT20) vs. Normal (HMEC+ BT20)	81	56	25
2	Invasive (HMEC+ BT20) vs. Non-invasive (MCF-10A + MCF-7) **(Normal+ cancer-related invasion)**	33	29	4
3	Cancerous invasive (BT20) vs. Normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7) **(Cancer-related invasion)**	52	35	17

3.2.4 Identification of differentially expressed proteins using mass-spectrometry

225 protein spots were picked for identification using MALDI ToF MS and/or LC-MS/MS. The proteins that were ≥ 3 fold up or down-regulated were analysed using LC-MS/MS for identification or to confirm identification from MALDI ToF MS. The criteria for positive identification of peptides were same as mentioned in 3.1.4. The location of identified proteins on a representative 2D-DIGE gel is shown in Figure 3.2.2. The gel showed the master number ID generated by Decyder 6.5 BVA module and refers to the specific protein IDs obtained for that particular protein spot.

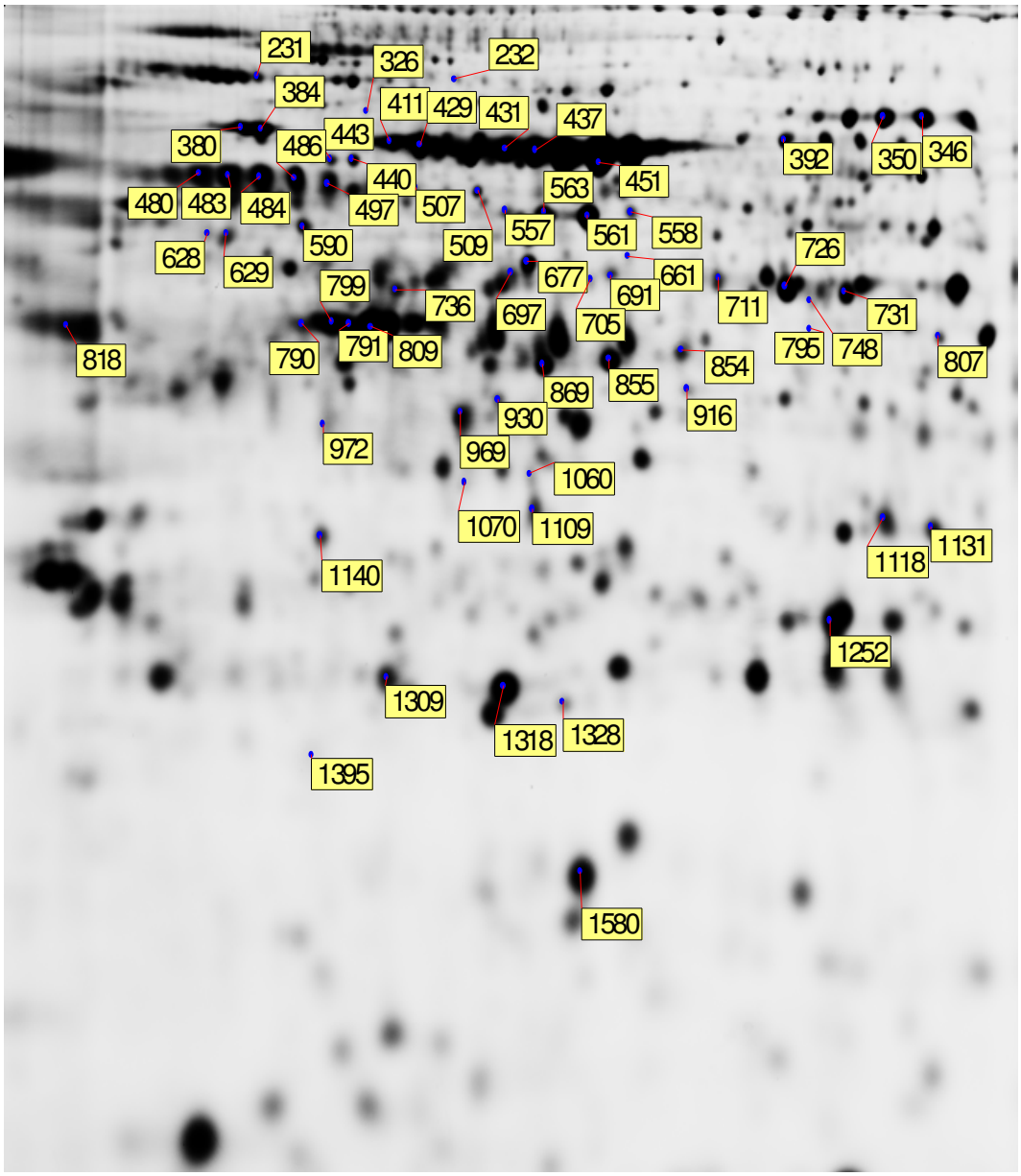


Figure: 3.2.2. Representative 2D-DIGE gel image of the Cy2-labelled pool of HMEC, MCF-10A, MCF-7 and BT20 conditioned media samples showing differentially expressed protein spots that have been successfully identified by MALDI-ToF MS and/or LC-MS/MS. Identified spots are represented on the gel using the Master Number ID generated by the Decyder software as a reference.

3.2.5 List of identified differentially regulated proteins in the cancerous vs. normal and invasive-related comparisons.

3.2.5.1 Cancerous (MCF-7+BT20) vs. normal (HMEC+MCF-10A)

A total of 42 proteins were identified in the comparison of cancerous (MCF-7+BT20) vs. normal (HMEC+MCF-10A) group (Table 3.2.2). Among these proteins, 25 proteins were up-regulated and 17 proteins were down-regulated in the cancer cell lines compared to the normal cells. The cell lines are compared from cancerous to normal and therefore proteins that are up-regulated are increased in the cancerous cell lines and are represented as positive fold changes. The proteins that are down-regulated have negative fold changes, as these proteins are expressed at lower levels in the cancerous cell lines compared to the normal cells. The identified secreted proteins are outlined in table 3.2.2 showing that dysfunction of these proteins/peptides could be associated with the cancerous phenotype.

- The comparison interestingly revealed a range of serine proteases inhibitors including SERPINA1, SERPINB1, SERPINB5, SERPINB7 and SERPINF1, which were 12.5-fold, 7.9-fold, 41.7-fold, 3.8-fold and 3.2-fold down-regulated in the cancer cell lines compared to the normal cells respectively. The secreted SERPINB5 has been reported as tumour suppressor in breast cancer (Dowling *et al.* 2007b, Kulasingam and Diamandis 2007).

- Three members of pro-cathepsins, which are cysteine proteinase proteases (Nakashima *et al.* 2004), were identified as differentially regulated. Pro-cathepsin A (CTSA), pro-cathepsin B (CTSB) and pro-cathepsin D (CTSD) were 2.1-fold, 31.2-fold and 2.4-fold down-regulated in the cancer cell lines compared to the normal cells respectively.

- Nucleobindin 1 precursor (NUCB1), involved in DNA binding and protein to protein interactions (Erickson 1989), were differentially regulated. NUCB1 was identified at 3 different spots, which could be due to post-translational modification, and were 12.5-fold, 12.5 and 14-fold up-regulated in the cancer cell lines compared to the normal cells.

- Ferritin, heavy polypeptide 1 (FHC), an iron-containing protein chain, was 3.6-fold down-regulated in the cancer cell lines compared to the normal cells.

- Protein disulfide isomerase family A, member 4 precursor (PDIA4) was found to be 37.8-fold up-regulated in the cancer cell lines compared to the normal cells.

On the basis of limited information in the literature regarding their potential role in cancer, this study demonstrates that PDIA4, SERPINB1, SERPINB7, NUCB1, CTSA and FHC are high potential protein targets that could have important roles in breast cancer and also could be potential biomarkers for breast cancer. Table 3.2.3 summarises the fold changes of these protein in the cancerous cell lines (MCF-7+BT20) compared to the normal cells (HMEC+MCF-10A).

Table-3.2.2. Proteins identified from the conditioned media from the comparison of cancerous (MCF-7+BT20) vs. normal (HMEC+MCF-10A) groups using MALDI ToF MS and/or LC-MS/MS. 2D-DIGE number refers to location of the identified protein on the gel shown in the Figure 3.2.2. '+' represents up-regulation and '-' represent down-regulation of protein in terms of fold change in the given comparison. (Supplementary data containing additional information of proteins identified: Appendix 5).

DIGE No.	**Gene Symbol**	**Protein Identification**	**Cancer vs. normal Fold Change**	**Biological function**
231	LAMC2	Laminin, gamma 2 isoform a precursor	-4.45	Cell adhesion
232	GSN	Gelsolin precursor	-2.53	Cell structure
	C1R	Complement C1r subcomponent precursor		Complement-mediated immunity
326	TPTE2	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase TPTE2	2.92	Phospholipid metabolism
346	BF	Complement Factor B	3.46	Complement-mediated immunity
350	BF	Complement factor B precursor	3.44	Complement-mediated Immunity
	RDX	Radixin		Cell structure
392	VIL2	Ezrin	5.86	Cell structure
431	PGM2	Phosphoglucomutase 2	9.3	Monosaccharide metabolism
	PLS3	Plastin-3		Cell structure

437	RNPEP	Aminopeptidase B	19.48	Fatty acid biosynthesis Proteolysis
451	PDIA4	Protein disulfide-isomerase A4 precursor	37.88	Protein disulfide-isomerase Reaction
480	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade A member 1	8.31	Proteolysis
483	NUCB1	Nucleobindin 1 precursor	12.55	Calcium ion homeostasis
484	NUCB1	Nucleobindin 1 precursor	12.56	Calcium ion homeostasis
486	NUCB1	Nucleobindin 1 precursor	14.04	Calcium ion homeostasis
497	HSPA6	Heat shock 70 kDa protein 6	8.47	Stress response
590	PDIA3	Protein disulfide-isomerase A3 precursor	3.82	Protein disulfide-isomerase reaction
628	TUBB	Tubulin beta	3.15	Cell structure Cell motility
629	NUCB2	Nucleobindin-2 precursor	3.66	Calcium ion homeostasis
661	CTSA	Lysosomal protective protein precursor (Cathepsin A)	-2.11	Cell surface receptor mediated signal transduction
691	SERPINF1	Pigment epithelium-derived factor precursor	-3.29	Proteolysis
697	CTSD	Cathepsin D preproprotein	-2.41	Proteolysis

731	SERPINB7	Serpin B7	-3.8	Proteolysis
790	ACTB	Beta actin	5.34	Cytokinesis Cell structure
791	gi|58082049	Hypothetical protein LOC123876	3.5	
795	INHBA	Inhibin beta A precursor	-2.86	Cell proliferation and differentiation
799	NUDC	Nuclear migration protein nudC	3.58	Cell proliferation and differentiation
	KRT18	Keratin, type I cytoskeletal 18		Cell structure
807	GLUL	Chain A, Glutamine Synthetase	1.68	Amino acid biosynthesis Nitrogen metabolism
809	ACTG1	Actin, gamma 1 propeptide	4.9	Cytokinesis, Cell structure
	KRT10	Keratin, type I cytoskeletal 10		Cell structure
818	VIM	Vimentin	-6.02	Cell structure
854	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade B, member 1	-7.98	Proteolysis
855	SERPINB5	Serpin B5 precursor (Maspin)	-41.75	Proteolysis
869	CTSB	Procathepsin B	-31.23	Proteolysis, Other oncogenesis
930	DDAH1	DDAH1 protein	2.59	Cell proliferation and differentiation

972	APOE	Apolipoprotein E precursor	-6.12	Lipid and fatty acid transport
1070	TSP	Thrombospondin-1 precursor	-1.7	Developmental process
1109	CAPZB	F-actin-capping protein subunit beta	1.72	Cell structure
1118	NP	Nucleoside phosphorylase	1.51	mRNA transcription regulation
1252	PRDX6	Peroxiredoxin 6	1.68	Antioxidation and free radical removal
1309	GSTP1	Glutathione S-transferase P	-21.77	Sulfur redox metabolism
1318	SERPINA1	Alpha-1-antitrypsin precursor	-12.58	Proteolysis
1328	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein precursor (fragment)	-5.05	Cell adhesion
1395	FHC	Ferritin, heavy polypeptide 1	-3.63	Oogenesis
1580	SOD1	Superoxide dismutase	2.62	Immunity and defense

Table 3.2.3: High priority secreted protein targets from the comparison of cancerous (MCF-7+BT20) vs. normal (HMEC+MCF-10A) group that might have a significant role in cancer.

2D-DIGE No.	Gene Symbol	Protein Identification	Fold Change
451	PDIA4	Protein disulfide-isomerase A4 precursor	37.88
486	NUCB1	Nucleobindin 1 precursor	14.04, 12.55 and 12.56
661	CTSA	Lysosomal protective protein precursor (Cathepsin A)	-2.11
691	SERPINB7	Serpin B7	-3.8
854	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade B, member 1	-7.98 and 8.31
1395	FHC	Ferritin, heavy polypeptide 1	-3.63

3.2.5.2 Invasive (HMEC+BT20) vs Non-invasive (MCF-10A+MCF-7) (Normal + cancer-related invasion)

Table 3.2.4 lists the proteins that have been identified in the N+C invasive (HMEC+BT20) vs. N+C non-invasive (MCF-10A+MCF-7) comparison. A total of 15 proteins were identified, of which 14 proteins were up-regulated and 1 protein was down-regulated in the N+C invasive cells compared to the N+C non-invasive cells (Table 3.1.4). The N+C invasive cells were compared to N+C non-invasive and therefore up-regulated proteins in the N+C invasive cells have been denoted by positive fold changes. The proteins that are down-regulated have negative fold changes as these proteins are expressed at lower levels in the N+C invasive cells compared to the N+C non-invasive cells. The proteins identified in this comparison may have a role in the invasion of normal and cancer cells.

Some well known proteins, like heat shock proteins (HSP90 and HSP5) and disulfide isomerase family A, member 3 precursor (PDIA3), that have been already correlated with invasion, were observed to be differentially expressed in this comparison.

- Heat shock proteins (HSP90 and HSP5), that are induced stress were found differentially expressed in this comparison. The heat shock protein 90-beta (HSP90) and heat shock 70kDa protein 5 (HSP5) were up-regulated in the N+C invasive cells compared to the N+C non-invasive cells by 3.1-fold and 2.9-fold, respectively.

- Protein disulfide isomerase-associated 3 precursor (PDIA3) is a soluble ER luminal protein that belongs to protein disulfide isomerase family. It was identified at 3 different spots in the gel and this could be due to post-translational modifications. PDIA3 was found to be 1.8-fold, 2-fold and 2.2-fold up-regulated in the N+C invasive cells compared to the N+C non-invasive cells.

Some proteins, like TALDO1 and GSS, were also identified from this comparison. The role of these proteins in breast cell invasion is poorly known.

- Transaldolase (TALDO1), involved in the pentose-phosphate pathway, was found to be 2.5-fold up-regulated in the N+C invasive cells compared to the N+C non-invasive cells.

- Glutathione synthetase (GSS), involved in synthesis of glutathione, was found to be 1.8-fold up-regulated in the N+C invasive cells compared to the N+C non-invasive cells.

On the basis of limited information in the literature regarding their potential role in breast invasion, TALDO1 and GSS proteins are potentially novel findings that could have important roles in the normal and cancer breast invasion.

Table 3.2.4**. Invasive vs. non-invasive (Normal + cancer-related invasion)**: Proteins identified from the comparison of Non-invasive (MCF-10A + MCF-7) vs. Invasive (HMEC+ BT20) group using MALDI ToF MS and/or LC-MS/MS. 2D-DIGE number refers to location of the identified protein on the gel shown in the Figure 3.2.2. '+' represents up-regulation and '-' represent down-regulation of protein in terms of fold change in the given comparison. (Supplementary data containing additional information of proteins identified: Appendix 5).

2D-DIGE No	**Gene symbol**	**Protein Identification**	**Invasive vs. Non-invasive**	**Biological function**
380	HSPA5	Heat shock 70kDa protein 5	2.92	Stress response
384	HSP90	Heat shock protein HSP 90-beta	3.11	Stress response
	PDIA3	Protein disulfide-isomerase A3 precursor		Protein disulfide-isomerase reaction
	BAT1	Spliceosome RNA helicase BAT1		Nucleic acid metabolism
557	FKBP4	FK506-binding protein 4	2.21	Protein folding
558	PDIA3	Protein disulfide isomerase-associated 3 precursor	1.84	Protein disulfide-isomerase reaction
561	PDIA3	Protein disulfide isomerase-associated 3 precursor	2.05	Protein disulfide-isomerase reaction
563	PDIA3	Protein disulfide isomerase-associated 3	2.29	Protein disulfide-isomerase

		precursor		reaction
677	GSS	Glutathione synthetase	1.82	Sulfur redox metabolism
705	CTSD	Cathepsin D preproprotein	-2.01	Proteolysis
	GDI2	Rab GDP dissociation inhibitor beta		Intracellular signaling cascade
	SSB	Lupus La protein		tRNA metabolism
711	AHCY	Adenosylhomocysteinase	2.43	Purine metabolism
726	PA2G4	Proliferation-associated protein 2G4	3.18	Cell proliferation and differentiation
	HSPA5	Heat shock 70kDa protein 5		Stress response
736	EIF4A	Eukaryotic initiation factor 4A-I	3.35	Translational regulation
748	CPA4	Chain A, Procarboxypeptidase A4	2.1	Proteolysis
916	TALDO1	Transaldolase 1	2.07	Pentose phosphate pathway
969	CAPZA	F-actin capping protein alpha-1 subunit	1.93	Cell structure
1060	ANXA3	Annexin A3	2.73	Lipid, fatty acid and steroid metabolism

3.2.5.3 Cancerous invasive (BT20) vs. Normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7) (Cancer-related invasion)

Table 3.2.5 outlines the identified proteins in the **Group 1** (cancer invasive cell line (BT20)) vs. **Group 2** (Normal invasive+ normal non-invasive+ cancerous non-invasive cells (HMEC+MCF-10A+MCF-7)) comparison. A total of 28 proteins were identified in this comparison group (Table 3.1.5). Among these proteins, 19 proteins were up-regulated and 9 proteins were down regulated in group 1 compared to group 2. The cell lines are compared from **Group 1** vs. **Group 2** and therefore proteins that are up-regulated in **Group 1** are represented as positive fold change. The proteins that are down-regulated in group 1 compared to group 2 have negative fold change.

Some well known proteins, like plastin-2 (LCP1) and PDIA3 that have already been correlated with cancer invasion were differentially expressed in this comparison.

- Plastin-2 (LCP1), an actin filament binding protein, was identified at 2 different spots which could possibly due to post-translational modification of this protein. LCP1 was found to be 9.8 and 7.9-fold up-regulated in group 1 compared to group 2.

- Protein disulfide isomerase-associated 3 precursor (PDIA3) was observed to be 1.8-fold up-regulated in group 1 compared to the group 2.

NUCB2, DDAH1 and HSPG2 were also identified in this comparison of the cancer invasive cell line (BT20) vs. normal invasive+ normal non-invasive+ cancerous non-invasive cells (HMEC+MCF-10A+MCF-7). The role of these proteins in breast cancer cell invasion is poorly known.

- Dimethylarginine dimethylaminohydrolase 1 (DDAH1) plays a role in nitric oxide generation by regulating cellular concentrations of methylarginines, which in turn inhibit nitric oxide synthase activity (Miura *et al.* 1992, Lin *et al.* 1998). DDAH1 was 2.93 fold up-regulated in group 1 compared to group 2.

- Nucleobindin 2 precursor (NUCB2), which is involved in DNA binding and protein to protein interactions (Cartwright *et al.* 1999), was found to be 5.2-fold up-regulated in group 1 compared to group 2.

- Fragment of Heparan sulfate proteoglycan 2 precursor (HSPG2), a basement protein involved in cell adhesion (Miura *et al.* 1992, Lin *et al.* 1998), was found to be 5.3-fold down-regulated group 1 compared to group 2.

On the basis of limited information in the literature regarding their potential role in breast cancer invasion DDAH1, HSPG2 and NUCB2 proteins are potentially novel findings that could have important role in breast cancer invasion.

Table 3.2.5. **Group 1** (BT20) **vs. Group 2** (MCF-10A+HMEC+MCF-7) **(Cancer-related invasion)**: Proteins identified from the Group 1 vs. Group 2 using MALDI ToF MS and/or LC-MS/MS. 2D-DIGE number refers to location of the identified protein on the gel shown in the Figure 3.2.2. '+' represents up-regulation and '-' represent down-regulation of protein in terms of fold change in the given comparison. (Supplementary data containing additional information of proteins identified: Appendix 5).

2D-DIGE No.	**Gene symbol**	**Protein Identification**	**Group 1 vs. Group 2**	**Biological function**
	GSN	Gelsolin precursor		Cell structure
232	C1R	Complement C1r subcomponent precursor	-3.22	Complement-mediated Immunity
326	TPTE2	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase TPTE2	2.46	Phospholipid metabolism
411	HSPA8	Heat shock 70kDa protein 8 isoform 1	2.36	Stress response
429	HSPA1B	Heat shock 70kDa protein 1B	3.01	Stress response
	PGM2	Phosphoglucomutase 2		Monosaccharide metabolism
431	PLS3	Plastin-3	4.37	Cell structure
437	RNPEP	Aminopeptidase B	5.37	Fatty acid biosynthesis Proteolysis
440	LCP1	Plastin-2	9.81	Cell structure

443	LCP1	Plastin-2	7.99	Cell structure
451	PDIA4	Protein disulfide-isomerase A4 precursor	5.74	Protein disulfide-isomerase Reaction
509	CCT8	T-complex protein 1 subunit theta	2.78	Protein folding
558	PDIA3	Protein disulfide isomerase-associated 3 precursor	1.85	Protein disulfide-isomerase Reaction
628	TUBB	Tubulin beta	3.02	Cell structure, Cell motility
629	N UCB2	Nucleobindin-2 precursor		Calcium ion homeostasis
677	GSS	Glutathione synthetase	1.72	Sulfur redox metabolism
691	SERPINF1	Pigment epithelium-derived factor precursor	-3.3	Proteolysis
697	CTSD	Cathepsin D preproprotein	-2.26	Proteolysis
705	CTSD	Cathepsin D preproprotein	-2.19	Proteolysis
736	HSPA5	Heat shock 70kDa protein 5	3.75	Stress response
	EIF4A	Eukaryotic initiation factor 4A-I		Translational regulation
763	CBS	Chain A, Recombinant Procathepsin B	-6.58	Proteolysis
807	GLUL	Chain A, Glutamine Synthetase	1.7	Sulfur redox metabolism
854	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade B, member 1	-9.95	Proteolysis
916	TALDO1	Transaldolase 1	2.5	Pentose-phosphate shunt

930	DDAH1	DDAH1 protein	2.93	Cell proliferation and differentiation
972	APOE	Apolipoprotein E precursor	-5.69	Lipid and fatty acid Transport
1131	TPI1	Triosephosphate isomerase	5.15	Glycolysis
1140	CLIC1	Chloride intracellular channel 1	1.83	Anion transport
1318	SERPINA1	Alpha-1-antitrypsin precursor	-11.21	Proteolysis
1328	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein precursor (fragment)	-5.33	Cell adhesion

3.2.6 Identification of invasion-related proteins by overlapping differentially expressed proteins from normal cells with cancerous cells

The aim of this analysis was to identify secreted proteins that are only differentially expressed in the invasive phenotypes or in the non-invasive phenotypes. Two lists of differentially expressed proteins from the comparison of the normal non-invasive vs. normal invasive cells (MCF-10A vs. HMEC) and the cancerous non-invasive vs. cancer invasive cell lines (MCF-7 and BT20) were generated (supplementary data: Appendix 2). The two generated differentially expressed protein lists were overlapped, determining the unique and common expressing proteins between the lists. A venn diagram outlining the distribution of proteins between the lists of differentially expressed protein in a comparison of cancerous non-invasive (MCF-7) vs. invasive (BT20) cell lines and normal-non-invasive (MCF-10A) vs. invasive (HMEC) cells is shown in figure 3.2.3.

The overlapping identified 9 unique proteins, including PRDX6, SOD1, ARP3, ANXA3, VIM, HC8, THBS1 and SERPINA1 in the normal comparison group (MCF-10A vs. HMEC) and alterations in the expression of these proteins could potentially be associated with normal-invasion in cells. Out of these 9 proteins PRDX6 and SOD1 were up-regulated in the normal non-invasive cells (MCF-10A) compared to the normal invasive cells (HMEC). 4 proteins (VIM, HC8, THBS1 and SERPINA1) were found down-regulated in the normal non-invasive cells (MCF-10A) compared to the normal invasive cells (HMEC).

A total of 27 proteins, including well known cancer and invasion-associated proteins (HSPA5, GNS, ACTB and HSP90) as well as other proteins (DDAH1, NUCB2 and SERPINB7) which are poorly known for their role in invasion, were found to be differentially expressed only in the cancer cell line comparison group (MCF-7 vs. BT20) and these proteins are possibly involved in the regulation of the invasive phenotype in breast cancer cells.

5 proteins were common in both lists of differentially expressed proteins (MCF-10A vs. HMEC and MCF-7 vs. BT20). One of these proteins, LAMC2 was down-regulated in both comparisons. The second protein spot contained 3 identifications (PDIA3/BAT1/ FKBP4)

so it is unclear at this stage which of these proteins is down-regulated in this comparison. The remaining 3 proteins include HSP60, CTSB and SERPINB1. HSP60 was up-regulated whereas CTSB and SERPINB1 were down-regulated in the cancer-invasive cell line (BT20) compared to the cancer-non-invasive cell line (MCF-7). HSP60 was down-regulated while CTSB and SERPINB1 were up-regulated in the normal-invasive cells (HMEC) compared to the normal-non-invasive cells (MCF-10A).

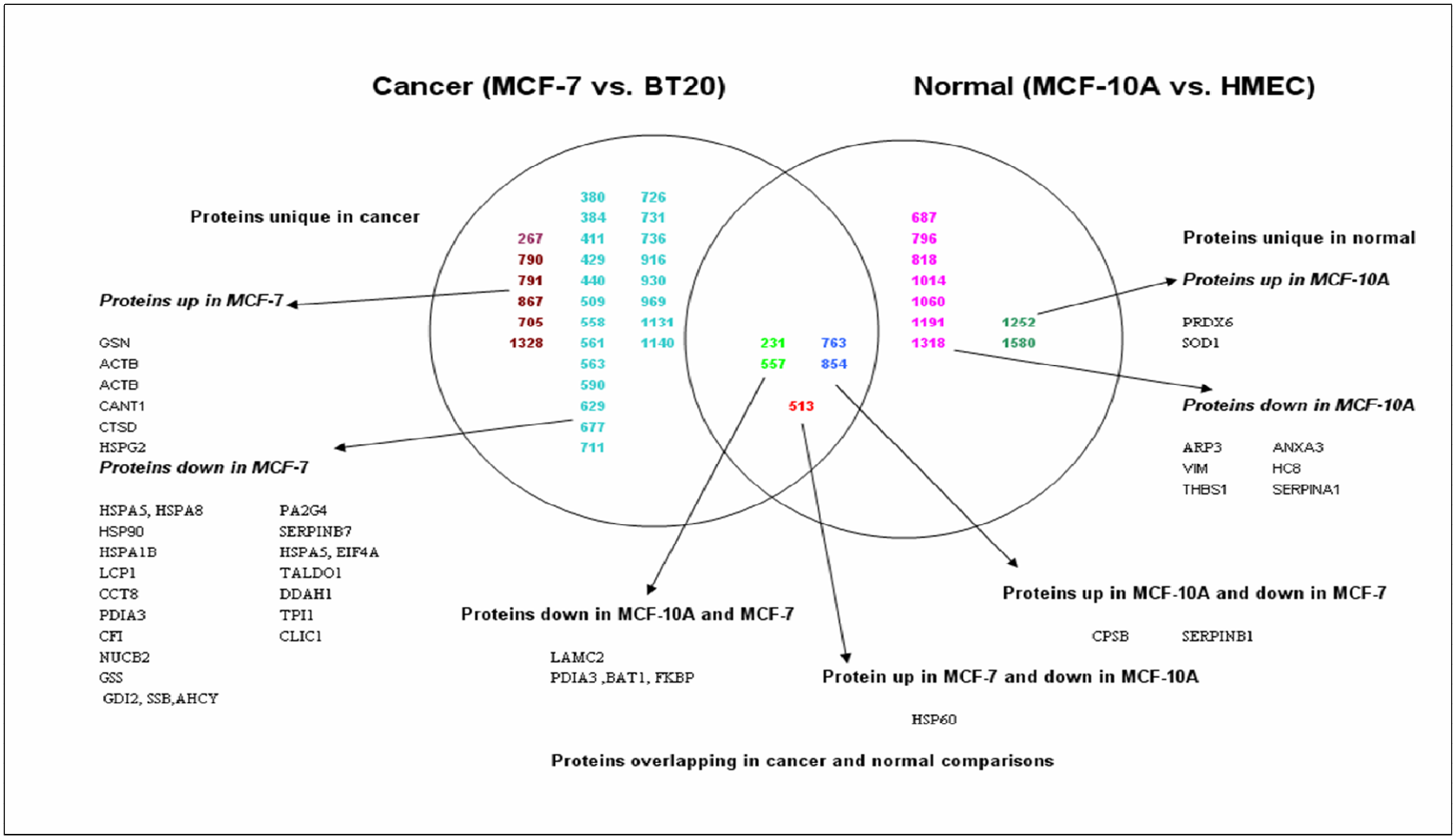


Figure 3.2.3. Venn diagram of differentially expressed overlapping secreted proteins of comparison in normal non-invasive (MCF-10A) vs. invasive (HMEC) cells and in cancer non-invasive (MCF-7) vs. Invasive (BT20). Proteins are represented by the 2D-DIGE number inside the circle and gene symbols outside the circle of protein.

3.2.7 Validation of Proteomics data by Western blot analysis

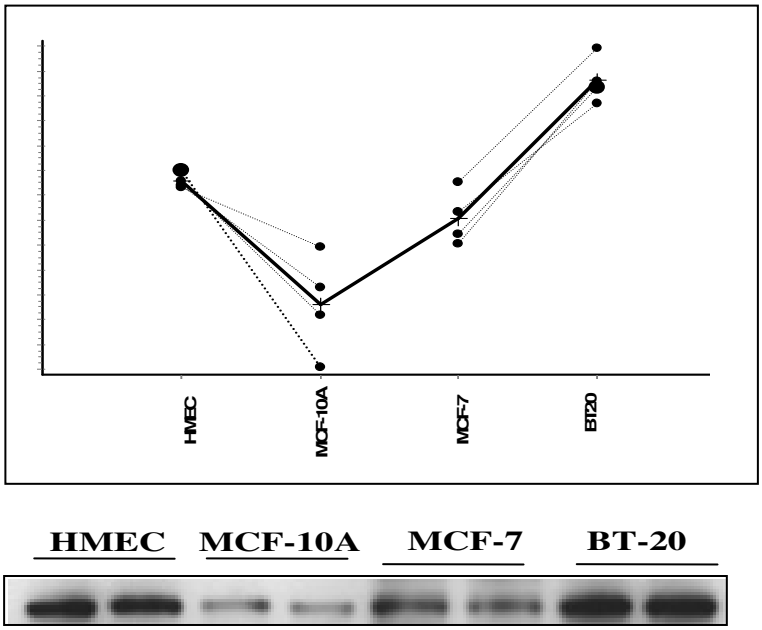
In order to validate the expression pattern as observed in 2D-DIGE analysis and identifications by MALDI-ToF and/or LC-MS/MS, Western blot analysis was carried out for selected proteins targets (Bip, Procathepsin B, Maspin, Nucleobindin 1 Precursor and Transaldolase-1) on conditioned media samples from the four breast cell lines (HMEC, MCF-10A, MCF-7 and BT20). Proteins from the conditioned media of biological duplicate samples were separated using 10% SDS gels and were blotted with antibodies as described in section 2.8.

Maspin (SERPINB5) and Procathepsin B were highly up-regulated in the normal cells (MCF-10A+HMEC) compared to the cancer cell lines (MCF-7+BT20). Nucleobindin 1 Precursor was also chosen for validation as it was highly down-regulated in this study in normal cell lines (HMEC+MCF-10A) compared to the cancer cell lines (MCF-7+BT20). Heat shock protein 70 protein 5 (BIP) and transaldolase-1 were chosen on the basis of antibody availability for them in the laboratory. Transaldolase-1 was also identified from 2D-DIGE of cell lysates from HMEC, MCF-10A, MCF-7 and BT20, and was validated by Western blotting (Figure 3.2.4-3.2.6), but in the conditioned media study, it showed a different expression pattern compared to the cell lysates study and therefore was chosen to validate its pattern in conditioned media samples using Western blot analysis.

Bip, Procathepsin B, Maspin, Nucleobindin 1 Precursor and Transaldolase-1 confirmed the trends observed in the proteomic analysis. Figures 3.2.4 to 3.2.6 show the expression pattern of these selected proteins observed using 2D-DIGE and Western blot analysis. Table 3.2.6 and 3.2.7 summarize the expression patterns observed in 2D-DIGE and western blot analysis for the targets.

Commassie stained gels was used to demonstrate as best as possible equal loading between the samples (Figure 3.2.7).

Heat shock protein 5 (BIP)



Procathepsin B

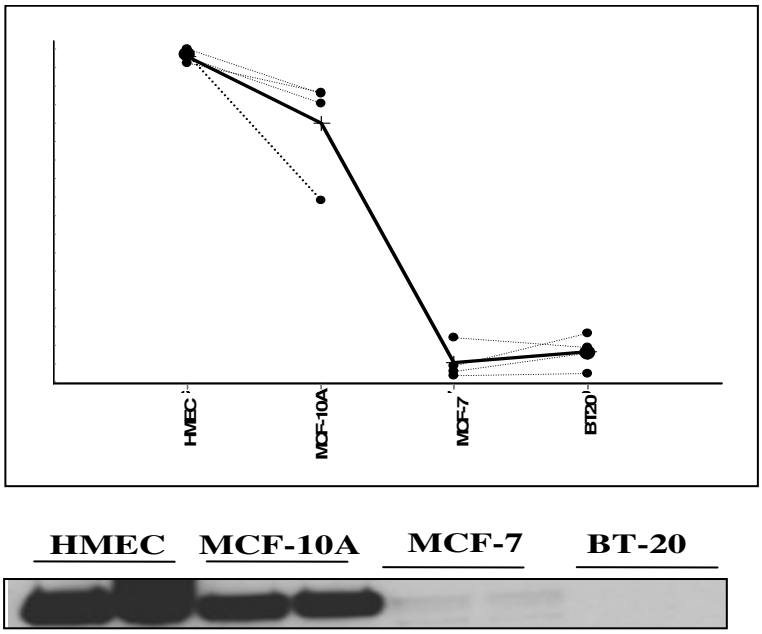
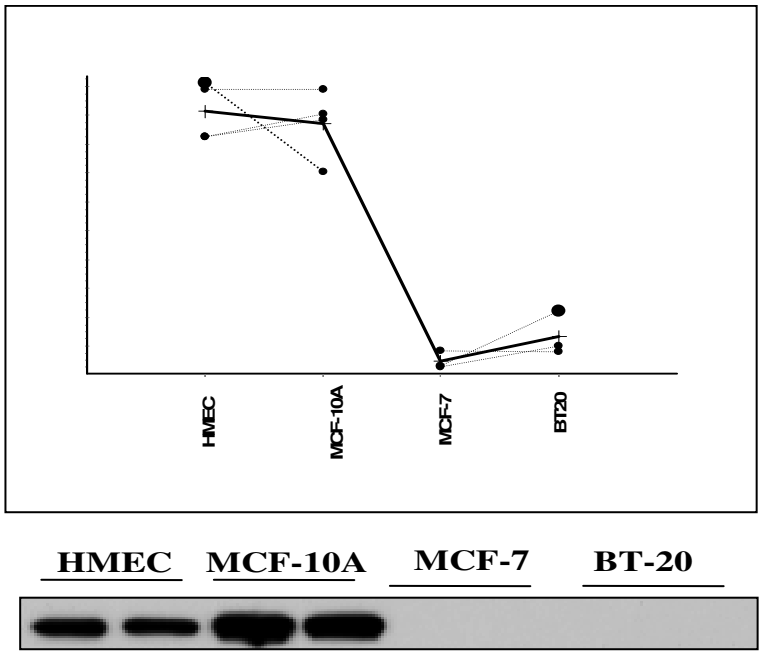


Figure 3.2.4. Standardized log abundance graph view (Decyder BVA module) and Western blot images of BIP and Procathepsin B protein expression from HMEC, MCF-10A, MCF-7 and BT20. Biological duplicate samples were used in this investigation.

Maspin



Nucleobindin 1 Precursor

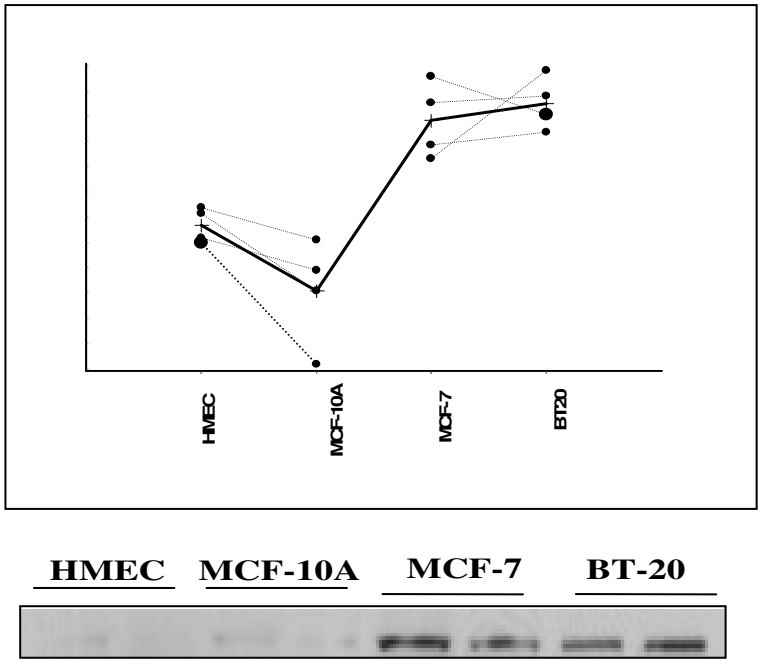


Figure 3.2.5. Standardized log abundance graph view (Decyder BVA module) and Western blot images of Maspin (SERPINB5) and Nucleobindin 1 Precursor protein expression from HMEC, MCF-10A, MCF-7 and BT20. Biological duplicate samples were used in this investigation.

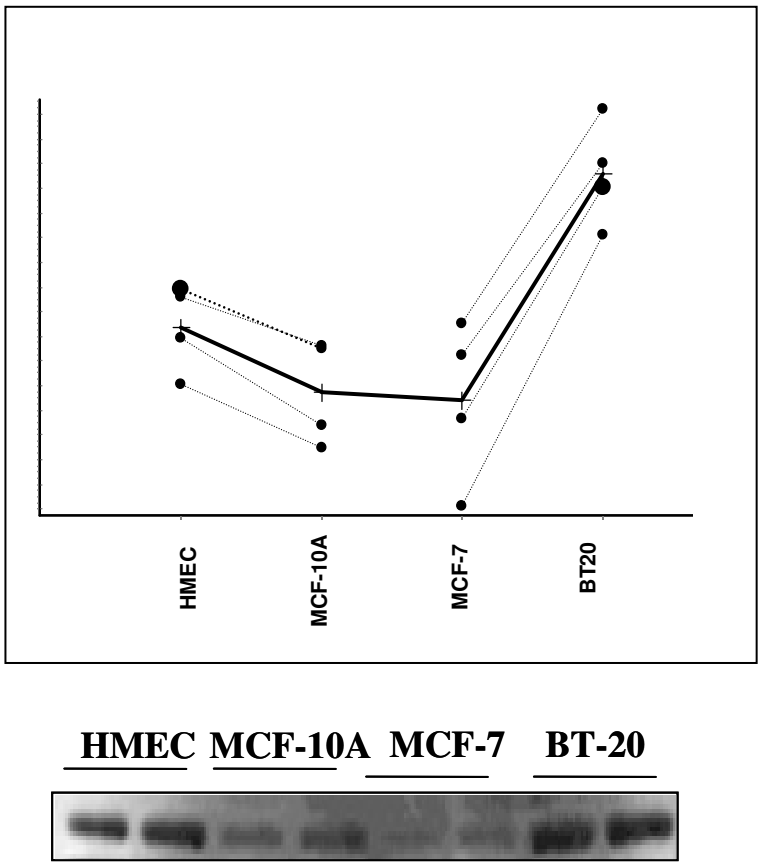


Figure 3.2.6. Standardized log abundance graph view (Decyder BVA module) and Western blot images of Transaldolase-1 protein expression from HMEC, MCF-10A, MCF-7 and BT20. Biological duplicate samples were used in this investigation.

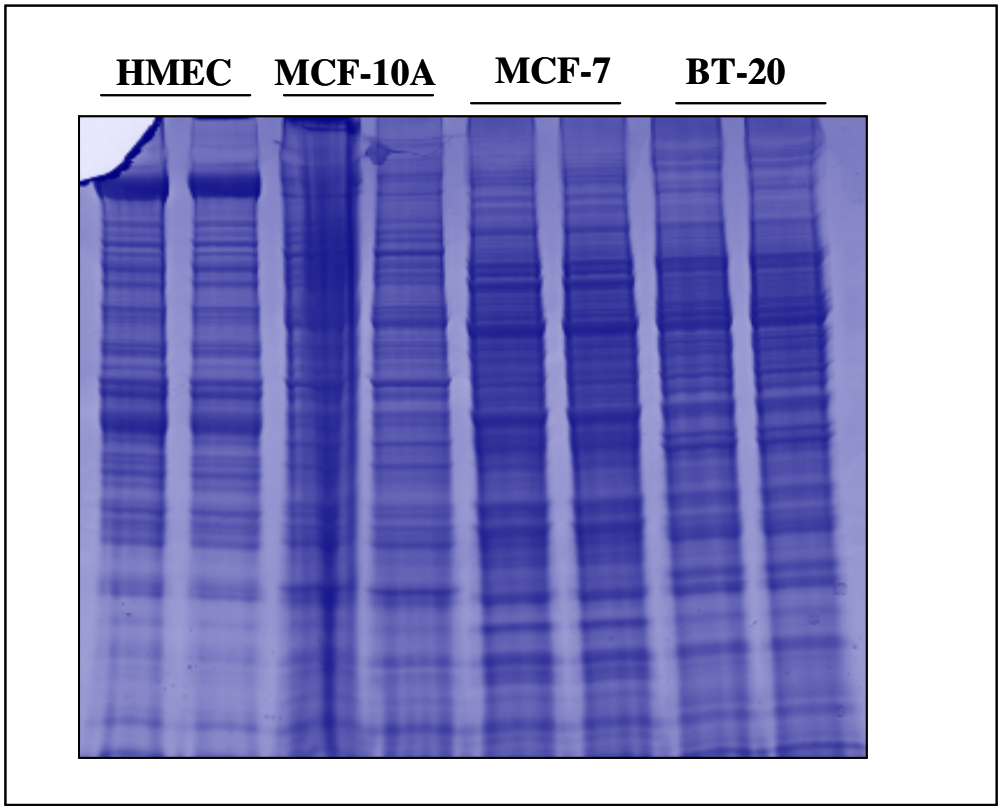


Figure 3.2.7. represents coomassie stained gel of conditioned media samples from HMEC, MCF-10A, MCF-7 and BT20 to demonstrate even loading between the samples.

The differential expression of proteins observed in 2D-DIGE and results observed in western blot analysis has been summarised in Table 3.2.7.1 and 3.2.7.2.

Table 3.2.6. Expression pattern of Procathepsin B, Maspin and Nucleobindin 1 Precursor observed in 2D-DIGE and WB analysis. (↑) up-regulated, (↓) down-regulated, (NDE) not significantly differentially expressed.

Protein name	**2D-DIGE expression pattern (cancer vs. normal)**	**WB expression pattern (cancer vs. normal)**
Procathepsin B	↓	↓
Maspin	↓	↓
Nucleobindin 1 Precursor	↑	↑

Table 3.2.7. Expression pattern of Nm23, Transaldolase-1 and STIP-1 observed in 2D-DIGE and WB analysis. (↑) up-regulated, (↓) down-regulated, (NDE) not significantly differentially expressed.

Protein name	**Analysis**	**MCF-10A vs. MCF-7**	**MCF-7 vs. BT20**	**HMEC vs. BT20**	**MCF-10A vs. HMEC**
BIP	2D-DIGE	↓	↓	↑	↓
	WB	↓	↓	↑	↓
Transaldolase-1	2D-DIGE	NDE	↓	↓	NDE
	WB	NDE	↓	↓	NDE

3.3 Proteomic analysis of isolated membrane fraction from HMEC, MCF-10A, MCF-7 and BT20

Membranes serve as a barrier between cells and the external environment. Invasive cancer cells have the ability to break signalling contact with neighbouring cells, degrade and penetrate the basement membrane. This process can disturb the normal function of a range of membrane and membrane-associated proteins such as adhesion molecules, transduction receptors and transport proteins. Therefore, investigation of protein expression changes in breast cell lines representative of normal, non-invasive and invasive phenotypes might be helpful to understand biological mechanisms leading to breast cancer. For this, differential proteomics study of isolated membrane fractions from HMEC, MCF-10A, MCF-7 and BT20 cells were carried out.

3.3.1 Experimental outline

Investigations were carried out to establish differential protein expression of membrane and membrane-associated proteins as previously discribed in section 3.1.2. For this, four biological samples from HMEC, MCF 10A, MCF-7 and BT20 were prepared separately. Each cell line sample was labelled with 2D-DIGE dyes (i.e. Cy3 and Cy5) as outlined in Table 3.1.2. A pooled internal control containing a mixture of all biological replicate samples in the experiment was labelled with Cy2 dye as discribed in section 2.7.3.2.

3.3.2 Analysis of differential expression of proteins using DeCyder

2D-DIGE protein expression maps (PEMs) for isolated membrane fractions from HMEC, MCF 10A, MCF-7 and BT20 were created. Analysis was carried out as described in section 3.1.3. After spot matching and filtering, a total of 614 protein spots were matched across all of the gels in the experiment (24 gels in total). Spots that were ±1.5 fold up or down-regulated with p-value $\leq$ 0.05 were considered significantly differentially expressed (DE).

3.3.2.1 Cancerous (MCF-7+ BT20) vs. Normal (HMEC+ BT20)

The objective was same for this analysis as previously described in sections 3.1.3.1 and 3.2.3.1 to identify membrane and membrane-associated proteins altered in the cancerous cell lines (MCF-7+BT20) in comparison to the normal cells (HMEC+ BT20). This comparison revealed altered expression of 78 proteins, of which 30 proteins were up-

regulated and 48 proteins were down-regulated in the cancerous cell lines (MCF-7+BT20) compared to the normal cells (HMEC+ BT20).

3.3.2.2 Invasive (HMEC+BT20) vs. Non-invasive (MCF-10A+MCF-7)

The objective was same for this analysis as previously described in section 3.1.3.2 and 3.2.3.2 to identify changes in membrane and its associated proteins from cell lines representing invasive (normal+cancerous (N+C)) and non-invasive (normal+cancerous(N+C)) phenotypes. 73 proteins were found differentially expressed in this comparison with 59 up-regulated and 14 down-regulated in the invasive cells (HMEC+BT20) compared to the non-invasive cells (MCF-10A+MCF-7). We have used **'normal + cancer related invasion**' term for this comparison in this thesis.

3.3.2.3 Cancerous invasive (BT20) vs. Normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7)

The objective was same for this analysis as previously described in section 3.1.3.3 and 3.2.3.3 to investigate membrane and membrane-associated protein changes in the cancerous invasive cell line (BT20) compared to the normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7). In this comparison, 83 proteins were found differentially expressed, of which 66 proteins were up-regulated and 17 proteins were down-regulated in BT20 compared to HMEC+MCF-10A+MCF-7. We used the **'Cancer-related invasion'** term for this comparison in this thesis.

Table 3.3.2 summaries the total number of differentially expressed proteins observed in the comparison of cancerous (MCF-7+BT20) vs. normal (HMEC+MCF-10A), invasive (HMEC+BT20) vs. non-invasive (MCF-10A+MCF-7) and cancerous invasive (BT20) vs. normal invasive + normal non-invasive + cancerous non-invasive (HMEC+MCF-10A+MCF-7) cells.

Table 3.3.1: List of total number of differentially expressed proteins in the comparison of cancerous (MCF-7+BT20) vs. normal (HMEC+MCF-10A), invasive (HMEC+BT20) vs. non-invasive (MCF-10A+MCF-7) and cancerous invasive (BT20) vs. normal invasive + normal non-invasive + cancerous non-invasive (HMEC+MCF-10A+MCF-7) groups and which pass the fold change ≥ 1.5 fold up/down regulated with t-test score of ≤ 0.05.

No.	**Comparison Groups**	**No. of differentially expressed proteins**	**Up-regulated**	**Down-regulated**
1	Cancerous (MCF-7+ BT20) vs. Normal (HMEC+ BT20)	95	41	54
2	Non-invasive (MCF-10A + MCF-7) vs. Invasive (HMEC+ BT20) **(Normal+cancer-related invasion)**	85	63	22
3	Cancerous invasive (BT20) vs. Normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7) **(Cancer-related invasion)**	109	87	22

3.3.3 Identification of differentially expressed proteins using mass-spectrometry

328 protein spots were picked from all four cell lines for identification using MALDI ToF MS and/or LC-MS/MS. The proteins that were ≥ 3 fold up or down-regulated were analysed using LC-MS/MS for identification or to confirm identification from MALDI ToF MS. The criteria for positive identification of peptides were same as discribed in section 3.1.4. The location of identified proteins on a representative 2D-DIGE gel is shown in Figure 3.3.1. The gel represents the master number ID generated by Decyder 6.5 BVA module which refers to the specific protein IDs obtained for that particular protein spot.

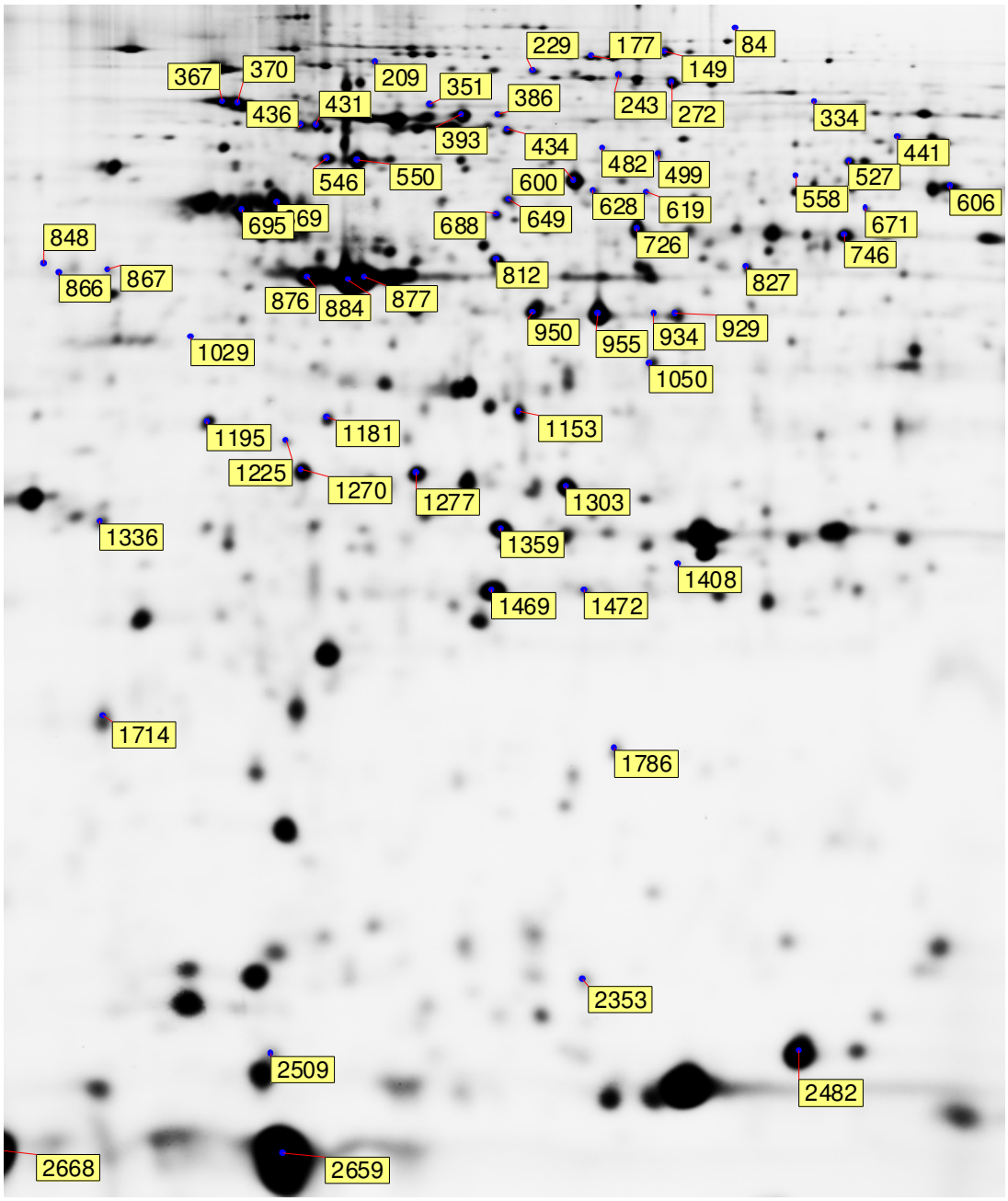


Figure: 3.3.1. Representative 2D-DIGE gel image of the Cy2-labelled pool of HMEC, MCF-10A, MCF-7 and BT20 membrane fraction samples showing differentially expressed protein spots that have been successfully identified by MALDI-ToF MS and/or LC-MS/MS. Identified spots are represented on the gel using the Master Number ID generated by the Decyder software as a reference.

3.3.4 List of identified proteins differentially regulated in the cancerous vs. normal and invasive-related comparisons.

3.3.4.1 Cancerous (MCF-7+BT20) vs. normal (HMEC+MCF-10A)

We identified 41 proteins which were found to be altered in the cancerous cell lines (MCF-7+BT20) in comparison to the normal cells (HMEC+MCF-10A) (Table 3.3.2). Among these proteins, 11 proteins were up-regulated and 30 proteins were down-regulated in the cancerous cell lines compared to the normal cells. The cells are compared from cancerous to normal and therefore proteins that are up-regulated are increased in the cancerous cell lines compared to the normal cells and are represented as positive fold changes. The proteins that are down-regulated have negative fold changes, as these proteins are expressed at lower levels in the cancerous cell lines compared to the normal cells.

- Interestingly, a number of mitochondrial membrane and membrane-associated proteins (mitochondrial Rho GTPases 2 (RHOT2), prohibitin (PHB), mitofilin (IMMT) and ATP synthase subunit beta, mitochondrial precursor (ATP5B) were observed to be altered in expression in the cancer cell lines compared to the normal cells. For examples, RHOT2 is a member of the novel subgroup of the Rho GTPases which are structurally different from Rho GTPases. The role of RHOT2 is in mitochondrial homeostasis and in apoptosis (Iozzo 1984). RHOT2 was found 4.2-fold down-regulated in the cancerous cell lines compared to the normal cells. Another protein, PHB, localized to the inner mitochondrial membrane which helps in the maintenance of mitochondrial function (Fransson *et al.* 2006), was found 2.7-fold up-regulated cancerous cell lines compared to the normal cells and it has been previously reported to be over-expressed in breast cancer cells (Mishra *et al.* 2005).

- A number of plasma membrane and membrane-associated S100 proteins were found to be down-regulated in the cancerous cell lines compared to the normal cells in this study. S100A2, which is well known to be associated with breast cancer (Jupe *et al.* 1996), was found to be 182.9-fold down-regulated in the cancerous cell lines compared to the normal cells. Other members of the S100 protein family including S100A6, S100A9, S100A14 and S100A16 were also found 18-fold, 1.5-fold, 3.3-fold and 2.2-fold down-regulated in the cancerous

cell lines compared to the normal cells, respectively. The roles of S100A6, S100A9, S100A14 and S100A16 in breast cancer are poorly known.

- SERPINB5 (maspin) was found to be 100.8-fold highly down-regulated in the cancerous cell lines compared to the normal cells. SERPINB5 was also found down regulated in the cancerous cell lines compared to the normal cells in the 2D-DIGE studies of cell lysates and conditioned media as described in sections 3.1.5 and 3.2.5.

A number of other proteins, for which very little information is available for the role of these proteins in breast cancer, were observed to be differentially expressed in this comparison. For example-

- FK506 binding protein 5 (FKBP5), also named as FKBP51, was observed to be 4.22 fold down regulated in the cancerous cell lines compared to the normal cells.

- Alpha-soluble NSF attachment protein (ASNAP1) a plasma membrane protein involved in intracellular protein traffic, was found to be 1.85-fold up-regulated in the cancerous cell lines compared to the normal cells.

On the basis of limited information in the literature regarding their potential role in breast cancer, RHOT2, IMMT, FKBP5, ASNAP1 and S100A14 proteins are potentially novel findings that could have important roles in breast cancer and also could serve as potential biomarkers for breast cancer. Table 3.3.3 summarises the fold changes of these protein in the cancerous cell lines compared to the normal cells.

Table-3.3.2. Proteins identified from the isolated membrane fraction for the comparison of cancerous (MCF-7+BT20) vs. normal (HMEC+MCF-10A) groups using MALDI ToF MS and/or LC-MS/MS. 2D-DIGE number refers to location of the identified protein on the gel shown in the Figure 3.3.1. '+' represents up-regulation and '-' represent down-regulation of protein in terms of fold change in the given comparison. (Supplementary data containing additional information of proteins identified: Appendix 6).

DIGE no	**Gene Symbols**	**Identification**	**Cancer vs. Normal Fold Change**	**Biological function**
229	RHOT2	Mitochondrial Rho GTPase 2	-4.2	Apoptosis, Cell adhesion
243	IMMT	Mitofilin	-4.2	Mitochondrial cristae maintaince
272	JUP	Junction plakoglobin	-1.87	Cell adhesion-mediated signalling
334	NT5E	5'-nucleotidase precursor	-4.38	Nucleic acid metabolism
367	ATP5B	ATP synthase subunit beta, mitochondrial precursor	-1.67	Electron transport
386	HSPA9	Stress-70 protein, mitochondrial precursor	-1.84	Stress response
393	HSPA9	Stress-70 protein, mitochondrial precursor	-2.03	Stress response
431	LMNB1	Lamin-B1	2.05	Cell structure
	HSPA8	Heat shock 70kDa protein 8 isoform 1		Stress response

436	LMNB1	Lamin-B1	1.98	Cell structure
527	CES1	Liver Carboxylesterase 1 precursor	33.25	Detoxification
606	G6PD	Structure Of A Deletion Variant Of Human Glucose 6- Phosphate Dehydrogenase Complexed With Structural And Coenzyme Nadp	5.36	Monosaccharide metabolism
619	FKBP5	FK506-binding protein 5	-4.22	Protein folding, Calcium mediated signalling
628	FKBP5	FK506-binding protein 5	-2.37	Protein folding, Calcium mediated signalling
649	KRT8	keratin 8	3.34	Cell structure
695	ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor	-1.77	Electron transport
746	GCN1L1	Translational activator GCN1	1.52	Protein biosynthesis
812	UQCRC1	Ubiquinol-cytochrome c reductase core protein I	-10.49	Electron transport
848	A2M	Alpha-2-macroglobulin precursor	-4.71	Ligand-mediated signalling
866	ACTA1	Actin, alpha skeletal muscle (Alpha-actin-1)	-5.13	Cytokinesis, Cell structure

867	ACTA1	Actin, alpha skeletal muscle (Alpha-actin-1)	-4.21	Cytokinesis, Cell structure
877	ACTB	Beta actin	-1.66	Cytokinesis, Cell structure
929	CAPG	Macrophage-capping protein	-7.09	Cell proliferation and differentiation
934	CAPG	Macrophage-capping protein	-4.69	Cell proliferation and differentiation
950	SERPINB5	Serpin B5 precursor	-9.68	Proteolysis
955	SERPINB5	Serpin B5 precursor	-100.88	Proteolysis
1029	NPM1	Nucleophosmin	-3.24	rRNA metabolism
1050	TALDO1	Transaldolase 1	2.84	Pentose-phosphate shunt
1153	ANXA3	Annexin A3	-3.51	Fatty acid and steroid metabolism
1181	ASNAP1	Alpha-soluble NSF attachment protein	1.85	Intracellular protein traffic
1195	ANXA5	Annexin A5	-2.16	Fatty acid and steroid metabolism
1277	PHB	Prohibitin	2.76	Cell proliferation and differentiation
1303	PSME1	Proteasome activator complex subunit 1	2.72	Proteolysis
1336	YWHAB	Chain A, 14-3-3 Protein Beta In Complex	-159	Signal transduction

		With Exoenzyme S Peptide		
1469	GSTP1	Glutathione S-Transferase P1- 1[v104] Complexed With (9r,10r)-9-(S-Glutathionyl)-10- Hydroxy-9,10-Dihydrophenanthrene	-20.69	Detoxification
1472	SAR1B	SAR1a gene homolog 2	1.82	General vesicle transport
1714	DIABLO	Diablo homolog, mitochondrial precursor	2.36	Apoptosis
2353	S100A9	S100-A9	-1.51	Cell communication
2482	S100A16	S100-A16	-2.24	Calcium ion binding
2509	S100A14	S100-A14	-3.37	Calcium ion binding
2659	S100A6	S100-A6	-18.04	Cell proliferation and differentiation
2668	S100A2	S100-A2	-182.99	Cell proliferation and differentiation

Table 3.3.3: High priority membrane and membrane-associated protein targets from the comparison of cancerous (MCF-7+BT20) vs. normal (HMEC+MCF-10A) that might have a significant role in breast cancer.

DIGE no	**Gene Symbols**	**Identification**	**Cancer vs. Normal Fold Change**
229	RHOT2	Mitochondrial Rho GTPase 2	-4.2
243	IMMT	Mitofilin	-4.2
367	ATP5B	ATP synthase subunit beta, mitochondrial precursor	-1.67
619	FKBP5	FK506-binding protein 5	-4.22, -2.37
1181	ASNAP1	Alpha-soluble NSF attachment protein	1.85
812	UQCRC1	Ubiquinol-cytochrome c reductase core protein I	-10.49
2509	S100A14	S100-A14	-3.37
2659	S100A6	S100-A6	-18.04

3.3.4.2 Invasive (HMEC+BT20) vs. non-invasive (MCF-10A+MCF-7) (Normal + cancer-related invasion)

Table 3.3.4 lists the proteins that have been identified in the N+C invasive (HMEC+ BT20) vs. N+C non-invasive (MCF-10A+MCF-7) comparisons. 27 proteins were identified in the invasive vs. non-invasive comparison group, of which 21 proteins were up-regulated and 6 proteins were down-regulated in the N+C invasive cells compared to the N+C non-invasive cells (Table 3.3.4). The invasive cells were compared to non-invasive cells and therefore up-regulated proteins in the invasive cells have been denoted by positive fold change. The proteins that are down-regulated have negative fold change as these proteins are expressed at lower levels in the N+C invasive cells compared to the N+C non-invasive cells.

This comparison revealed three mitochondrial membrane and membrane- associated proteins as being differentially expressed, include 60 kDa heat shock protein 1, mitochondrial precursor (HSPD1); NADH dehydrogenase (ubiquinone) Fe-S protein 1, precursor (NDUFS1); ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor (ATP5B).

- ATP5B, involved in the glycolytic pathway, was found to be 1.52-fold down-regulated in the normal and cancer invasive cells (HMEC+BT20) compared to the normal and cancer non-invasive cells (MCF-10A+MCF-7).

- NDUFS1 which transfers electrons from NADH to the respiratory chain (Liu *et al.* 2000a), was 1.61 fold down-regulated in the N+C invasive cells compared to the N+C non-invasive cells.

- HSPD1, also known as heat shock 60 protein, has been reported over- expressed in invasive breast tissues and this protein was found to be down-regulated (1.5 and 2.2-fold) at two different locations on the gel in the N+C invasive cells compared to the normal and cancer N+C non-invasive cells.

Two S100 proteins, S100A9 and S100A16 were differentially expressed in this comparison. S100A9 was found 1.8 fold down-regulated while S100A16 was up-

regulated in the N+C invasive cells compared to the normal and cancer N+C non-invasive cells.

Some well known proteins, like Non-metastatic cells 1 (NME1), involved in pyrimidine metabolism, is known to be correlated with invasion. NME1 was found 2.6-fold down-regulated in the N+C invasive cells compared to the N+C non-invasive cells.

Some other proteins like RuvB-like-2 and Neutral alpha-glucosidase AB precursor were also identified from this comparison. The role of these proteins in breast cell invasion is not yet known.

- WD repeat protein 1 (WDR1), involved in protein-protein interactions and cell motility (Ricci *et al.* 2004), was found to be 2.4-fold up-regulated in the N+C invasive cells compared to the N+C non-invasive cells .
- RuvB-like 2 (RUVBL2) involved in ATPase and DNA helicase activities (Verma *et al.* 2004), was found to be 4.0-fold up-regulated in the N+C invasive cells compared to the N+C non-invasive cells.
- Adipocyte plasma membrane-associated protein (C20ORF3), involved in adipocyte differentiation, was found to be 1.9-fold up-regulated in the N+C invasive cells compared to the N+C non-invasive cells.
- Neutral alpha-glucosidase AB precursor (GANAB), involved in protein modification (Bal *et al.* 2005), was found to be down-regulated (2.3, 2.2 and 2.6-fold) at three different locations on the gel in the N+C invasive cells compared to the N+C non-invasive cells.

On the basis of limited information in the literature regarding their potential roles in breast invasion, GANAB, RUVBL2, WDR1 and C20ORF3 proteins are potentially novel findings that could have important roles in invasion process of normal and cancer breast cells.

Table 3.3.4: **Invasive vs. Non-invasive (Normal + cancer-related invasion)**: Proteins identified from the comparison of Non-invasive (MCF-10A + MCF-7) vs. Invasive (HMEC+ BT20) group using MALDI ToF MS and/or LC-MS/MS. 2D-DIGE number refers to location of the identified protein on the gel shown in the Figure 3.3.1. '+' represents up-regulation and '-' represent down-regulation of protein in terms of fold change in the given comparison. (Supplementary data containing additional information of proteins identified: Appendix 6).

DIGE no	Gene Symbol	Identification	Invasive vs. non-invasive Fold Change	Biological function
84	GANAB	Neutral alpha-glucosidase AB precursor	2.37	Protein modification
177	GANAB	Neutral alpha-glucosidase AB precursor	2.69	Protein modification
209	HSPD1	60 kDa heat shock protein 1, mitochondrial precursor	2.27	Protein folding
351	NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1, precursor	-1.61	Electron transport
370	HSPA5	Heat shock 70kDa protein 5	1.57	Stress response
431	LMNB1	Lamin-B1	-1.52	Cell structure
	HSPA8	Heat shock 70kDa protein 8 isoform 1		Stress response
434	RUVBL2	RuvB-like 2	4.06	mRNA transcription regulation
441	WDR1	WD repeat-containing protein 1	2.47	Cell motility

481	CCT6A	T-complex protein 1 subunit zeta	1.83	Protein folding
499	TCP1	T-complex protein 1 subunit alpha	1.78	Protein folding
546	HSPD1	60 kDa heat shock protein 1, mitochondrial precursor	1.59	Protein folding
558	PDIA3	Protein disulfide-isomerase A3 precursor	2.92	Protein disulfide-isomerase reaction
600	PDIA3	Protein disulfide-isomerase A3 precursor	1.96	Protein disulfide-isomerase reaction
669	TUBA1C	Tubulin alpha 6	1.51	Cell structure
671	RUVBL1	RuvB-like 1	1.65	mRNA transcription regulation
695	ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor	-1.52	Electron transport
726	C20ORF3	Adipocyte plasma membrane-associated protein	-1.71	Other carbohydrate metabolism
827	AHCY	Adenosylhomocysteinase	2.02	Purine metabolism
876	ACTB	Actin, gamma 1 propeptide	1.72	Cytokinesis, Cell structure
884	ACTB	Actin, gamma 1 propeptide	1.57	Cytokinesis, Cell

				structure
1053	ANXA3	Annexin A3	1.54	Fatty acid and steroid metabolism
1270	CLIC4	Chloride intracellular channel protein 4	1.62	Anion transport
1336	YWHAB	Chain A, 14-3-3 Protein Beta In Complex With Exoenzyme S Peptide	1.94	Signal transduction
1359	HSPB1	Heat shock protein beta-1	4.64	Stress response
2353	S100A9	S100-A9	1.8	Cell communication
1786	NME1	Non-metastatic cells 1, protein	-2.65	Pyrimidine metabolism
2482	S100A16	S100-A16	-2.32	Calcium ion binding

3.3.4.3 Cancerous invasive (BT20) vs. Normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7) (Cancer-related invasion)

Table 3.3.4.4 outlines the 35 identified proteins in the **Group 1** (cancer invasive cell line (BT20)) vs. **Group 2** (Normal invasive+ normal non-invasive+ cancerous non-invasive cells (HMEC+MCF-10A+MCF-7)) comparison. Among these proteins, 25 proteins were up-regulated and 10 proteins were down regulated in the group 1 compared to the group 2. The cell lines are compared from **Group 1** vs. **Group 2** and therefore proteins that are up-regulated in Group 1 are represented as positive fold change. The proteins that are down-regulated in group1 have negative fold change. These proteins could be involved in breast cancer invasion.

This analysis showed that six mitochondrial membrane and membrane-associated protein were found altered in group 1 compared to group 2. HSPD1, UQCRC1 and NDUFS1 were 2.13, 3.39 and 2.67-fold down-regulated in group 1 compared to group 2, respectively. HSPD1 was identified from two spots and was 1.82-fold and 1.67-fold up-regulated in group 1 compared to group 2. Prohibitin, ATP5B and DIALBO were found to be up-regulated in group 1 compared to group 2 by 1.85 fold, 2.13 and 2.23 fold, respectively.

From the S100 protein family, S100A2, S100A14 and S100A16 were found to be 155-fold, 3.5-fold and 4-fold down-regulated in group 1 compared to group 2 respectively.

This comparison list also revealed some other proteins that have not been investigated extensively to date for their involvement in cancer invasion and could have a significant role in breast cancer disease

- Liver Carboxylesterase 1 precursor (CES1), involved in detoxification, was found to be 45.2-fold over-expressed in group 1 compared to group 2.

- Adenosylhomocysteinase (AHCY), is an enzyme which converts S-adenosylhomocysteine to homocysteine, was found to be 2.7-fold up-regulated in group 1 compared to group 2.

- RuvB-like 2 (RUVBL2) involved in ATPase and DNA helicase activities (Totani *et al.* 2006), was found to be up-regulated (5.57 and 2.0-fold) at two different locations on the gel in group 1 compared to group 2.

- Transaldolase 1, involved in the pentose-phosphate pathway, was found to be 4.2-fold up-regulated in group 1 compared to group 2.

On the basis of limited information in the literature regarding their potential role in breast cancer invasion, ATP5B, CES1, AHCY, RUVBL2 and TALDO1 proteins are intersting findings as they have not been previously linked with breast cancer invasion and could be associated with the breast cancer invasive phenotype.

Table 3.3.5: **Group 1/Group 2 (Cancer-related invasion)**: Proteins identified from the **Group 1 vs. Group 2** using MALDI ToF MS and/or LC-MS/MS. 2D-DIGE number refers to location of the identified protein on the gel shown in the Figure 3.3.1. '+' represents up-regulation and '-' represent down-regulation of protein in terms of fold change in the given comparison. (Supplementary data containing additional information of proteins identified: Appendix 6).

DIGE no	**Gene symbol**	**Identification**	**Group 1 vs. group 2 Fold change**	**Biological function**
84	GANAB	Neutral alpha-glucosidase AB precursor	2.66	Protein modification
177	GANAB	Neutral alpha-glucosidase AB precursor	2.71	Protein modification
149	GANAB	Neutral alpha-glucosidase AB precursor	1.59	Protein modification
209	HSPD1	60 kDa heat shock protein, mitochondrial precursor	2.67	Protein folding
351	NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1, precursor	-1.61	Electron transport
370	HSPA5	Heat shock 70kDa protein 5	1.62	Stress response
434	RUVBL2	RuvB-like 2	5.57	mRNA transcription regulation
441	WDR1	WD repeat-containing protein 1	2.11	Cell motility
499	TCP1	T-complex protein 1 subunit alpha	2.03	Protein folding
527	CES1	Liver Carboxylesterase 1 precursor	45.2	Detoxification

546	HSPD1	60 kDa heat shock protein, mitochondrial precursor	1.82	Protein folding
550	HSPD1	60 kDa heat shock protein, mitochondrial precursor	1.67	Protein folding
558	PDIA3	Protein disulfide-isomerase A3 precursor	3.91	Protein disulfide-isomerase reaction
600	PDIA3	Protein disulfide-isomerase A3 precursor	2.33	Protein disulfide-isomerase reaction
606	G6PD	Structure Of A Deletion Variant Of Human Glucose 6-Phosphate Dehydrogenase Complexed With Structural And Coenzyme Nadp	3.73	Monosaccharide metabolism
669	TUBA1C	Tubulin alpha 6	1.6	Cell structure
688	RUVBL2	RuvB-like 2	2.05	mRNA transcription regulation
695	ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor	-2.13	Electron transport
726	C20ORF3	Adipocyte plasma membrane-associated protein	-1.97	Other carbohydrate metabolism
746	GCN1L1	Translational activator GCN1	1.9	Protein biosynthesis
812	UQCRC1	Ubiquinol-cytochrome c reductase core protein I	-3.39	Electron transport
827	AHCY	Adenosylhomocysteinase	2.78	Purine metabolism

866	ACTA1	Actin, alpha skeletal muscle	2.47	Cytokinesis, Cell structure
929	CAPG	Macrophage-capping protein	-3.36	Cell proliferation and differentiation
934	CAPG	Macrophage-capping protein	-4.79	Cell proliferation and differentiation
1050	TALDO1	Transaldolase-1	4.29	Pentose-phosphate shunt
1225	PSME2	Proteasome activator complex subunit 2	3.61	Proteolysis
1270	CLIC4	Chloride intracellular channel protein 4	1.8	Anion transport
1277	PHB	Prohibitin	1.85	Cell proliferation and differentiation
1408	HSPB1	Heat shock protein beta-1	-4.81	Stress response
1714	DIABLO	Diablo homolog, mitochondrial precursor	2.23	Apopotosis
1786	NME1	Non-metastatic cells 1, protein	-1.78	Pyrimidine metabolism
2482	S100A16	S100-A16	-4.02	Calcium ion binding
2509	S100A14	S100-A14	-3.53	Calcium ion binding
2668	S100A2	S100-A2	-155.42	Cell proliferation and differentiation

3.3.5 Identification of invasion-related proteins by overlapping differentially expressed proteins from normal cells with cancerous cells

The objective of overlapping two lists was to identify membrane and membrane-associated proteins that are only differentially expressed in the invasive phenotypes or in the non-invasive phenotypes. Two lists of differentially expressed proteins between normal non-invasive vs. normal invasive cell lines (MCF-10A vs. HMEC) and cancerous non-invasive vs. cancer invasive (MCF-7 and BT20) cell lines were generated (supplementary data: Appendix 3). The two generated differentially expressed protein lists were overlapped determining unique and common expressing proteins between the lists. A venn diagram outlining the distribution of proteins between lists of differentially expressed proteins in a comparison of cancerous non-invasive (MCF-7) vs. invasive (BT20) cells, and normal-non-invasive (MCF-10A) vs. invasive (HMEC) cells.

The overlapping identified 29 proteins, including GANAB, LANB1, ITGA3, KRT8, SERPINB5, S100A14 and ANXA3, in the normal comparison group and alterations in the expression of these proteins could potentially be associated with normal-invasion in cells. Out of these 29 proteins, 24 proteins were up-regulated in the normal non-invasive cell line (MCF-10A) suggesting reduced levels of thse poteins in the normal invasive cells (HMEC). 5 proteins were found down-regulated in the non-invasive cell line (MCF-10A).

28 proteins were found differentially expressed in the cancer non-invasive and invasive cell lines and these proteins are possibly involved in the invasive phenotype of breast cancer cells. Only 3 proteins (PNPO, HSPB1 and hypothetical protein) were found to be up-regulated in the cancer non-invasive cell line (MCF-7), however 25 proteins were found to be down-regulated including TCP, TALDO1, ALDH1A3, S100A9 and S100A6.

A total of 7 proteins were observed common in both comparison groups indicating the proteins involved in normal and cancer invasion. Dysfunction of these proteins could be involved in the development of the invasive phenotype of normal cells as well as cancer cells. Out of these 7 proteins, 2 proteins (C20ORF3 and SA100A16) were up-regulated and 2 were down-regulated (GANAB, RUVBL2) in both the non-invasive cell lines compared

to the normal and cancer invasive cell lines. 3 proteins were observed differentially expressed with the opposite expression pattern. PHB, HSPB1 and DIABLO proteins have been reported up-regulated in many types of cancer but the role of GANAB, RUVBL2, C20ORF3 and SA100A16 proteins in invasion especially in breast cancer are not clear yet.

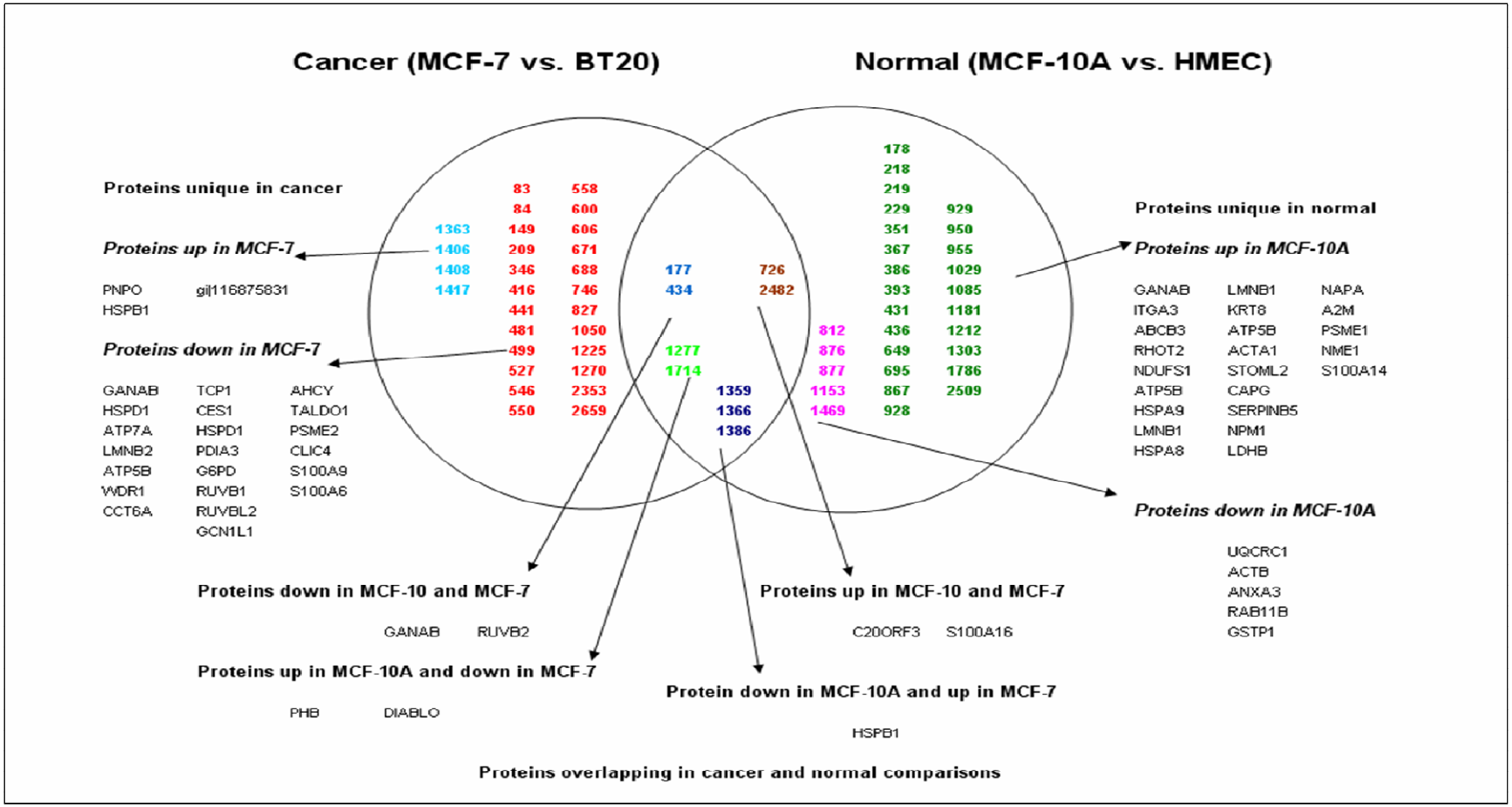


Figure 3.3.2. Venn diagram of differentially expressed overlapping membrane and membrane-associated proteins of comparison in normal non-invasive (MCF-10A) vs. invasive (HMEC) cells and in cancer non-invasive (MCF-7) vs. Invasive (BT20). Proteins are represented by the 2D-DIGE number inside the circle and gene symbols outside the circle of protein.

3.4 Investigation of low molecular weight secreted proteins from human cell lines using Surface-enhanced laser desorption/ionization (SELDI-ToF MS)

Melanoma is among the most serious cancers and originates from the melanocytes of the epidermis. Invasion and metastasis are intimately involved in the pathology of melanoma, but the biological and molecular natures of these phenomena remain poorly understood. Our objective of this study was to identify potential biomarker (s) for either melanoma or melanoma invasion. There have been various reports linking the development of resistance to chemotherapeutic drugs, such as doxorubicin, methotrexate and melphalan resulting in an increase in the invasive properties of cell lines. This indicates that a possible association exists between multiple drug resistance and the invasive potential of carcinoma cells. Previously in this laboratory, selection of the human drug sensitive and invasive melanoma cell line (MDA-MB-435S-F) with the chemotherapeutic agents paclitaxel and doxorubicin, resulted in the development of drug-resistant cell lines displaying enhanced invasion. Therefore, the identification of proteins/peptides which are differentially regulated in drug-sensitive and drug-resistance cells could be helpful to identify altered proteins/peptides that are associated with invasion (Bal *et al.* 2005). We used SELDI-ToF MS to monitor differential expression patterns from the melanoma cell lines MDA-MB-435S-F and its paclitaxel-resistant variant, MDA-MB-435S-F/Taxol-10p4p which display a greater invasive potential compared to the parental cell line (Glynn *et al.* 2004).

3.4.1 Serum concentration

To optimize the serum concentration that is suitable to ensure maximum binding of proteins/peptides to the chip surface, conditioned media from MDA-MB-435S-F and MDA-MB-435S-F/Taxol1p4pSI cells grown in 10% FCS, 0.5% FCS and serum-free conditioned media were analysed on Q10 (strong anion-exchange) chip surface using 50 mM Tris pH 8.0 binding buffer as described in section 2.10.9. Figure 3.4.1 A & B shows the comparative map of conditioned media from MDA-MB-435S-F and MDA-MB-435S-F/Taxol1p4pSI cultured in 10% and 0.5% FCS supplemented culture medium. The results indicate that the peaks between m/z 1010 to 1050 were possibly inhibited by the binding of other proteins/peptides from serum in conditioned media. However conditioned media from cells grown in serum-free media showed separate binding of all the proteins/peptides between m/z 1010 to 1200 as shown in Figure

3.4.1C. Therefore serum-free culture was considered as the optimal condition for further investigation.

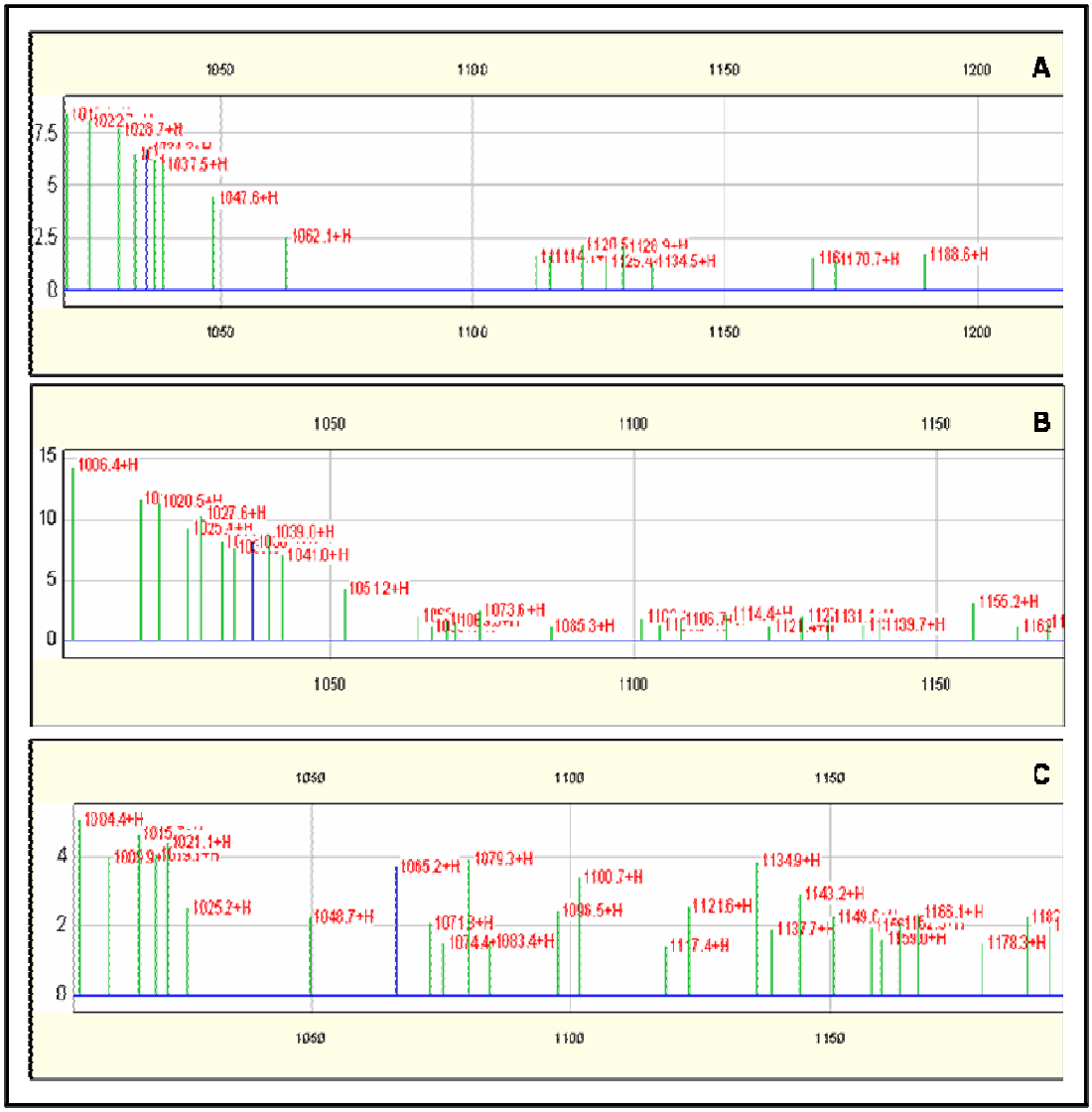


Figure 3.4.1: Comparative peak maps on Q10 chip surface of conditioned media from MDA-MB-435S-F and MDA-MB-435S-F/Taxol1p4pSI cells. (A) grown in 10% FCS, (B) grown in 0.5% FCS, (C) grown in serum-free conditions.

3.4.2 Selection of chip surface

Prior to full profiling analysis, optimization studies were performed using 10 µg of extracted protein from serum-free conditioned media samples representing 72hrs of culture, on four different chromatographic surfaces to see which surface of Protein Chip array gives optimal results as described in section 2.10. Conditioned media samples from melanoma cell lines, MDA-MB 435S-F and MDA-MB 435S-F/Taxol10p4p, were concentrated using 5000 MW and 10000MW cut-off centricons (Millipore). The following Protein Chip arrays were selected, an IMAC30 (immobilized metal affinity capture) activated with copper ions, CM10 (weak cation-exchange) with 20 mM Tris pH 4.5, Q10 (strong anion-exchange) with 50 mM Tris pH 8.0 and H50 (reversed-phase hydrophobic surface) with 10% acetonitrile/0.1% trifluoroacetic acid. The IMAC30 ProteinChip 8-spot arrays and 5000MW centricon fractionation provided better profiles under these conditions in terms of number and resolution of peaks and therefore was selected for the final profiling studies. Table 3.4.1 summarises the number of peaks discovered by profiling conditioned media on the four different ProteinChip arrays (CM10, Q10, H50 and IMAC).

Table 3.4.1: The number of peaks observed by profiling serum-free conditioned media from MDA-MB 435S-F and MDA-MB 435S-F/Taxol10p4p on four chromatographic chip surfaces. (*) Peaks had signal to noise ratio ≥ 3.5 in the first pass; signal to noise ratio ≥ 2.0 in the second pass.

Array type	**Total peaks discovered * using 5000MW centricons with mass range 5-20000kDa**	**Total peaks discovered * using 10000MW centricons with mass range 20-60000kDa**
IMAC30	41	19
Q10 pH 8.0	24	23
CM10 pH 4.5	37	29
H50	36	22

3.4.3 SELDI ToF MS analysis of conditioned media from MDA-MB-435S-F and MDA-MB-435S-F/Taxol10p4pSI

Conditioned media from MDA-MB-435S-F and MDA-MB-435S-F/Taxol10p4pSI were analysed in triplicate using IMAC-Cu^{2+} chip surface. Results were cross-checked for their reproducibility by using different biological sample and replicates on different chip batches as described in section 2.10. Figure 3.4.2 (A) shows the comparative chromatographs of mass signals and Figure 3.4.2 (B) shows the one-dimensional gel view from the conditioned media samples analyzed on IMAC-Cu^{2+} ProteinChip arrays. A total of 166 peaks were detected and the most prominent differentially expressed peak identified was at m/z 7653. The peak intensity of the m/z 7653 was 4-fold greater in the MDA-MB-435S-F/Taxol10p4pSI cell line with a statistically significant p-value of < 0.04. To further investigate m/z 7653, conditioned media samples representing 24, 48 and 72 hrs of culture in serum-free media were profiled and the results indicated that the intensity of the peak gradually decreased with time (Figure 3.4.3).

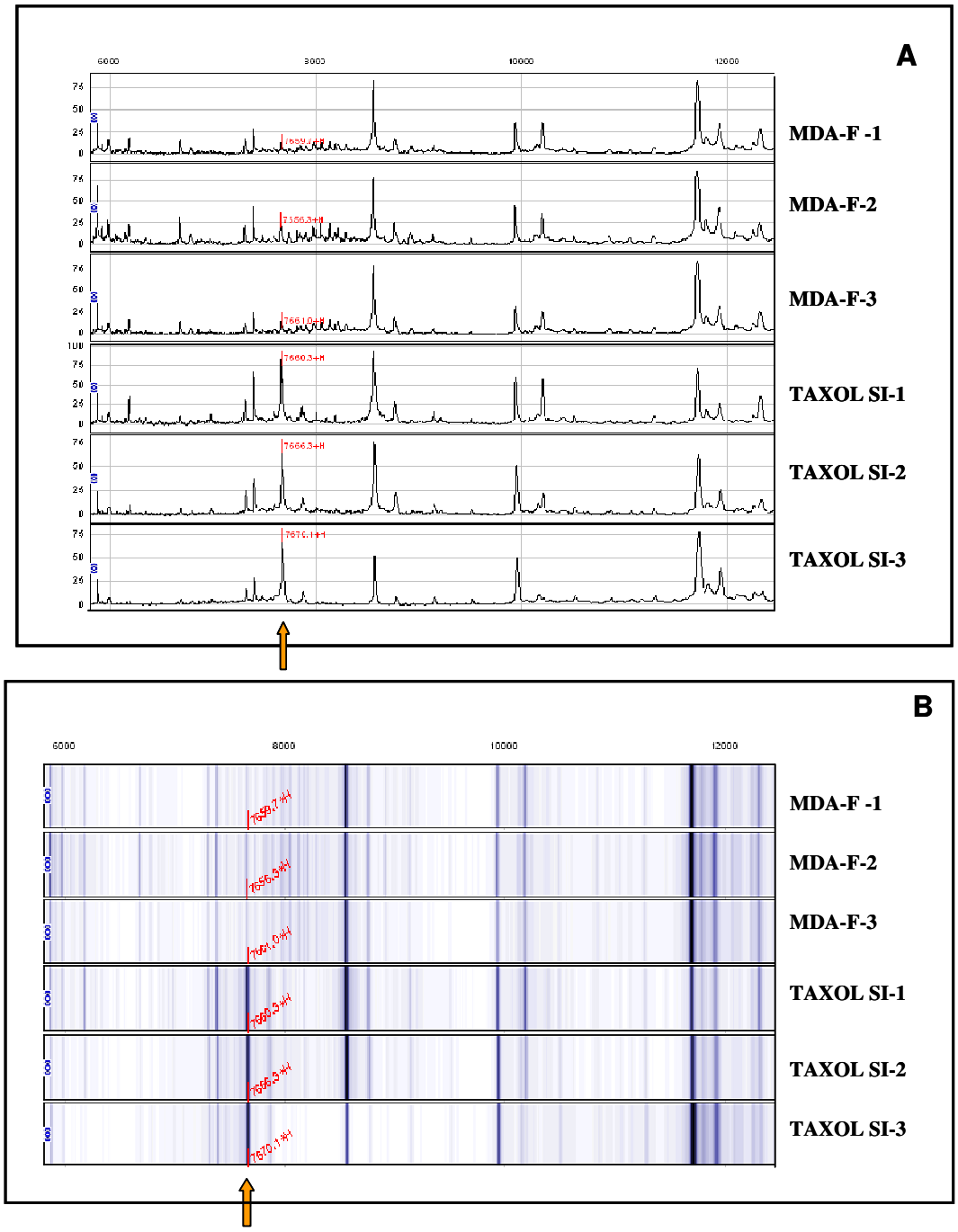


Figure 3.4.2 (A) Chromatographic view of the conditioned media from the human cancer drug-sensitive phenotype MDA-MB-435-S-F (MDA-F) and its paclitaxel-resistant MDA-MB-435S-F/Taxol10p4pSI (TAXOL SI) run in triplicate on IMAC-Cu^{2+} ProteinChip Arrays. The arrow indicates the position of a differentially expressed peak of m/z 7653, (B) 1D Gel view of the corresponding chromatographs.

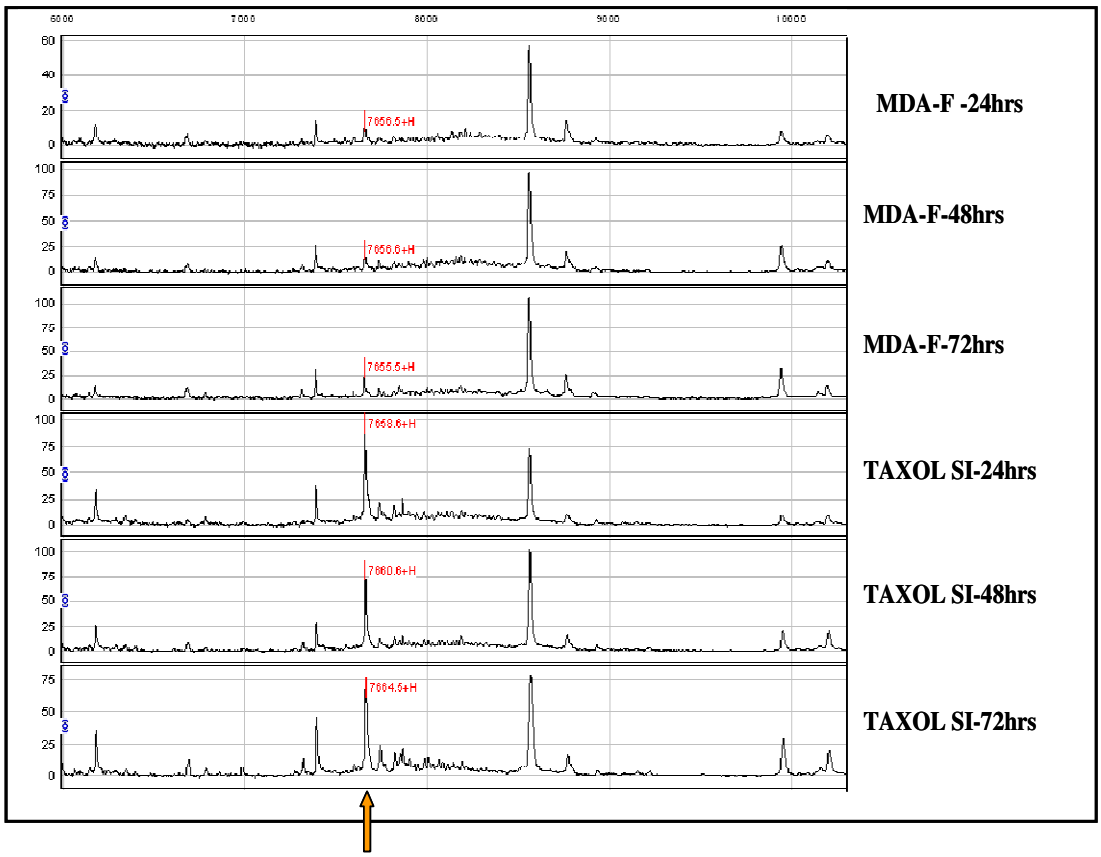


Figure 3.4.3: Chromatographic view of the conditioned media from the human cancer drug-sensitive phenotype MDA-MB-435-S-F (MDA-F) and its paclitaxel-resistant variant MDA-MB-435S-F/Taxol10p4pSI (TAXOL SI) collected at 24hrs, 48hrs and 72hrs. Conditioned media from the different time intervals were analysed on IMAC-Cu^{2+} ProteinChip Arrays. The arrow indicates the position of a differentially expressed peak of m/z 7653.

3.4.4 SELDI-ToF MS analysis of conditioned media from MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI and MDA-MB-435S-F/Adr-10p10pSI

The conditioned media from the drug resistance variant (Doxorubicin) were also analysed to see measure the levels of the m/z 7653 peak. For this, conditioned media from MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI and MDA-MB-435S-F/Adr-10p10pSI were analysed in duplicate on IMAC-Cu^{2+} chip surface using SELDI-ToF MS. The conditioned media from the doxorubicin-resistant variant of MDA-MB-435S-F (MDA-MB-435S-F/Adr-10p10pSI) also showed a 2.6-fold greater peak intensity of m/z 7653 in comparison to the MDA-MB-435S-F parental cell line (Figure 3.4.4).

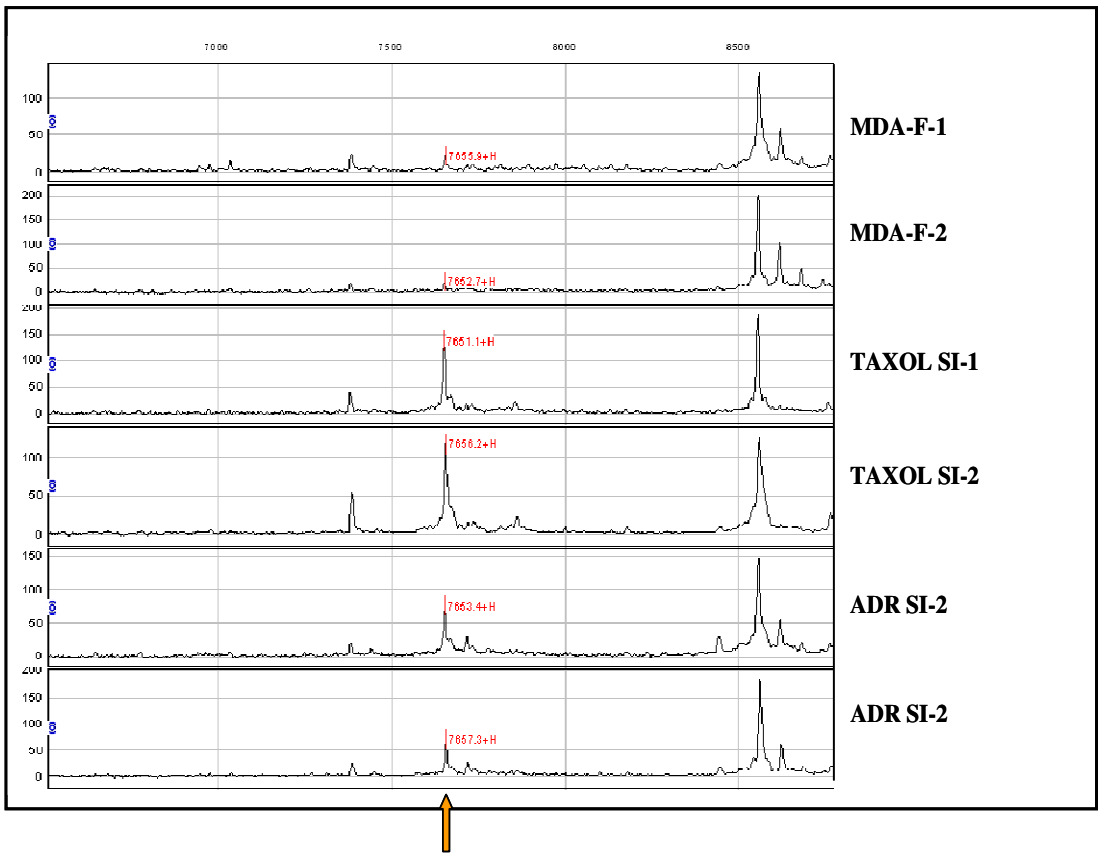


Figure 3.4.4: Chromatographic view of the conditioned media from the human cancer drug-sensitive and invasive phenotype MDA-MB-435-S-F (MDA-F), its paclitaxel-resistant superinvasive variant MDA-MB-435S-F/Taxol10p4pSI (TAXOL SI) and its doxorubicin-resistant superinvasive variant MDA-MB-435S-F/Adr-10p10pSI (ADR SI) run in duplicate on IMAC-Cu^{2+} ProteinChip Arrays. The arrow indicates the position of a differentially expressed peak at m/z 7653.

3.4.5 Purification of the m/z 7653 marker candidate

The IMAC HyperCel sorbent has the same binding capacity as an IMAC ProteinChip Array and is used for the enrichment of the target protein. The conditioned media from MDA-MB-435S-F/Taxol-10p4pSI cells was fractionated using BioSepra IMAC-Cu^{2+} HyperCel spin columns as described in section 2.11. All seven fractions collected from unbound peptides/proteins through to 500 mM Imidazole were analysed on NP20 ProteinChip Arrays. Figure 3.4.5 A shows the chromatograph of all seven fractions collected from unbound peptides/proteins through to 500 mM Imidazole indicating that the greatest concentration of the m/z 7653 peak elutes in the 250 mM fraction with the majority of the contaminating proteins removed in earlier fractions. The 250mM fraction was run on a 4-12% NuPAGE Bis-Tris 1D gels using a MES/SDS running buffer (as described in section 2.11.2). The MES/SDS running buffer is specially formulated for greater resolution in the lower molecular weight range. Silver staining of the gel allowed a band at m/z 7653 to be excised (Figure 3.4.5 B).

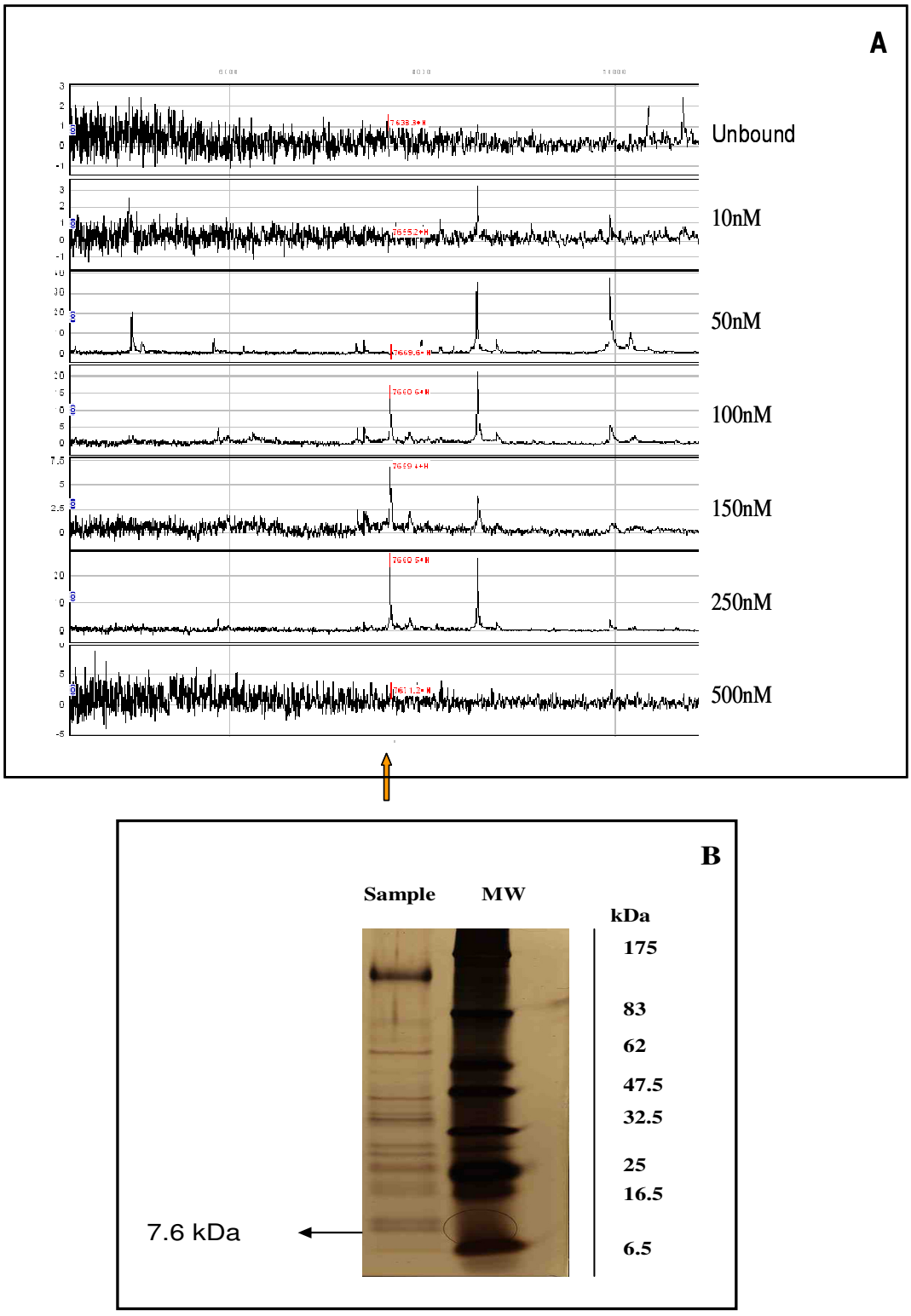


Figure 3.4.5: Chromatograph on NP20 ProteinChip surface from all seven fractions collected from unbound peptides/proteins through to 500 mM Imidazole by IMAC-Cu^{2+} Hypercel fractionation of MDA-MB-435S-F/Taxol10p4pSI using increasing concentrations of Imidazole. The arrow indicates the position of the m/z 7653 peak. (B) location of the 7.6kDa band in the gel.

3.4.6 Identification of the m/z 7653 marker candidate

In-gel digestion of proteins was carried out using a modified version of Schevchenko et al, 1996 (Glynn *et al.* 2004). Tryptic digests of the m/z 7653 band were subject to MALDI ToF/ToF analysis (as described in section 2.11.3). All MS and MS/MS experiments were carried out in positive reflectron mode. Five precursor ions for MS/MS were selected automatically on the basis of intensity from the MS spectra. The MS and MS/MS data were combined and searched against a number of databases and identified m/z 7653 as a bovine transferrin protein. The molecular weight of the identified transferrin protein was 79.8kDa suggesting that the m/z 7653 peak is a fragment of transferrin protein. The accession number of the protein was gi|29135265 and its pI was 6.75. Figure 3.4.6 shows the spectra generated from MALDI ToF/ToF MS.

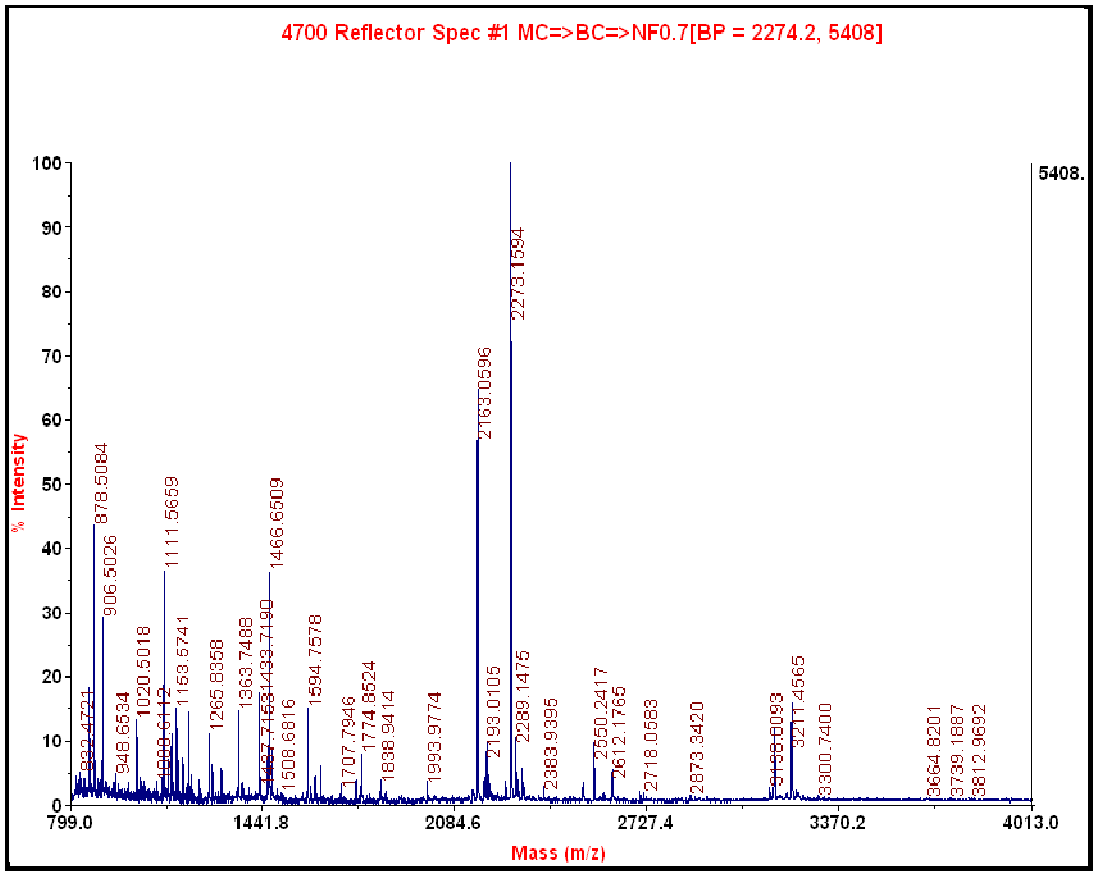


Figure 3.4.6: MALDI-ToF/ToF generated spectrum from tryptic digest of the m/z 7653 peak. Two matched peptides were at m/z 1466.6434 and m/z 1594.7573 and m/z 7653 peak was positively identified as a possible protein fragment of bovine transferrin.

3.4.7 Gelatin Zymography analysis

The increased levels of m/z 7653 in the conditioned media of MDA-MB-435S-F/Taxol10p4pSI possibly indicates that bovine transferrin protein was being cleaved by proteases. Zymography was used to assess the levels of proteolytic activity of different proteinase enzymes in the conditioned medium from the melanoma cells as described in section 2.12. Gelatin is a substrate for matrix metalloproteinases (MMPs), serine and cysteine proteinases. Gelatin zymography analysis of the conditioned media from MDA-MB-435S-F and MDA-MB-435S-F/Taxol10p4pSI showed increased protease activity in the MDA-MB-435S-F/Taxol10p4pSI cells compared to MDA-MB-435S-F. Using a panel of protease inhibitors the protease type was narrowed down to metalloproteinases. The panel included EDTA, Aprotinin, Pepstatin A, E-64 and 1,10 Phenanthroline. EDTA and 1,10 Phenanthroline are metalloproteinase inhibitors and addition of EDTA and 1,10 Phenanthroline to the substrate buffer eliminates expression of all protease bands, indicating the gelatin degrading proteases secreted by MDA-MB-435S-F/Taxol10p4pSI cells are probably members of the matrix metalloproteinase family. Aprotinin, Pepstatin A and E-64 are protease inhibitors that cover cysteine and serine proteases and were found to have no effect on activity. Figure 3.4.7 shows the gelatin zymogram of secreted proteases of MDA-MB-435S-F (MDA-F) and MDA-MB-435S-F/Taxol10p4pSI (TAXOL SI) in the presence or absence of protease inhibitors (EDTA, 1,10 Phenanthroline, Aprotinin, Pepstatin A and E-64). The results indicate that EDTA and 1,10 Phenanthroline were able to inhibit the activity of the MMPs. The two secreted bands are possibly MMP-2 and MMP-9 due to the approximate molecular weight of the bands; MMP-2 has a molecular weight of approximately 62kDa while MMP-9 has a molecular weight of 92kDa.

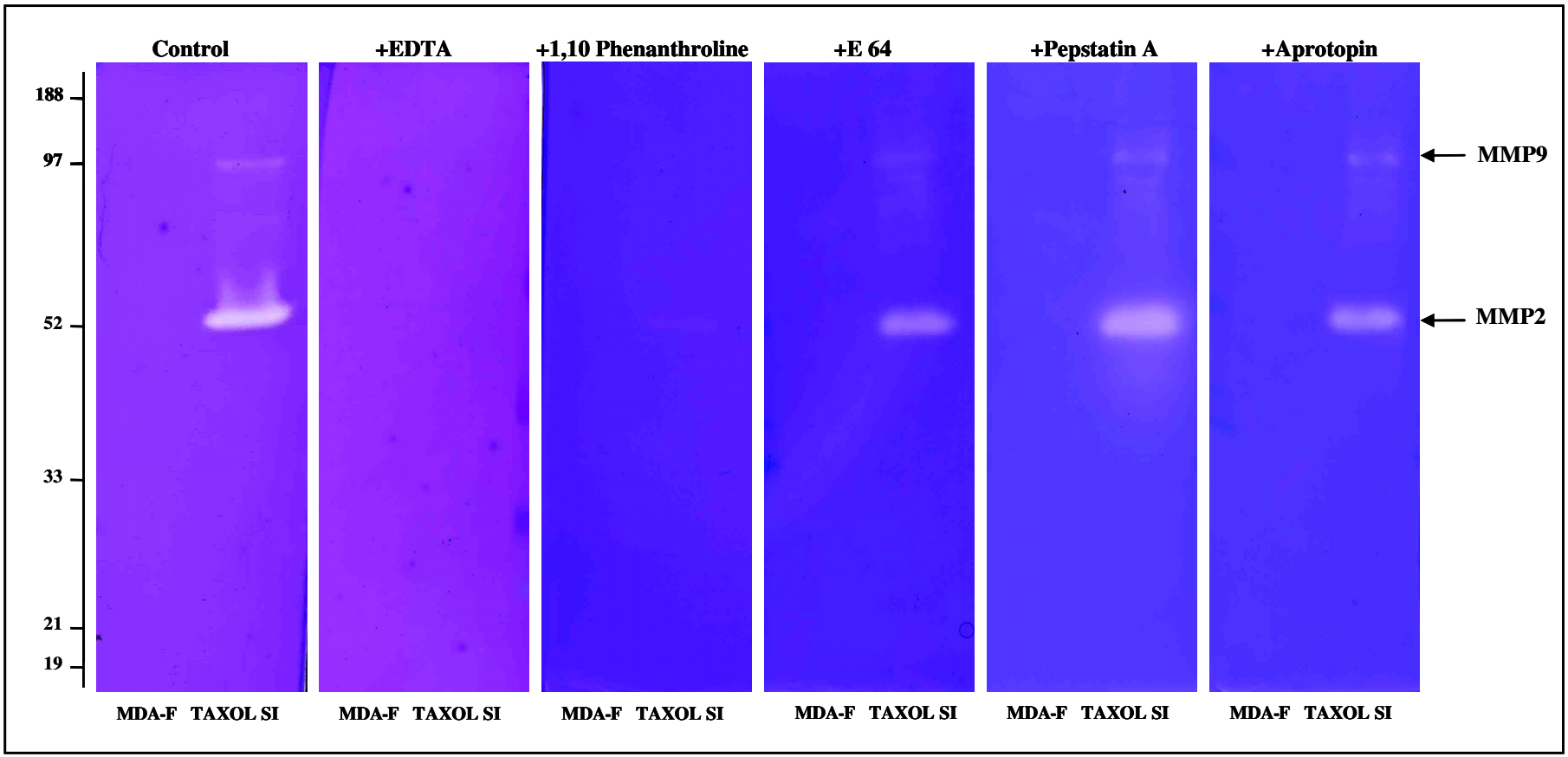


Figure 3.4.7: Gelatin zymograms of secreted proteases of MDA-MB-435S-F (MDA-F) and MDA-MB-435S-F/Taxol10p4pSI (TAXOL SI) in the presence or absence of protease inhibitors (EDTA, 1,10 Phenanthroline, Aprotinin, Pepstatin A and E-64). These gels are representative of repeated experiments.

3.4.8 Treatment with MMP inhibitors

Three biological samples of conditioned media from cells treated with MMP 2 and MMP 9 inhibitors and untreated control cultures were profiled using SELDI-ToF MS to investigate the effect of MMP inhibitors (as described in section 2.13) on the peak at m/z 7653. Figure 3.4.8 (A) shows the chromatograph of conditioned media on IMAC-Cu^{++} ProteinChip arrays from MDA-MB-435S-F/Taxol10p4pSI cultured untreated and treated with MMP 2 or MMP 9 inhibitors. Figure 3.4.8 (B) shows the relative intensity graphs of the m/z 7653 and m/z 8579 peaks. The m/z 7653 peak was 2.18 fold (p= 0.00016) down-regulated in cells treated with the MMP 9 inhibitor and 3.97 fold (p= 0.00010) down-regulated in cells treated with the MMP 2 inhibitor as compared to untreated cells. The expression of the m/z 8579 peak served as a control for this experiment and the expression level were found unaltered following treatment with the MMP inhibitors. The mean intensities of the m/z 8579 peak was similar in samples from MDA-MB-435S-F/Taxol10p4pSI treated with MMP 2 and MMP 9 inhibitors and untreated samples. This suggests that the decrease observed in the intensity of the m/z 7653 peak intensity is specifically due to effects of MMP 2 and MMP 9 inhibition.

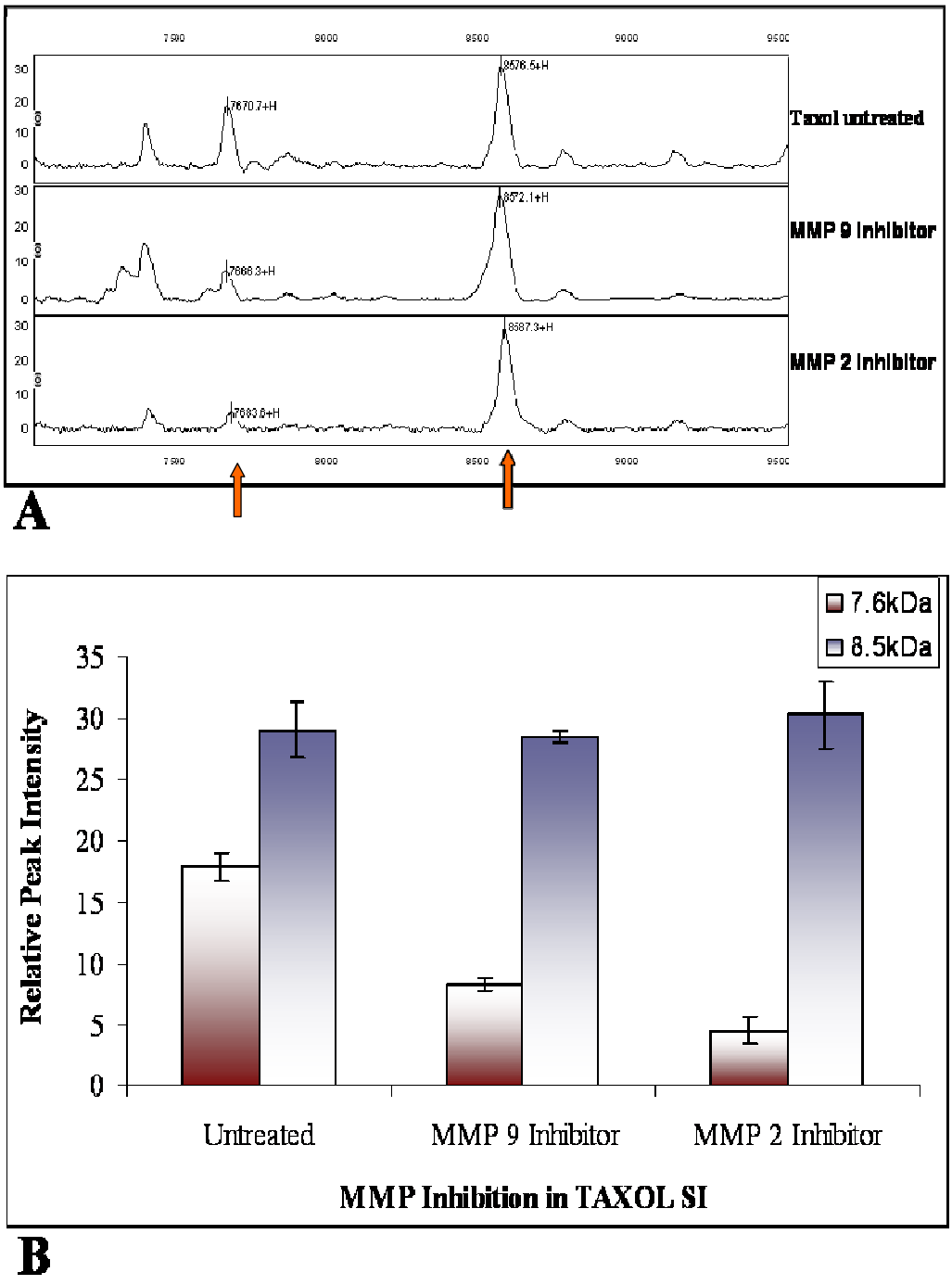


Figure 3.4.8 (A) SELDI-ToF MS spectra of conditioned media on IMAC-Cu^{++} ProteinChip arrays from MDA-MB-435S-F/Taxol10p4pSI cultured with MMP 2 or MMP 9 inhibitors. The arrow indicates the position of the m/z 7653 (7.6kDa) and m/z 8579 (8.5kDa) peak species. (B) Graph showing the comparison of the average peak intensities for the m/z 7653 peak from MDA-MB-435S-F/Taxol10p4pSI cultured with MMP 2 or MMP 9 inhibitors. The error bars represent the standard deviation calculated from the three independent biological replicate samples.

3.4.9 Prediction of possible cleavage sites for MMPs in transferrin

The transferrin protein was scanned for the presence of potential MMP cleavage sites using the bioinformatics tool ProfileDB (http://protease.burnham.org/www/tools/cgi-bin/specdb/specdb_home.html). The predicted cleavage sites for MMP 2 were P-X-X-[Hy], [IL]-X-X-[Hy], [Hy]-S-X-L, H-X-X-[Hy] and for MMP 9 were P-X-X-[Hy]-[ST], P-R-[ST]-[Hy]-[ST], G-L-[KR], R-R-[Hy]-L, R-X-L. No MMP cleavage sites were predicted in the full transferrin sequence by this software. The identified fragment was also overlapped with the full transferrin protein sequence in an effort to identify potential MMP cleavage sites. The identified peptide was observed to be lying towards the C-terminus of transferrin (Figure 3.4.9). The fragment of transferrin was observed at m/z 7653 in SELDI analysis therefore it could be suspected that MMPs might be cleaving transferrin at ~69 amino acids from the C-terminus resulting in the 7.6kDa fragment.

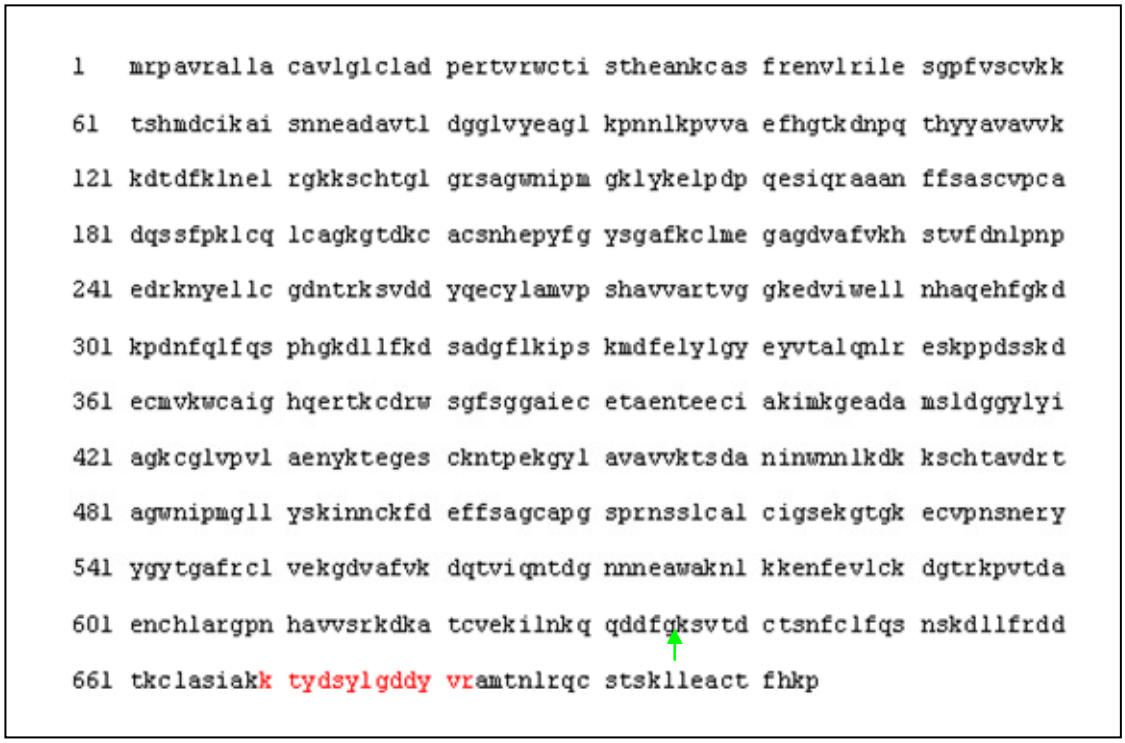


Figure 3.4.9. Investigation for possible cleavage site for MMP 2 and MMP 9 in transferrin. Red denotes the peptide identified by MALDI ToF/ToF for the transferrin fragment and the arrow indicates a possible cleavage site for these MMPs calculated based on molecular weight (7.6kDa).

The amino acid sequence of bovine transferrin was also aligned with the sequence of human transferrin using ClustalW (Figure 3.4.9 B). The results indicated that bovine transferrin shares 81% sequence homology with human transferrin. The similarity around the predicted MMP2 and MMP9 cleavage site was nearly similar. At 635 amino acid, K (Lysine) was replaced with S (Serine) in human transferrin, whereas 2 amino acids on each side of K were conserved. This is important because MMP2 and MMP9 have cleavage site motifs comprised of ~4-6 amino acids with conserved boundary amino acids suggesting that human transferrin could possibly also be target for MMP2 and MMP9, although human transferrin does not show significant sequence similarity at C-terminus region. Thus there is possibility that the expression of 7.6 kDa could be detected in human clinical samples (e.g. serum) from melanoma.

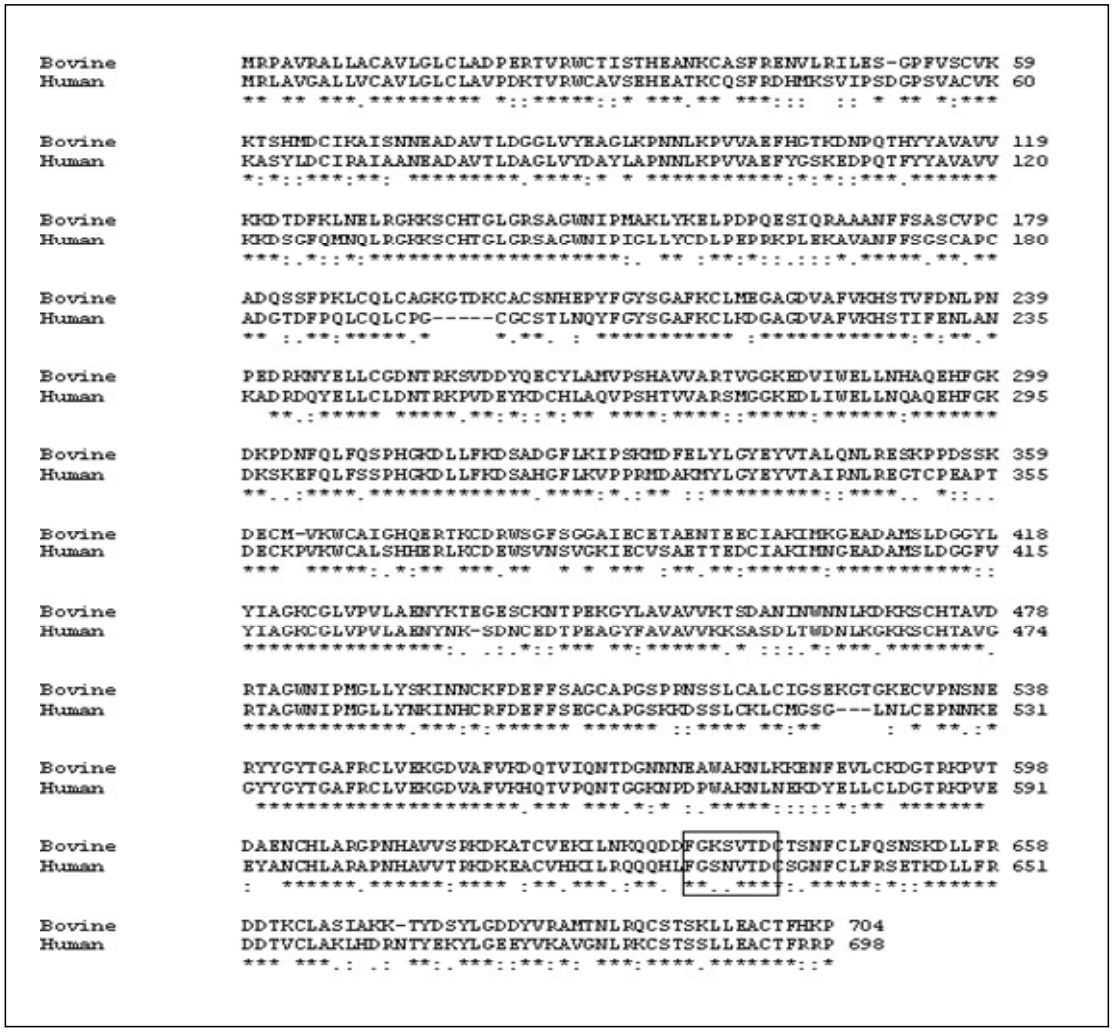
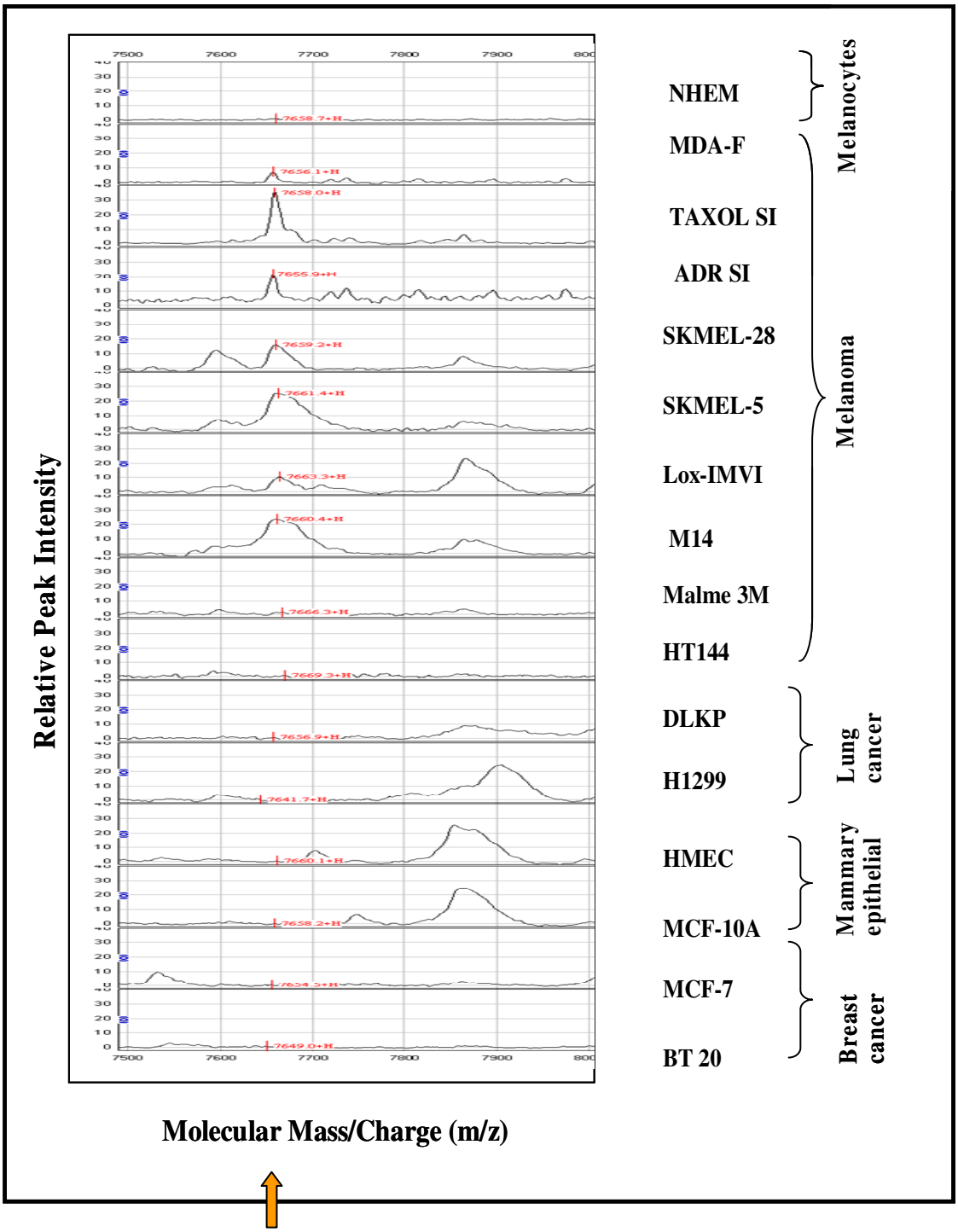


Figure 3.4.9 B: Sequence alignment of bovine and human transferrin using ClustalW. The box shows conserved boundary amino acids around the predicted cleavage site (K) for MMP2 and MMP9 in bovine transferrin.

3.4.10 Investigation of m/z 7653 peak in conditioned media from sixteen cell lines with different tissues origin by SELDI-ToF MS

This analysis of the profiles was aimed to investigate the levels of the transferrin fragment (m/z 7653) as a potential biomarker in melanoma or if it can be related to melanoma invasion. SELDI-ToF MS was used to profile protein/peptides in the conditioned media of 16 cell lines including melanocytes (NHEM), 9 melanoma (M14, HT144, SKMEL-28, SKMEL-5, Malme-3M, Lox-IMVI, MDA-MB-435S-F, MDA-MB-435S-F/ Taxol10p4pSI and MDA-MB-435S-F/ Adr-10p10pSI), 2 normal mammary epithelial (HMEC and MCF-10A), 2 breast cancer (MCF-7 and BT20) and 2 lung cancer (DLKP and H1299) cell lines with different invasive phenotypes. For this purpose, four biological replicate samples of conditioned media from all 16 cell lines were profiled on IMAC30 Cu^{++} ProteinChip arrays. Figure 3.4.10 (A) shows a representative SELDI-ToF MS spectra in a mass/charge (m/z) range from 7500 to 7900. Figure 3.4.10 B is a graphical representation of the m/z 7653 peak and also includes the statistical analysis of the expression of the m/z 7653 peak. Figure 3.6.10 (A) clearly indicates that the m/z 7653 peak is detected in all melanoma cell lines (M14, SKMEL-28, SKMEL-5, Malme-3M, Lox-IMVI, MDA-MB-435S-F, MDA-MB-435S-F/ Taxol10p4pSI and MDA-MB-435S-F/ Adr-10p10pSI) except HT144 with statistical significance ($p= 3x10^{-5}$). However the m/z 7653 was absent in melanocytes (NHEM), normal mammary epithelial (HMEC and MCF-10A), breast cancer (MCF-7 and BT20) and lung cancer (DLKP and H1299) cell lines. The m/z 7653 peak was 8.5-fold higher in MDA-MB-435S-F, 34.2-fold in MDA-MB-435S-F/ Taxol10p4pSI, 17.8 fold in MDA-MB-435S-F/ Adr-10p10pSI, 15.8-fold in SKMEL-28, 22.9-fold in SKMEL-5, 12.6-fold in Lox-IMVI, 30.2-fold in M14 and 1.68-fold in Malme 3M cells compared to melanocytes. This analysis clearly indicates that the m/z 7653 is detected in all melanoma cell lines analysed, except HT144.


Relative Peak Intensity
Molecular Mass/Charge (m/z)
NHEM
MDA-F
TAXOL SI
ADR SI
SKMEL-28
SKMEL-5
Lox-IMVI
M14
Malme 3M
HT144
DLKP
H1299
HMEC
MCF-10A
MCF-7
BT 20
Melanocytes
Melanoma
Lung cancer
Mammary epithelial
Breast cancer

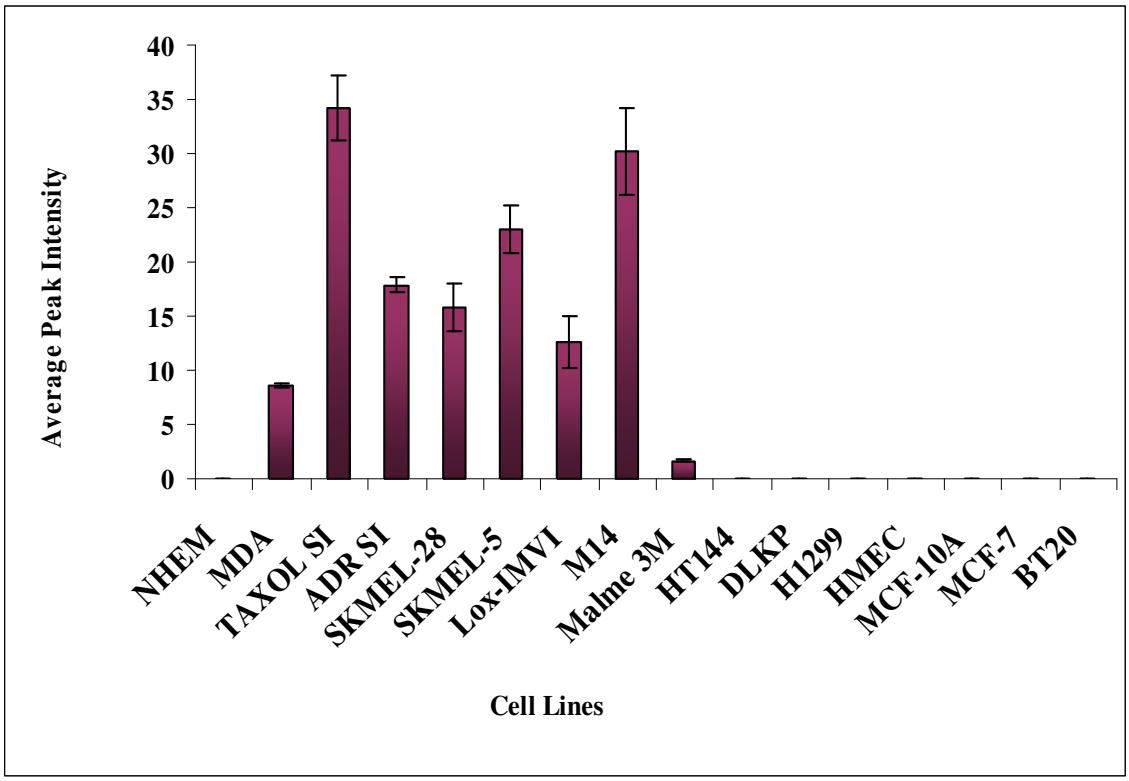


Figure 3.4.10 (A) SELDI-ToF MS spectra of the m/z 7653 peak on IMAC-Cu^{++} ProteinChip Arrays from conditioned media from melanocytes (NHEM), melanoma (MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI, MDA-MB-435S-F/Adr-10p10pSI, M14, HT144, SKMEL-28, SKMEL-5, Malme-3M and Lox-IMVI), normal mammary cells (HMEC and MCF-10A), lung (DLKP and H1299) and breast (MCF-7 and BT20) cancer cell lines. The arrow indicates the position of the m/z 7653 peak. (B) Graph showing the comparison of the average peak intensities for the m/z 7653 peak on the IMAC-Cu^{++} array in different cell lines. The error bars represent the standard deviation calculated from the four independent biological replicates from each cell lines.

3.4.11 Reproducibility of the experiment

The reproducibility of the SELDI-ToF MS spectra for the m/z 7653 peak, i.e., mass and intensity intra-experiment and inter-experiment, was also determined. For this analysis four biological replicate samples of conditioned media from 6 melanoma cell lines (M14, HT144, SKMEL-28, SKMEL-5, Malme-3M and Lox-IMVI) were profiled independently on IMAC-Cu^{++} chip surfaces. The intra and inter-experiment mean CV for the mass of the m/z 7653 peak were 0.03% and 0.05% respectively, whereas the intra and inter experiment mean CV for the normalized intensity of the peak were 11% and 22% respectively, demonstrating the reproducibility of the technique.

3.4.12 Invasion assay of melanocytes and melanoma cell lines

The invasion status of melanocytes and all of the melanoma cell lines was analysed to determine if the expression of the m/z 7653 could be correlated with the invasion status of the cell lines. The invasion assays were performed for melanocytes (NHEM) and melanoma cell lines (MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI, MDA-MB-435S-F/Adr10p10pSI, HT144, M14, SKMEL-5, Lox-IMVI, Malme-3M and SKMEL-28) over 48hrs of culture (section 2.6). The graph was plotted on the basis of the total number of cells that cross the matrigel membrane (Figure 3.4.11). In this investigation SKMEL-5 was found to be non-invasive and other melanoma cell lines (HT144, Malme-3M, M14, Lox-IMVI, MDA-MB-435S-F/Adr10p10pSI, MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI and SKMEL-28) including melanocytes were invasive (Figure 3.4.11). SKMEL-28, MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI and melanocytes (normal cells) cells were highly invasive compared to Lox-IMVI, MDA-MB-435S-F/Adr10p10pSI. However HT144, Malme-3M and M14 were low invasive compared to SKMEL-28, MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI.

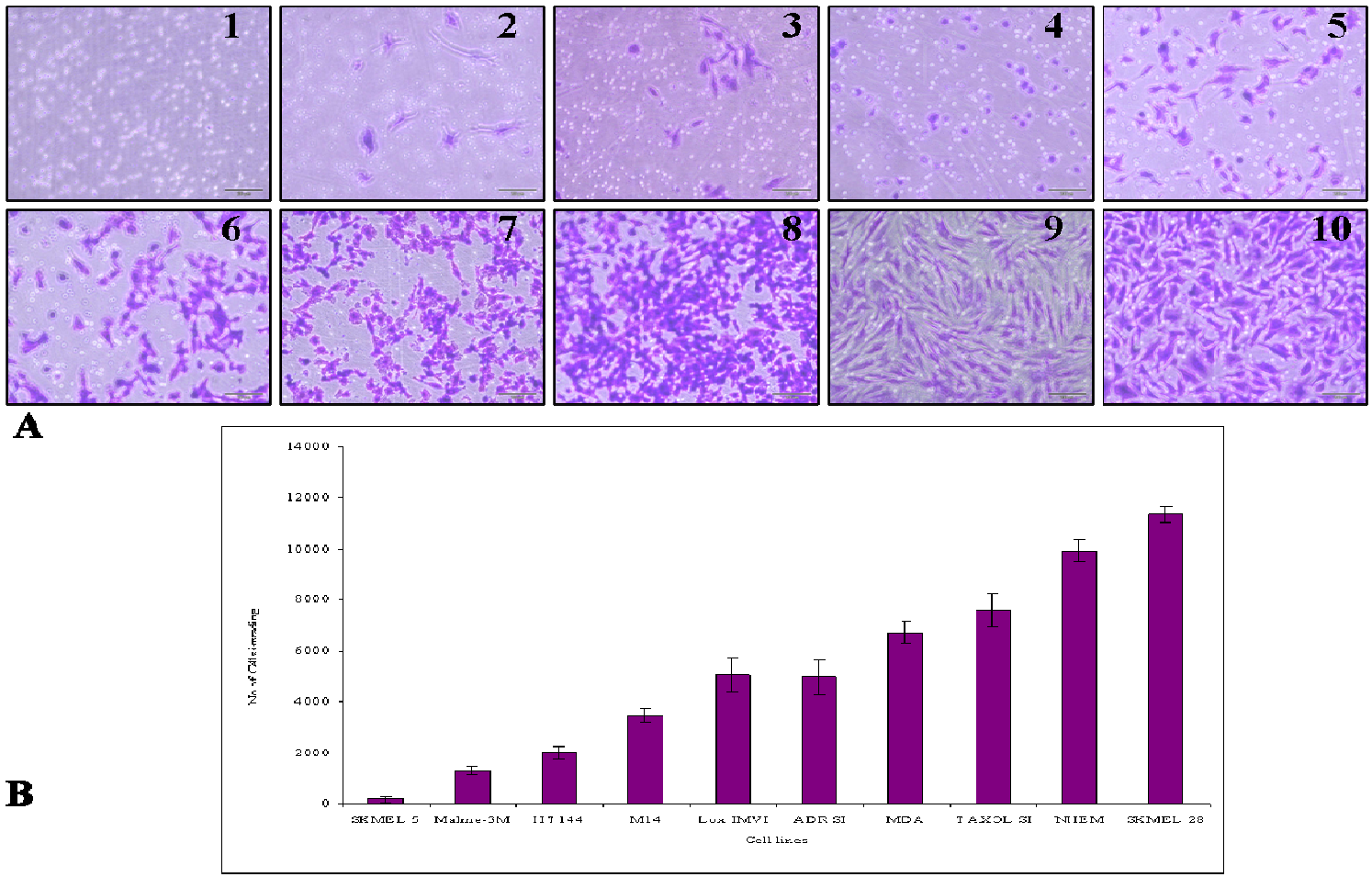


Figure 3.4.11: Investigation of the invasion capabilities of melanocytes and melanoma cells. **A.** Images from the invasion assay- (1) SKMEL-5, (2) Malme-3M, (3) HT144, (4) M14, (5) LovIMVI, (6) MDA-MB-435S-F/Adr10p10pSI, (7) MDA-MB-435S-F, (8) MDA-MB-435S-F/Taxol10p4pSI, (9) NHEM and (10) SKMEL-28. **(B)** Graphical representation of comparative levels of invasion in the cells tested. Magnification= 200x, Scale bar=200µm. Error bars represent the standard deviation calculated from data obtained from three biological samples.

3.4.13 Relation to invasion status

Figure 3.4.12 shows the images from a 48 hour invasion assay of melanocytes (NHEM) and all melanoma cell lines (MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI, MDA-MB-435S-F/Adr-10p10pSI, M14, HT144, SKMEL-28, SKMEL-5, Malme-3M and Lox-IMVI) and from the figure it is clear there is no correlation in the levels of expression of the transferrin fragment with the levels of invasion as SKMEL-5 is non-invasive and expresses high levels of the m/z 7653 species. Malme 3M is invasive but expresses low levels of the the m/z 7653 species. Also melanocytes are invasive and there was no expression of the m/z 7653 species. Therefore, our results concluded that the m/z 7653 species could possibly be a biomarker for melanoma. Table 3.4.2 summarises the invasion status and m/z 7653 expression in all of the melanoma cell lines.

Table 3.4.2: Relation of invasion status with the levels of m/z 7653 peak in melenocytes and melanoma cell lines.

Cell lines	**Invasion status**	**Levels of m/z 7653 Peak (Fold change compared to melanocytes)**
NHEM	Invasive	Not detected
MDA-MB-435S-F	Invasive	8.5
MDA-MB-435S-F/Taxol10p4pSI	Invasive	34.2
MDA-MB-435S-F/Adr-10p10pSI	Invasive	17.8
M14	Low invasive	30.2
HT144	Low invasive	Not detected
SKMEL-28	Invasive	15.8
SKMEL-5	Non-invasive	22.9
Malme-3M	Invasive	1.68
Lox-IMVI	Invasive	12.6

3.4.14 Western blot analysis of NHEM and MDA-MB-435S-F/Taxol10p4pSI cells

There is no commercially available bovine transferrin antibody which can recognize the transferrin fragment from conditioned media. Transferrin receptor is a carrier protein for transferrin protein therefore it could be possible that transferrin receptor expression is also altered between melanocytes and melanoma cell lines. Thus western blot analysis of whole cell lysates from melanocytes (NHEM) and one melanoma cell line (MDA-MB-435S-F/Taxol10p4pSI), which has the highest level of truncated transferrin, using an antibody against the transferrin receptor was carried out. Figure 3.4.12A shows that the transferrin receptor at approximately 190kDa was found to be increased in the MDA-MB-435S-F/Taxol10p4pSI cell line compared to the melanocytes. The expression of the internal control protein α-tubulin was un-changed. Densitometry analysis showed that the transferrin receptor was 35.9 fold up-regulated in the melanoma cell line (MDA-MB-435S-F/Taxol10p4pSI) compared to melanocytes. This 35.9-fold increase in the expression of transferrin receptor possibly corresponds to the 34.2-fold increase in the level of truncated transferrin identified by SELDI-ToF MS analysis in the MDA-MB-435S-F/Taxol10p4pSI conditioned media (Figure 3.4.13B).

The expression of transferrin receptor also showed a marked increase in abundance MDA-MB-435S-F/Taxol10p4pSI cell line compared to its parental cell line MDA-MB-435S-F. The transferrin receptor was found to be approximately 3.5-fold increased in the MDA-MB-435S-F/Taxol10p4pSI compared to the parental cell line (Figures 3.4.14B). This 3.5-fold increase in the receptor possibly corresponds to a 4-fold increase in the transferrin fragment identified by SELDI analysis found in the MDA-MB-435S-F/Taxol10p4pSI conditioned media (Figure 3.4.14C). The commassie stained gels was used to demonstrate as best as possible equal loading between the samples (Figures 3.4.14A).

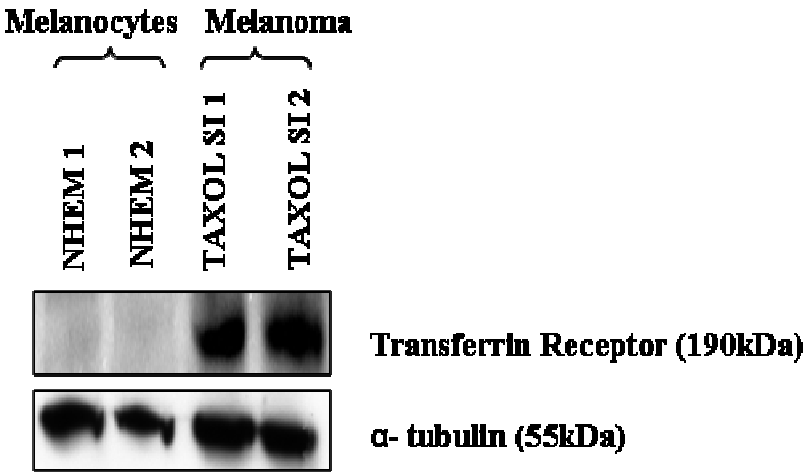


Figure 3.4.12 (A): Western blot analysis of transferrin receptor in melanocytes and the melanoma cell line, MDA-MB-435S-F/Taxol10p4pSI. Cell lysates from NHEM and MDA-MB-435S-F/Taxol10p4pSI (TAXOL SI) in duplicate were separated on an SDS-PAGE gel, transferred on to nitrocellulose membranes, probed with anti-transferrin receptor and anti-α-tubulin antibody. An increase in the expression of transferrin receptor in melanoma cell lines compared to melanocytes was observed. α-tubulin was used as a loading control and no significant change in expression of α-tubulin was observed.

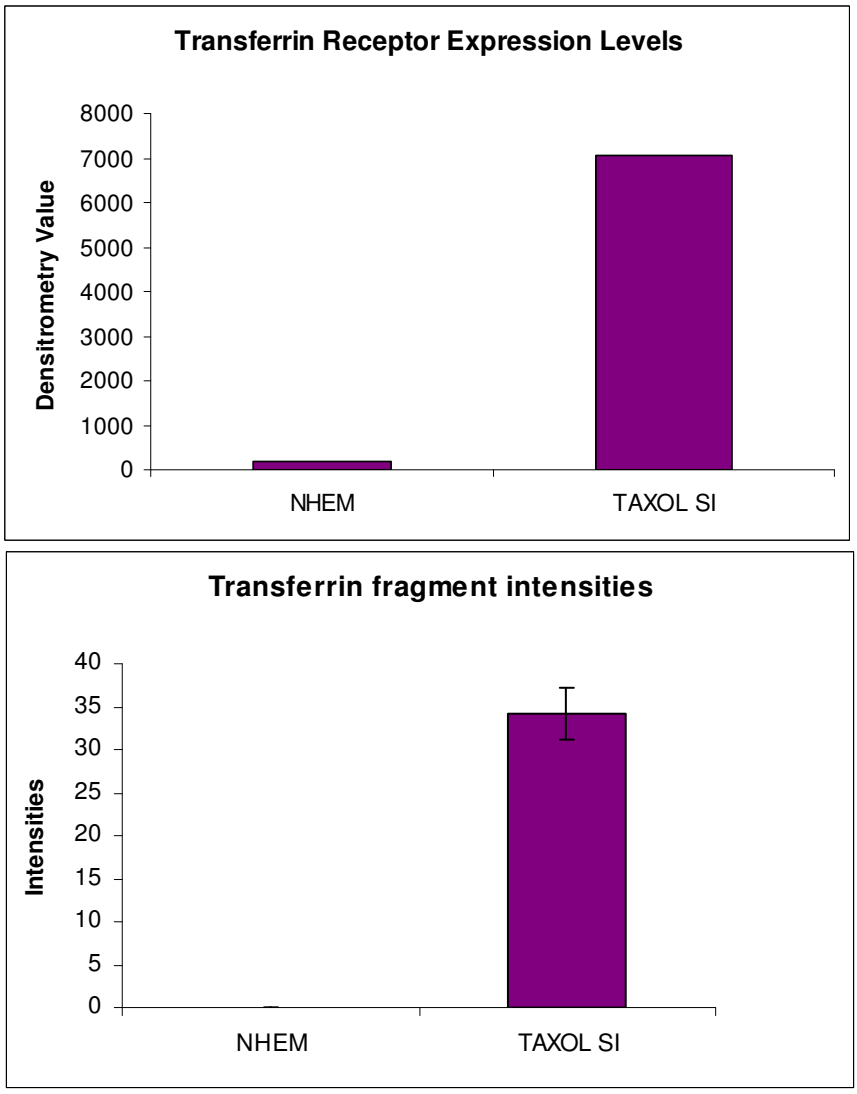


Figure 3.4.13(B) Upper panel- Statistical analysis of the transferrin receptor protein levels from melanocytes (NHEM) and the melanoma cell line MDA-MB-435S-F/Taxol10p4pSI. Lower panel- Statistical analysis for the intensity levels of the m/z 7653 peak from SELDI-ToF MS found secreted in conditioned media by the melanoma cell line MDA-MB-435S-F/Taxol10p4pSI.

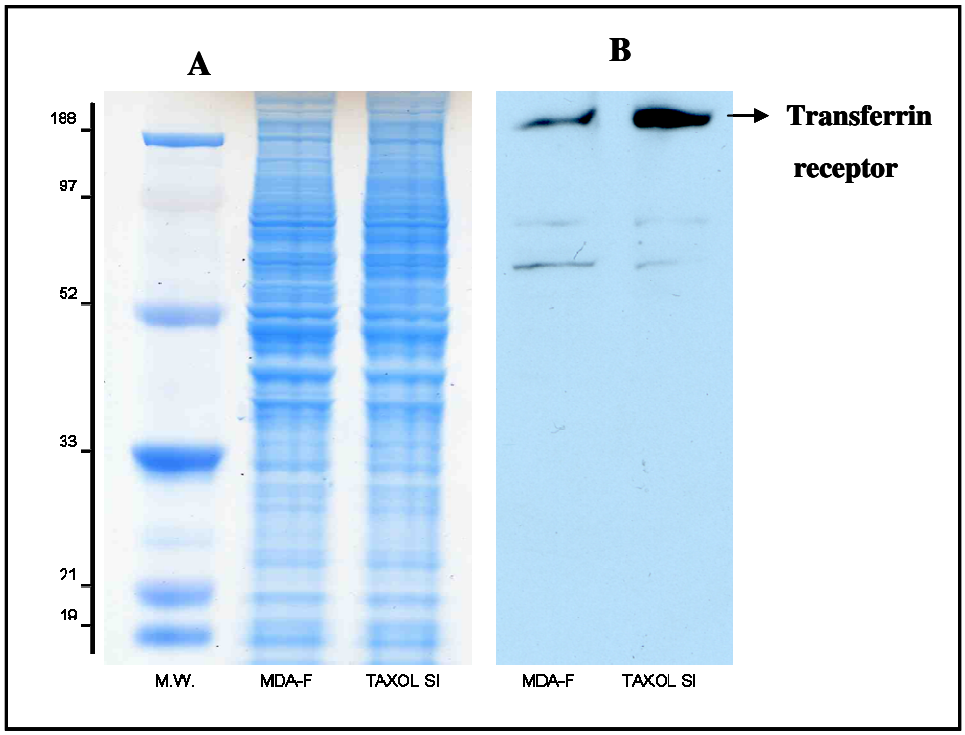


Figure 3.4.14 (A) Coomassie blue stained gel showing equal protein loading from both MDA-MB-435S-F and its paclitaxel-resistant superinvasive variant MDA-MB-435S-F/Taxol10p4pSI. (B) Western blot analysis of MDA-MB-435S-F and MDA-MB-435S-F/Taxol10p4pSI probed with a transferrin receptor antibody showing the increase in abundance of the 190kDa protein in the MDA-MB-435S-F/Taxol10p4pSI phenotype. This Western blot is representative of repeat experiments.

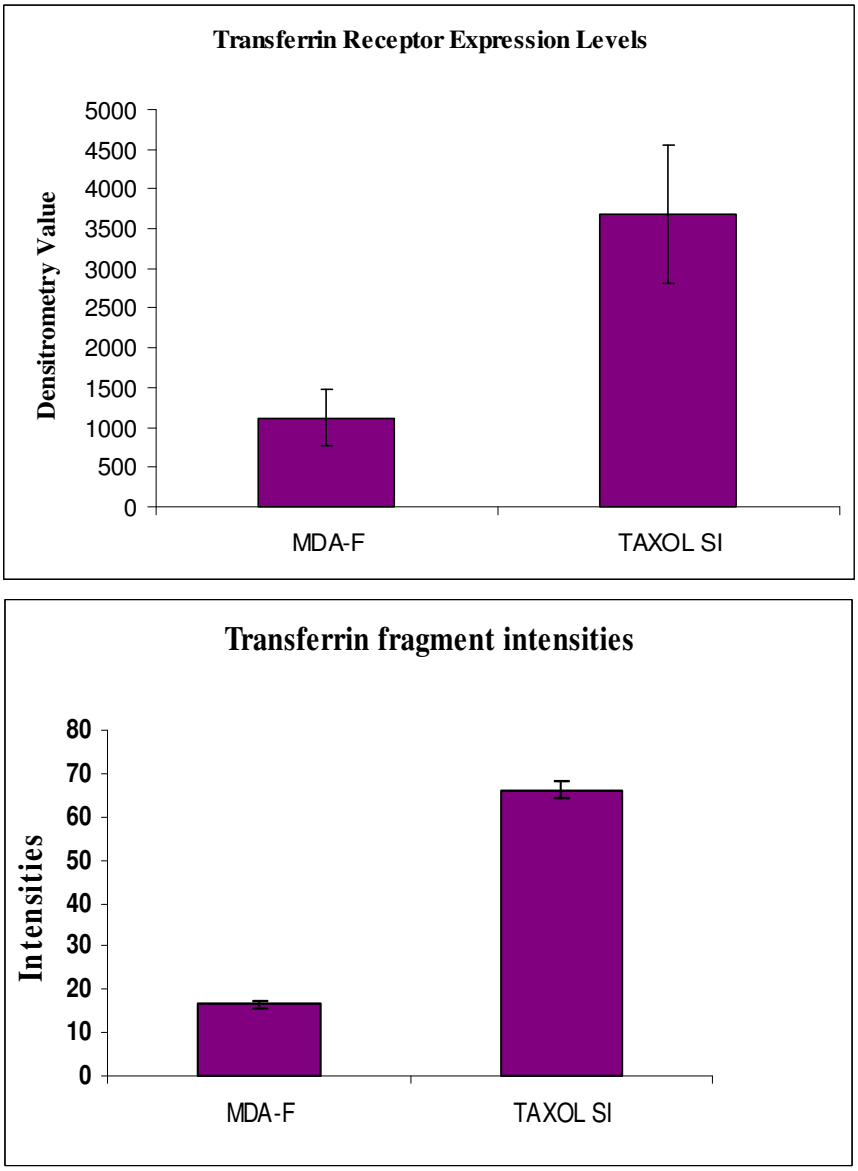


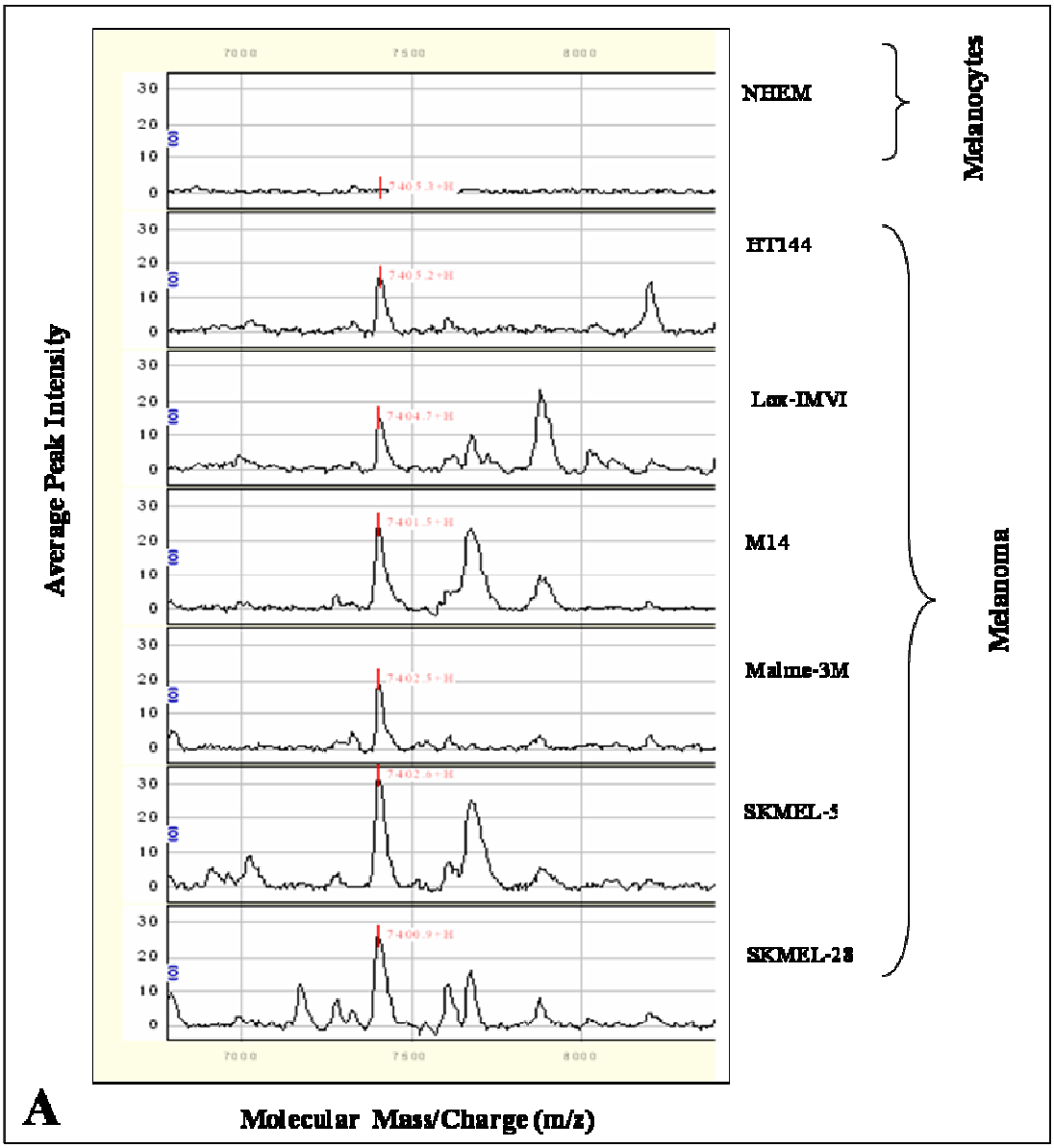
Figure 3.4.14 (C) Upper panel- Statistical analysis of the transferrin receptor from melanoma cell line MDA-MB-435S-F and MDA-MB-435S-F/Taxol10p4pSI. Lower panel- Statistical analysis for the intensity levels of the m/z 7653 peak from SELDI-ToF MS found secreted in conditioned media by melanoma cell lines MDA-MB-435S-F/Taxol10p4pSI and MDA-MB-435S-F.

3.5 Differential expression of proteins/peptides from media conditioned by melanocytes and melanoma cell lines

3.5.1 SELDI-ToF analysis analysis

This study aimed to investigate differential expression profiling of melanocytes and melanoma cell lines to discover potential biomarkers for melanoma that could be helpful to understand the process of transformation of melanocytes cells into melanoma. For this, conditioned media from melanocytes (NHEM) and six melanoma cell lines (HT144, Lox-IMVI, M14, Malme-3M, SKMEL-5 and SKMEL-28) were analysed by SELDI-ToF MS. For this purpose, four biological replicate samples of conditioned media from melanocytes and melanoma cell lines were profiled on IMAC30 Cu^{++} ProteinChip arrays. Four peaks at m/z 7406, 8584, 11959 and 12081 were found differentially expressed.

Figure 3.5.1 A shows a representative SELDI-ToF MS spectra of m/z 7406. Figure 3.5.1 B is a graphical representation of intensities of the m/z 7406 peak and also includes the statistical analysis of the peak. The m/z 7406 peak is dominantly expressed in all melanoma cell lines (HT144, Lox-IMVI, M14, Malme-3M, SKMEL-5 and SKMEL-28) with statistical significance ($p= 3.5x10^{-7}$) (Figure 3.7.1B). The m/z 7406 peak was up-regulated by 15.2-fold in HT144, 19.2-fold in Lox-IMVI, 35.6-fold in M14, 21-fold in Malme 3M, 35.5-fold in SKMEL-5, and 37.5-fold in SKMEL-28 cells compared to melanocytes.


A
Average Peak Intensity
Molecular Mass/Charge (m/z)
NHEM
HT144
Lox-IMVI
M14
Malme-3M
SKMEL-5
SKMEL-28
Melanocytes
Melanoma

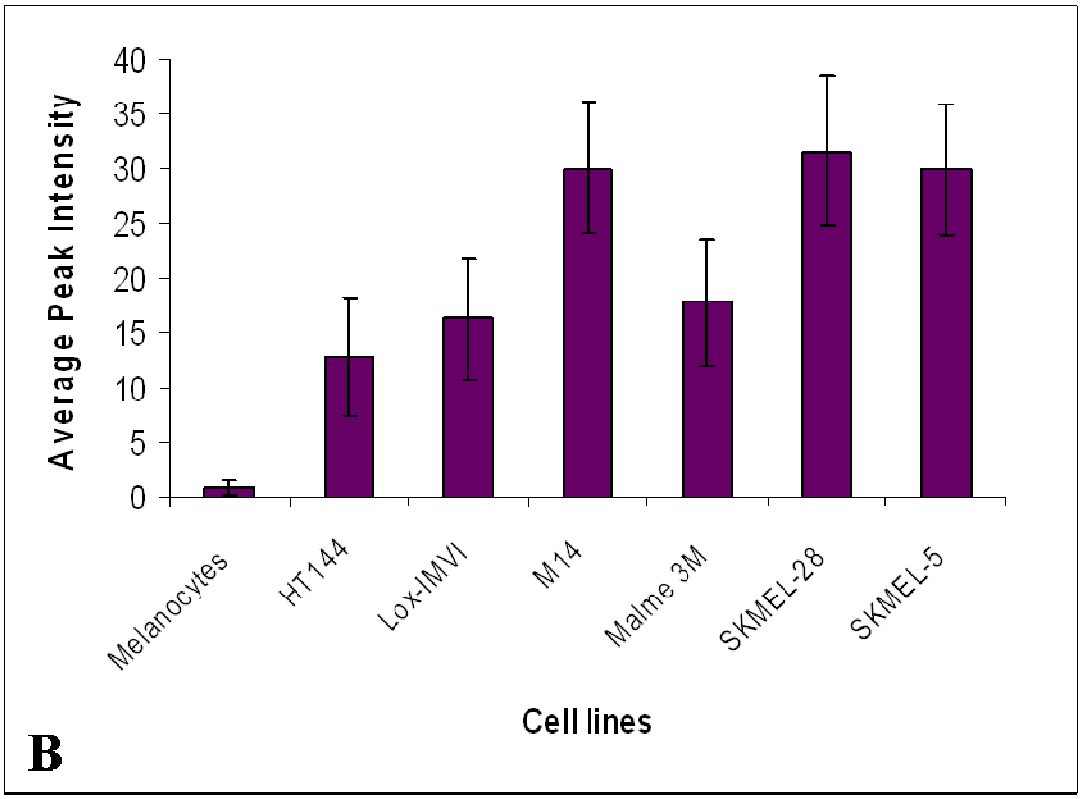
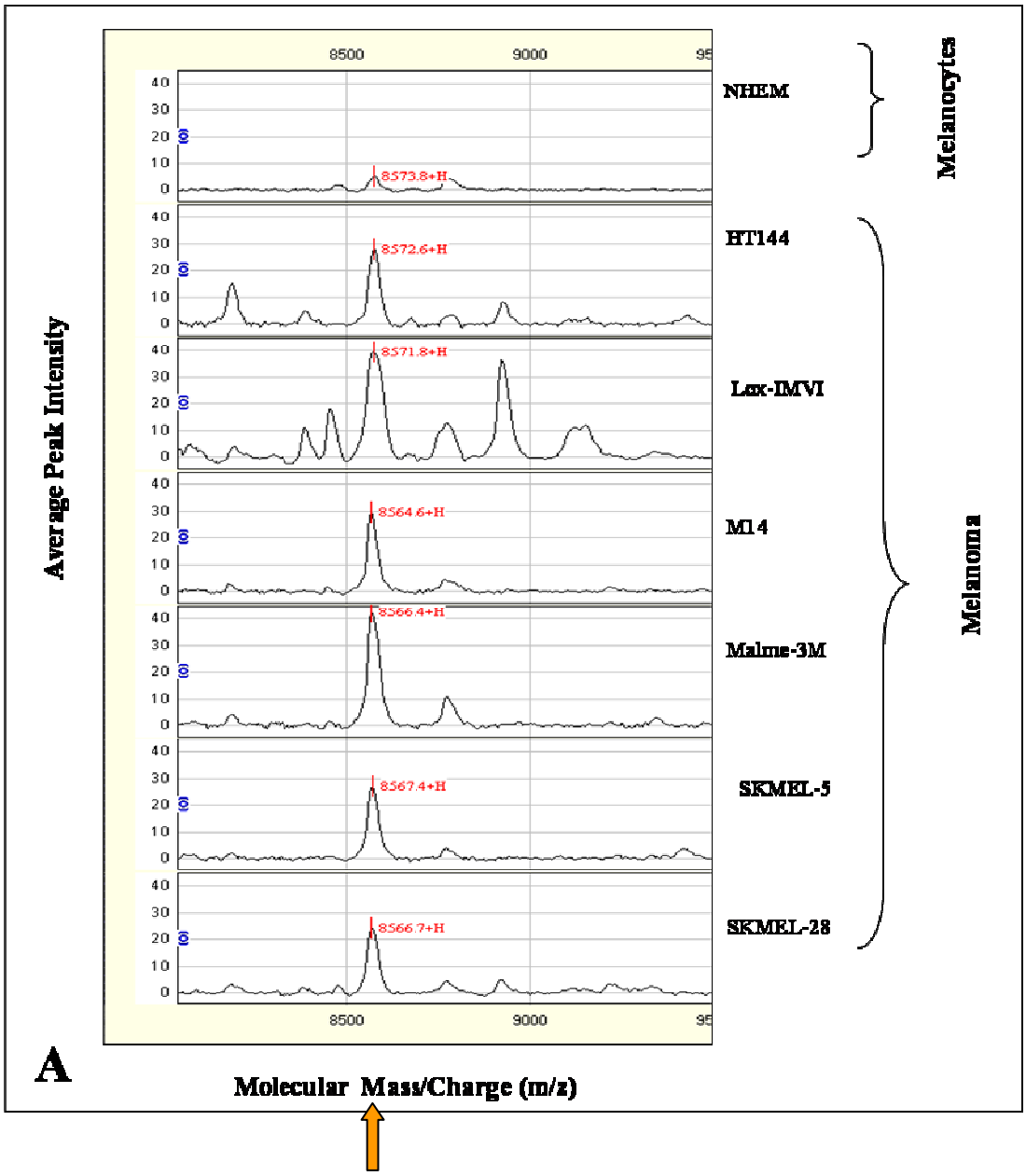


Figure 3.5.1 (A) SELDI-ToF MS spectra of the m/z 7406 peak on IMAC-Cu^{++} ProteinChip Arrays from conditioned media from the melanocytes (NHEM), melanoma (HT144, Lox-IMVI, M14, Malme-3M, SKMEL-5 and SKMEL-28) cell lines. (B) Graph showing the comparison of the average peak on the IMAC-Cu^{++} array in different cell lines. The error bars represent the standard deviation calculated from the four independent biological replicates from each cell line.

Figure 3.5.2 A shows a representative SELDI-ToF MS spectra of m/z 8584 peak. Figure 3.5.2 B is a graphical representation of the intensities of m/z 8584 and also includes the statistical analysis of the peak. The m/z 8584 peak is dominantly expressed in all melanoma cell lines (HT144, Lox-IMVI, M14, Malme-3M, SKMEL-5 and SKMEL-28) with statistical significance ($p= 9.3x10^{-7}$) (Figure 3.7.2B). The m/z 8584 peak was up-regulated by 5-fold in HT144, 10.6-fold in Lox-IMVI, 8.6-fold in M14, 10.4-fold in Malme 3M, 7.2-fold in SKMEL-5, and 7.9-fold in SKMEL-28 cells compared to melanocytes.



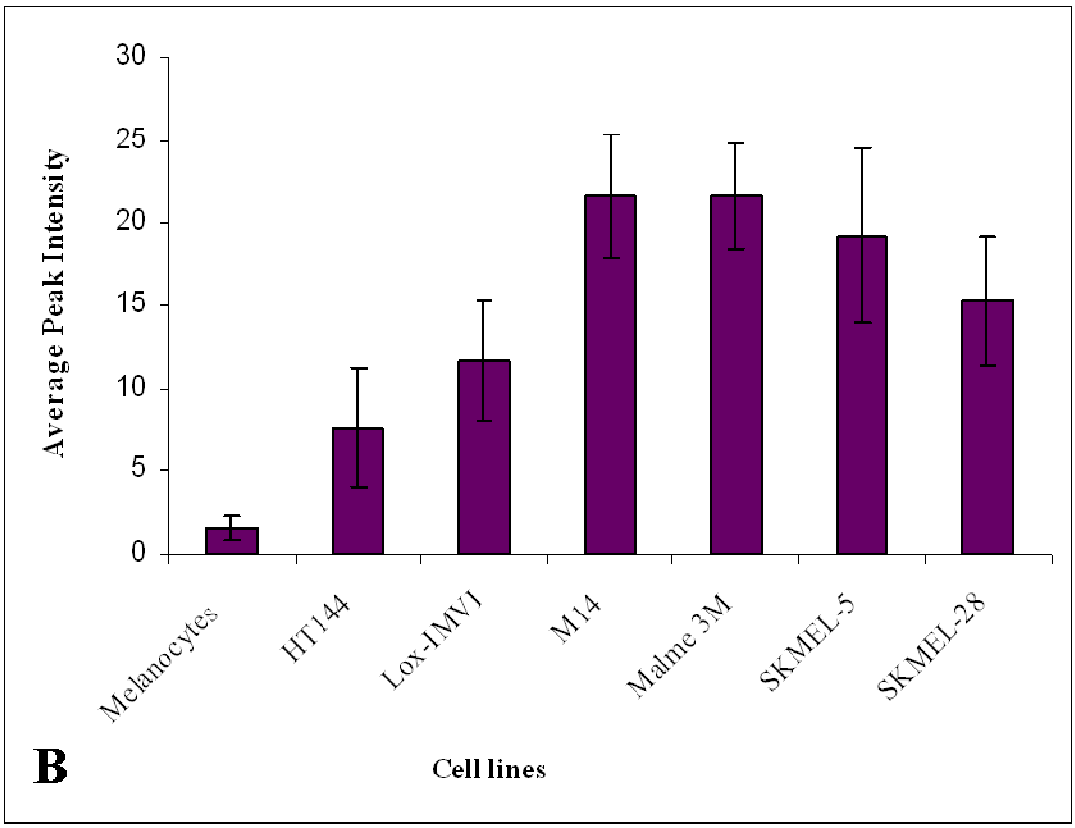


Figure 3.5.2 (A) SELDI-ToF MS spectra of the m/z 8584 peak on IMAC-Cu^{++} ProteinChip Arrays from conditioned media from the melanocytes (NHEM), melanoma (HT144, Lox-IMVI, M14, Malme-3M, SKMEL-5and SKMEL-28) cell lines. The arrow indicates the position of a differentially expressed peak at m/z 8584. (B) Graph showing the comparison of the average peak intensities for the m/z 8584 peak on the IMAC-Cu^{++} array in different cell lines. The error bars represent the standard deviation calculated from the four independent biological replicates from each cell line.

Figure 3.5.3 A shows a representative SELDI-ToF MS spectra of the m/z 11959 and 12081 peaks. Figure 3.5.3 B is a graphical representation of the intensities of m/z 11959 and 12081 peaks and also includes the statistical analysis of the peaks. Figure 3.5.3 B1 clearly indicates that the m/z 11959 peak is dominantly expressed in all melanoma cell lines (HT144, Lox-IMVI, M14, Malme-3M, SKMEL-5 and SKMEL-28) with statistical significance ($p= 2.2x10^{-6}$). The m/z 11959 peak was up-regulated by 4.9 fold in HT144, 7.4-fold in Lox-IMVI, 13.8-fold in M14, 13.7-fold in Malme 3M, 12.3-fold in SKMEL-5 and 9.7-fold in SKMEL-28 cells compared to melanocytes. Figure 3.5.3 B2 clearly indicates that the m/z 12081 peak is dominantly expressed in all melanoma cell lines (HT144, Lox-IMVI, M14, Malme-3M, SKMEL-5 and SKMEL-28) with statistical significance ($p= 1.0x10^{-8}$). The m/z 12081 peak was up-regulated by 11.3-fold in HT144, 3.5-fold in Lox-IMVI, 4.8-fold in M14, 13.9-fold in Malme 3M, 4.9-fold in SKMEL-5 and 22.1-fold in SKMEL-28 cells compared to melanocytes.

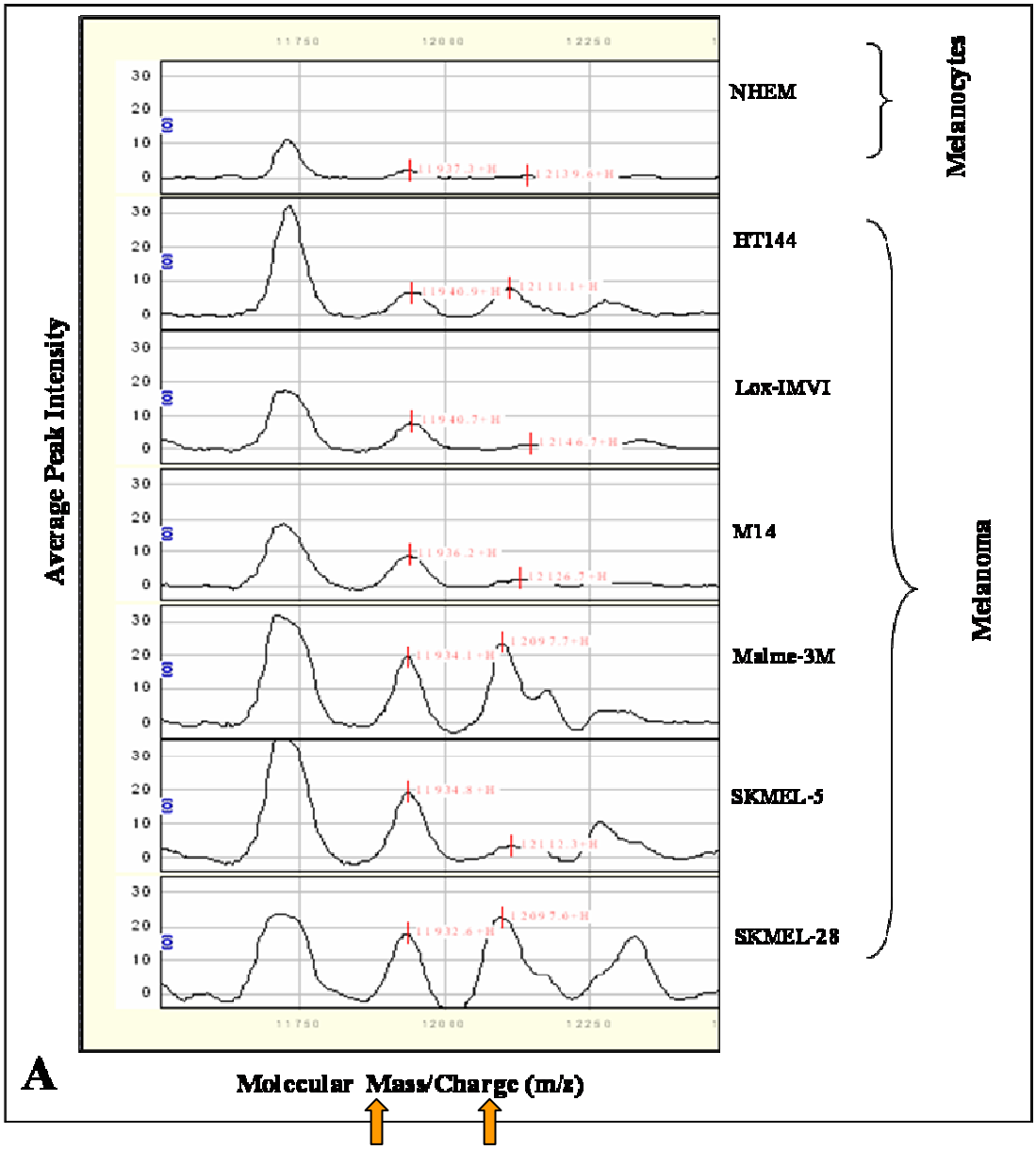
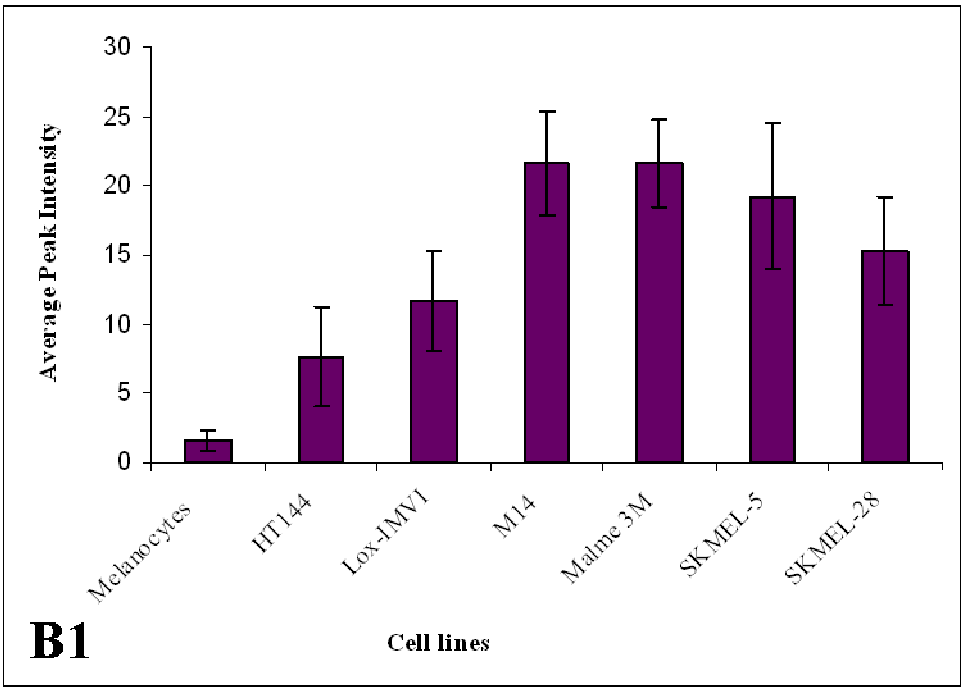


Figure 3.5.3 (A) SELDI-ToF MS spectra of the m/z 11959 and 12081 peaks on IMAC-Cu^{++} ProteinChip Arrays from conditioned media from melanocytes (NHEM), melanoma (HT144, Lox-IMVI, M14, Malme-3M, SKMEL-5and SKMEL-28) cell lines. The arrow indicates the position of differentially expressed peaks at m/z 11959 and 12081.



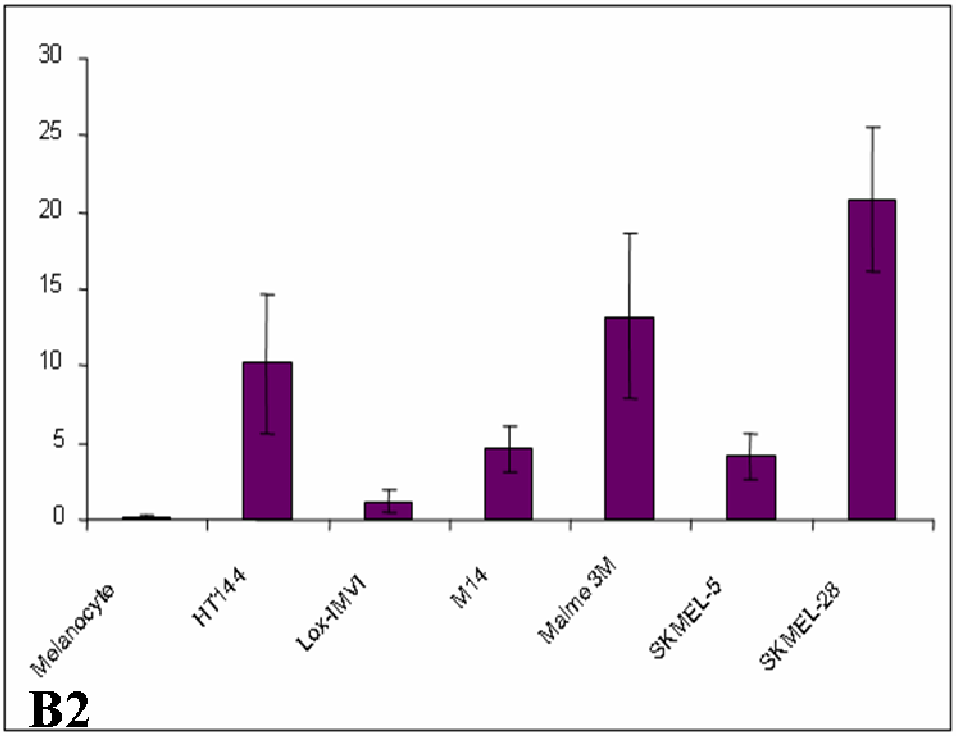


Figure 3.5.3 (B1 and B2) Graph showing the comparison of the average peak intensities for the m/z 11959 and 12081 peak respectively on the IMAC-Cu^{++} array in different cell lines. The error bars represent the standard deviation calculated from the four independent biological replicates from each cell line.

3.5.2 Reproducibility of the experiment

The reproducibility of the SELDI-ToF MS spectra for m/z 7404, 8584, 11959 and 12081 peak, i.e., mass and intensity was also determined. For this analysis four biological replicate samples of conditioned media from melanocytes and 6 melanoma cell lines (M14, HT144, SKMEL-28, SKMEL-5, Malme-3M and Lox-IMVI) were profiled independently on IMAC-Cu^{++} chip surfaces. The inter-experimental mean CV for the mass of the m/z 7404, 8584, 11959 and 12081 peaks were 0.04, 0.03, 0.009 and 0.03 % respectively, whereas the inter-experimental mean CV for the normalized intensity were 12.7, 15.2, 12.8 and 20.2% respectively, demonstrating the reproducibility of the technique.

3.5.3 Relation to invasion status

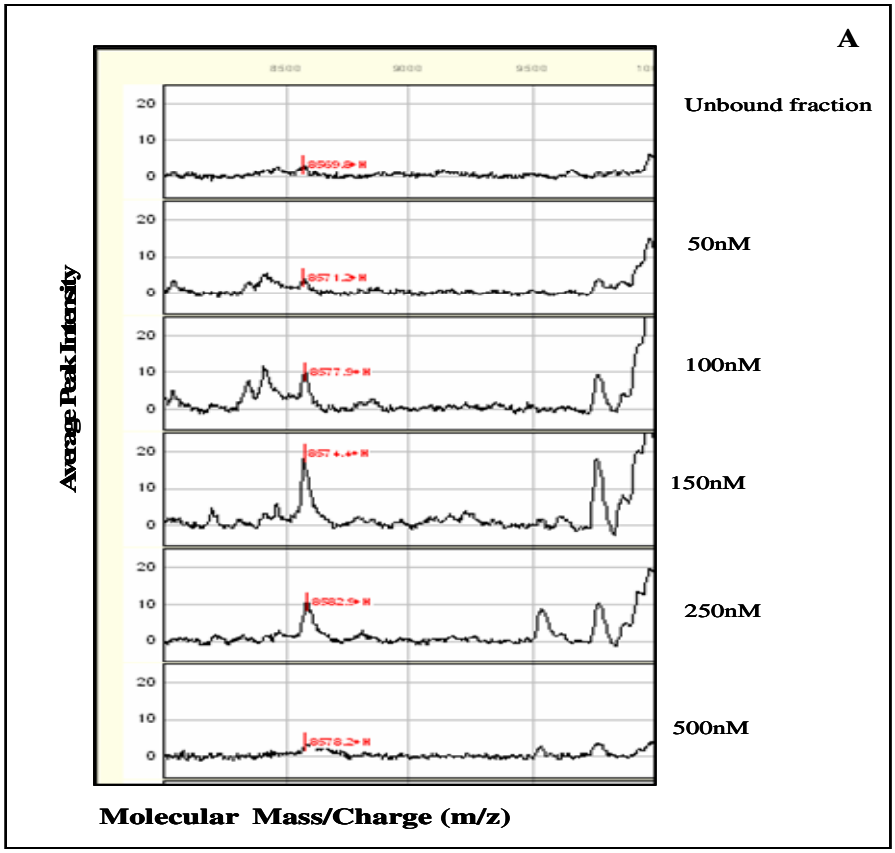
We were also interested to see if the expression of the m/z 7404, 8584, 11959 and 12081 peaks have any correlation with the invasion status of the cell lines. To determine correlation with the invasion status, the expression levels of the m/z 7404, 8584, 11959 and 12081 peaks were compared with the invasion assay results from the melanocytes and melanoma cell lines as outlined in result section 3.4.12. No correlation was seen as SKMEL-5 is non-invasive cell line expression and all of four peaks (m/z 7404, 8584, 11959 and 12081 peaks) were observed to be expressed in the conditioned media of this cell line. Table 3.5.3.1 summarises the invasion status and expression of the m/z 7404, 8584, 11959 and 12081 peaks in the conditioned media of melanocytes and melanoma cell lines.

Table 3.5.1 : Relation of invasion status with the levels of the m/z 7406, 8584, 11959 and 12081 peaks expression in melanocytes and melanoma cell lines.

Cell lines	**Invasion status**	**Levels of m/z 7406 (Fold change compared to melanocytes)**	**Levels of m/z 8584 (Fold change compared to melanocytes)**	**Levels of m/z 11959 (Fold change compared to melanocytes)**	**Levels of m/z 12081 (Fold change compared to melanocytes)**
NHEM	Invasive	-	-	-	-
HT144	Low invasive	15.2	5	4.9	11.3
Lox-IMVI	Invasive	19.2	10.6	7.4	3.5
M14	Invasive	35.6	8.6	13.8	4.8
Malme-3M	Low invasive	21	10.5	13.7	13.9
SKMEL-5	Non-invasive	35.5	7.2	12.3	4.9
SKMEL-28	Invasive	37.5	7.9	9.7	22.1

3.5.4 Purification of the Potential marker candidate

Although the m/z 7404, 8584, 11959 and 12081 peaks did not show any correlation with invasion but was highly expressed in melanoma cell lines therefore attempts were made to identify these proteins as they could play an important role in the transformation of melanocytes to melanoma. To identify differentially expressed peaks, conditioned media from SKMEL-28 cells was fractionated using BioSepra IMAC-Cu^{2+} HyperCel spin columns. The 150mM imidazole fraction showed the highest concentration of the 8.5kDa protein along with the least amount of contaminating peaks (section 2.11.1). We tried to fractionate the m/z 7404, 11959 and 12081 peaks but we were not able to purify them. This possibly needs more advanced fractionation methods to be applied to purify them from the complex mixture of proteins from the conditioned media. Figure 3.5.4 shows the chromatograph from all of the seven fractions collected from unbound peptides/proteins through to 500 mM Imidazole. All fractions were run on a 16% Tricine 1D-gel. Coomassie staining of the gel allowed a band at approximately 8.5kDa to be excised. Figure 3.5.4 B shows the coomassie stained gel and the position of the 8.5kDa protein.



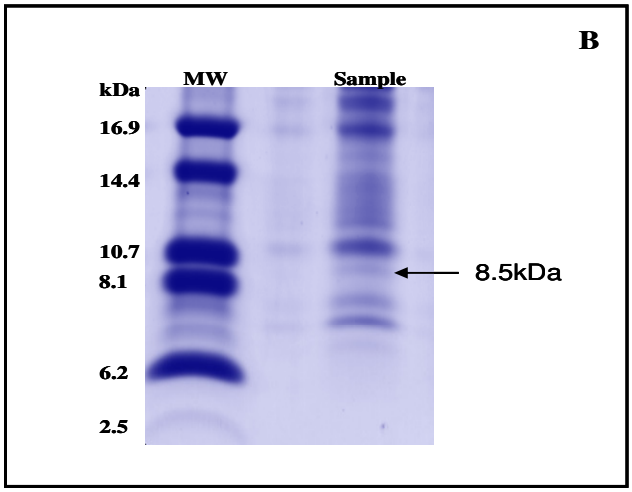


Figure 3.5.4 (A): IMAC-Cu^{2+} Hypercel fractionation of SKMEL-28 using increasing concentrations of imidazole. All seven fractions were collected from unbound peptides/proteins through to 500 mM imidazole and were analysed on NP20 ProteinChip Arrays. (B) Coomassie stained gel and the arrow indicates the approximately position of the 8.5kDa protein/peptide.

3.5.5 Identification of the 8.5kDa marker candidate

In-gel digestion was carried out for the gel plug of that equivalent to the 8.5kDa protein/peptide band. Tryptic digests of the proteins were subject to MALDI ToF analysis. The expectation value for the protein was determined by Ettan MALDI-ToF Pro evaluation software (GE Healthcare) using the ProFound database search engine for peptide mass fingerprints (Section 2.7.3.10). The identified proteins by MALDI ToF MS had an expectation value of 0.09 (a value of 0 denotes 100% confidence in the database match, a value of, 0.09 denotes a 9% or less chance that the identification is random) with 61.8% coverage of the sequence of the identified protein. The MS data were combined and searched against a number of databases and identified as ubiquitin protein. A total of 11 peptides were generated by MALDI ToF MS after tryptic digestion and 5 peptides matched for ubiquitin protein (gil51703339). The molecular weight of identified ubiquitin was 8.55 and pI was 6.6. Figure 3.5.5 shows the spectra generated from MALDI ToF MS.

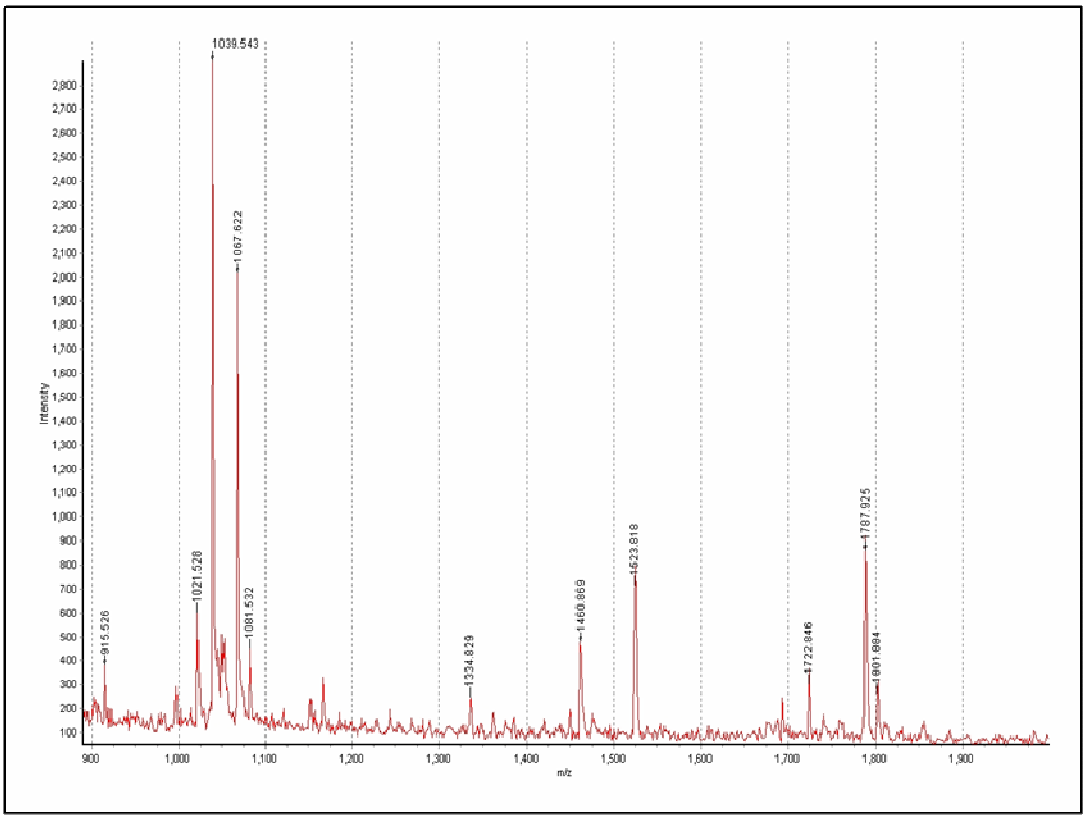


Figure 3.5.5: MALDI-ToF generated spectrum from a tryptic digest of the 8.5kDa peak. The 8.5kDa peak was positively identified as ubiquitin protein.

3.5.6 Validation of ubiquitin protein in conditioned media by western blot

In order to the validate the expression pattern and the identification of the 8.5kDa peak observed by SELDI ToF MS, western blot analysis was carried out. Immunoblot analysis of conditioned media from melanocytes (NHEM) and one melanoma cell line (SKMEL-28), which has the highest expression of ubiquitin, using an antibody against ubiquitin showed a marked increase in the abundance levels of the ubiquitin protein in SKMEL-28 at 8.5kDa. Figure 3.5.6 shows ubiquitin was found to be increased in the melanoma cell line compared to the melanocytes.

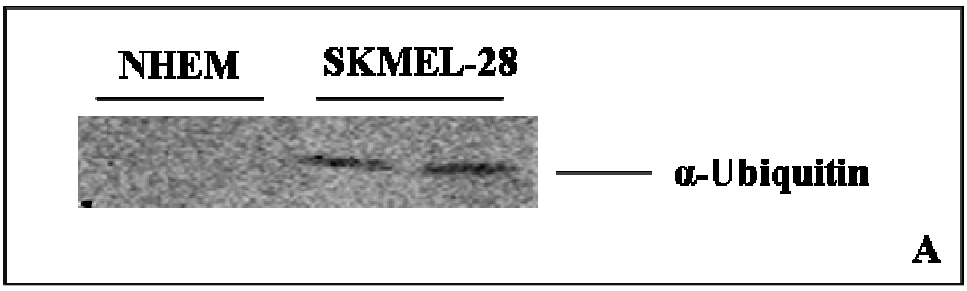


Figure 3.5.6. Western blot images for the expression of ubiquitin protein in the conditioned media from NHEM and SKMEL-28. Biological duplicate samples were used in this investigation.

Section 4

Discussion

4.1 Breast Cancer

Breast cancer is a leading cause of death among women. It is a disease of the middle and late ages of life as 75% of breast cancer is diagnosed in women over the age of 50. Although breast cancer is less common at a young age, younger women tend to have a more aggressive form of the disease than older women. The 5-year survival rate is close to 97% when the cancer is confined to the breast (Shevchenko *et al.* 1996). Currently mammography is the most commonly employed method for breast cancer screening despite its disadvantages such as high false positive and negative rates, hazardous exposure, and patient discomfort (Jemal *et al.* 2004).

Breast cancer is a complex disease that reflects the genetic as well as protein changes within a cell. Gene expression data gives us limited relevant information since proteins are the main functional units performing all biological process in the cell or organism and may have post-transcriptional event(s) and post-translational modification(s) that contribute towards biological activity of proteins. Proteomic technologies allow for identification of the protein changes caused by the disease process in a relatively accurate manner. The inherent advantage afforded to proteomics is that the identified protein is itself the biological endpoint. At the protein level, distinct changes occur during the transformation of a healthy cell into a neoplastic cell, including altered expression, differential protein modification, changes in specific activity and aberrant localization, and all of which may affect cellular function. Therefore, we used 2D-DIGE followed by MALDI-ToF MS and LC-MS/MS mass spectrometry in this investigation to identify the changes in protein expression in breast cell lines with normal and cancerous, non-invasive and invasive phenotypes.

Proteins redistribute in response to a variety of physiological stimuli. Activation of many cellular regulatory pathways is accompanied by the translocation of key proteins from one region of the cell to another such as membrane and extracellular regions. The main objective of this part of the work was to establish differential protein profiling followed by identification of differently expressed proteins from three fractions (cell lysates, conditioned media and membrane fraction) of four breast cell lines.

4.1.1 Differential Analysis of protein profiles of total cell lysates from HMEC, MCF-10A, MCF-7 and BT20 cells

Protein expression maps (PEMs) were created for total cell lysates from normal-invasive (HMEC), normal-non-invasive (MCF-10A), cancer-invasive (BT20) and cancer-non-invasive (MCF-7) cells. Since the cellular proteins could play an essential role in cancer and invasion in breast cells, PEMs were compared using DeCyder 6.5 software to identify cancer, normal+cancer-related invasion and cancer-related invasion-associated changes in protein expression in the cell lysates. The data was compared in three groups (Box 1)

Box 1: To identify cancer and invasion-associated proteins, protein profiles were created for HMEC, MCF-10A, MCF-7 and BT20 cells and three lists of differentially expressed proteins were generated;

- Cancer associated differentially expressed proteins from the comparison of cancer (MCF-7+BT20) vs. normal (HMEC+MCF-10A),
- Normal+cancer invasion-related differentially expressed proteins from the comparison of the invasive (HMEC+BT20) vs. non-invasive (MCF-10A+MCF-7)
- Cancer invasion-related differentially expressed proteins from the comparison of cancer invasive (BT20) vs. normal invasive+ normal non-invasive+ cancerous non-invasive (HMEC+MCF-10A+MCF-7) *(Group1 vs. group 2).*

4.1.1.1 Cancer (MCF-7+BT20) vs. Normal (HMEC+MCF-10A)

Investigation of the alterations of protein expression in breast cancer cells (MCF-7 and BT20) compared to normal cells (HMEC+MCF-10A) can be very useful in understanding the changes in the functional pathways and thus the fundamental mechanisms of cancer development. A total of 89 protein spots were observed differentially expressed in this comparison group, of which 42 were up-regulated and 47 were down-regulated in the cancerous cell lines in comparison to the normal cells (Table 3.1.3). A total of 44 protein spots were identified using MALDI-ToF MS and/or LC-MS/MS from this comparison, among these 27 proteins were up-regulated while 17 protein spots were down-regulated in the cancer cell lines (Table 3.1.4). In some cases, multiple protein identities were also observed for single protein spots for a number of differentially expressed protein spots, which could be due to the poor resolution of proteins that have the similar pI and molecular weight using 2D-gel electrophoresis-based investigations. For example, G6PD and CCT-β were identified at DIGE No. 1050 (Table 3.1.6, Appendix 4).

This cancer vs. normal comparison study revealed some proteins which are well established in the literature as having a potential role in breast cancer; for example, ANXA1 and SERPINB5. We also identified five interesting proteins whose role in breast cancer is not yet clear. These include eIF4AIII, UQCRC1, SERPINB1, SOD1 and DCI as mentioned in Tables 3.1.4 and 3.1.5.

4.1.1.1.1 Annexin 1 (ANXA1)

ANXA1 was identified at two different locations on the 2D-gels and was observed to be down-regulated by 3.8-fold and 26.8-fold in the cancer cells compared to the normal cells. ANXA1 participates in the regulation of membrane trafficking and cellular adhesion and alteration of these processes could result in the development of cancer (Leitch 1995, Esserman *et al.* 2002). Down-regulation of ANXA1 was also reported in many types of cancers, including head and neck (Gerke and Moss 2002), esophageal (Garcia Pedrero *et al.* 2004), gastric (Paweletz *et al.* 2000), breast (Hippo *et al.* 2001) and prostate (Ou *et al.* 2008). The down-regulation of ANXA1 expression in multiple types of cancers suggests that ANXA1 may be a negative regulator of cancer cell growth (Xin *et al.* 2003). However, a few reports have suggested that ANXA1 is actually over expressed in some malignancies

including breast cancer (Gerke and Moss 2002). The reduction of ANXA1 protein expression has been suggested to occur at an early stage of malignant transformation (Ahn *et al.* 1997).

4.1.1.1.2 SERPINB5

SERPINB5, also called maspin, is a non-classical serine protease inhibitor (serpin) with tumour suppressive activity. This protein was identified at 3 locations on the 2D-gel and found to be 98.7, 20.1 and 7.3-fold down-regulated in the the cancer cells compared to normal cells. SERPINB5 could be cytosolic, secretory or membrane in localization (Shen *et al.* 2005). SERPINB5 is strongly down-regulated in various cancers (Toillon *et al.* 2007, Khalkhali-Ellis *et al.* 2008, Pemberton *et al.* 1997) and the level of SERPINB5 has been inversely correlated with the stage of malignancy during breast cancer progression (Khalkhali-Ellis *et al.* 2008, Machtens *et al.* 2001). Four different variants of SERPINB5 possibly due to post-translation modifications (phosphorylation) have been already observed in fibroblast cells (Vecchi *et al.* 2008, Maass *et al.* 2001a & b). This suggests SERPINB5 as an important transduction molecule in cancer. A recent study showed that inhibition of miR-21 can increase the expression of SERPINB5 in MDA-MB-231 cells (Horswill *et al.* 2008, Odero-Marah *et al.* 2002). miR-21 has been observed to exert anti-apoptotic effects and is over expressed in cancers (Zhu *et al.* 2008). Therefore, down-regulation of SERPINB5 in the cancer cells in this investigation is in agreement with the published literature.

4.1.1.1.3 Translation initiation factor 4A subunit 3 (eIF4AIII)

eIF4AIII was 2.2-fold up-regulated in the cancer cells compared to the normal cells. Eukaryotic initiation factor 4A (eIF4A) is an ATP-dependent RNA helicase that facilitates translation with other initiation factors (eIF4F, eIF4E and eIF4G) (Chan *et al.* 2005). eIF4A has 3 isoforms, eIF4AI, eIF4AII and eIF4AIII. eIF4AI and eIF4AII are known to regulate translation, but the role of eIF4AIII in translation has not been characterized yet (Li *et al.* 1999). eIF4AIII has been shown to inhibit translation in a rabbit reticulocyte lysate when added in large amounts (Li *et al.* 1999). eIF4AIII has been suggested to facilitate ribosomal entry site (IRES)-dependent translation of XIAP mRNA that codes for an inhibitor of

apoptosis (Li *et al.* 1999) which could be facilitating survival of the cancer cells. It has also been found up-regulated in gastric cancer (Marissen and Lloyd 1998, Clemens *et al.* 1998, Pestova *et al.* 1996, Holcik *et al.* 1999).

4.1.1.1.4 Ubiquinol-cytochrome-c reductase complex core protein 1 (UQCRC1)

UQCRC1 is a nuclear-encoded protein localized to the inner mitochondrial membrane. UQCRC1 was 18-fold up-regulated in the cancer compared to the normal cells. UQCRC1 plays a key role in mitochondria-to-nucleus retrograde response and has been observed to be highly expressed in breast and ovarian tumours (Karam *et al.* 2008). However the mechanisms of UQCRC1 in breast cancer are not clear.

4.1.1.1.5 SERPINB1

SERPINB1, also known as monocyte/neutrophil elastase inhibitor, is a member of the serpin superfamily and was 2.9-fold up-regulated the cancer cells compared to the normal cells. SERPINB1 was characterized initially as a fast-acting inhibitor of neutrophil elastase (Kulawiec *et al.* 2006). SERPINB1 functions as a physiological inhibitor of the serine proteases of neutrophil azurophil (primary) granules, enzymes that are important in antimicrobial defense (Remold-O'Donnell 1985). SERPINB1 has overlapping target protease specificity with SERPINA1 but it is a member of the intracellular serpin family and is located in the cytoplasm. It is therefore more likely to be involved in the control of proteases that inadvertently leak from granules. The role of SERPINB1 in the regulation of cell growth is still unclear, however it has been observed at relatively high levels in early haemopoietic progenitor cells compared to mature cells (Cooley *et al.* 2001).

4.1.1.1.6 Chain J, Superoxide Dismutase Mutant With Lys 136 Replaced By Glu, Cys 6 Replaced By Ala and Cys 111 Replaced By Ser (SOD1)

This SOD1 mutant was 2.3-fold up-regulated in the cancer cells compared to the normal cells. More than 20 mutants of SOD1 have been observed to date and many of which have been associated with various phenotypes including cytoskeleton maintenance, cell death and growth (Missen *et al.* 2006). The superoxide dismutases (SOD's) constitute a family of antioxidant enzymes that catalyze the conversion of superoxide anions to oxygen and

hydrogen peroxide (Rosen *et al.* 1993, Juarez *et al.* 2008, Vigilanza *et al.* 2008). They include the manganese-containing SOD (SOD2) in the mitochondria, and the copper-zinc-containing SOD (SOD1) in the cytoplasm (Noor *et al.* 2002), with a small fraction in the mitochondria intermembrane space (Gregory *et al.* 1974). The over expression of SOD1 has been observed to be associated with alterations in the ubiquitin-proteasome system which may lead to accumulation of ubiquitinated proteins and various diseases including cancer and amyotrophic lateral sclerosis (Sturtz *et al.* 2001). Inhibition of SOD1 has been observed to inhibit EGF-, IGF-1- and FGF-2-mediated phosphorylation of ERK1/2 which results in reduced cell proliferation (Cheroni *et al.* 2009). The down regulation of SOD1 has also been associated with a decrease in actin and beta-tubulin content leading to altered cell morphology and induced cell death (Juarez *et al.* 2008). This suggests that SOD1 may be associated with increased growth and reduced death in breast cancer cells. However, the role of a mutant form of SOD1, which was identified by mass-spectrometry in our study, in cancer is unknown.

4.1.1.1.7 3,2-trans-enoyl-CoA isomerase (DCI)

3,2-trans-enoyl-CoA isomerase (DCI) was observed to be 3.1-fold up-regulated in the cancer cell lines compared to the normal cells in our study. DCI is also known as dodecenoyl-CoA isomerase or Delta(3),Delta(2)-enoyl-CoA isomerase or D3,D2-enoyl-CoA isomerase. DCI has been reported to be involved in the biochemical reactions of lipids and fatty acid and localized in the mitochondrion (Vigilanza *et al.* 2008). 2,3-trans-Enoyl-CoA isomerase was found reduced in metastatic breast cancer patients compared to non-malignant breast cancer patients (Janssen and Stoffel 2002). The role for DCI in breast cancer is not yet known.

4.1.1.1.8 Summary

The fact that ANXA1 and SERPINB5 are differentially expressed in our study and also in the published literature validates our experimental approach and techniques used. This approach also enabled us to identify eIF4AIII, UQCRC1, SERPINB1, SOD1 and DCI which have not previously been associated with breast cancer as they were found altered in breast cancer vs. breast normal cells in-vitro in our study.

4.1.1.2 Invasive (HMEC+BT20) vs. non-invasive (MCF-10A+MCF-7)

A total of 31 protein spots were observed differentially expressed in this comparison group, of which 20 were up-regulated and 11 were down-regulated in the normal+cancer (N+C) invasive cells (HMEC+BT20) compared to N+C non-invasive cells (MCF-10A+MCF-7) (Table 3.1.3). From these, 15 proteins were identified out of which 8 were up-regulated and 7 were down-regulated in the invasive cells (Table 3.1.6). A number of N+C invasion-related proteins were identified in this study. This comparison also revealed a number of known invasion-associated proteins such as NME1 as well as three proteins, PSME1, CLIC1 and RAD23B, which have not been previously associated with the normal+cancer-invasion and therefore may be involved in the regulation of invasion process in normal and cancer breast cells**.**

4.1.1.2.1 Non-metastatic protein 1 (NME1)

NME1, a metastasis-suppressor, was 2.4-fold down-regulated in N+C invasive vs. N+C non-invasive cells. NME1 is a heterodimeric protein and acts as a nucleoside diphosphate (NDP) kinase which is involved in the synthesis of nucleoside triphosphates. NME1 is well known for its involvement in cancer and invasion of breast (Vydra *et al.* 2008). The reduced expression of NME1 has been correlated with high tumour metastatic potential in several human breast carcinomas (Ouatas *et al.* 2003). An *in vitro* study showed that when a metastatic breast cancer cell line MDA-MB-435 (which is now considered as a melanoma cell line, see section 4.2.1) with low endogenous NME1 expression was transfected with NME1, the cells recovered their normal morphology and patterns of growth (Stahl *et al.* 1991, Heimann *et al.* 1998). The mechanisms of action of NME1 are still unknown; however it has been shown that the histidine protein-kinase activity correlates tightly with the ability of NME1 to suppress cell motility (Howlett *et al.* 1994).

4.1.1.2.2 Proteosome activator subunit 1 (PSME1)

PSME1 was 3-fold down-regulated in the N+C invasive vs. N+C non-invasive cells. The proteasomes are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway (Hartsough and Steeg 1998). An essential function of a modified proteasome, the immunoproteasome, is the processing of class I MHC peptides (Mattsson *et al.* 2001). PSME1 is an alpha

isoform of PA28 unit of a proteasome. PA28 is reduced in hepatocarcinoa tumours compared to the non-tumour tissues (Rivett and Hearn 2004). PA28 selectively up-regulates the presentation of viral MHC class I epitopes in tumour cells and this results in impaired presentation of a human TRP2 tumour antigen and ultimately in tumourigenesis (Zhang *et al.* 2007). Impaired expression of proteasome subunits has also been observed to be involved in the loss of HLA class I expression in human colon cancer cells (Sijts *et al.* 2002). However the involvement of PSME1 (PA28 alpha) in breast cancer is unclear.

4.1.1.2.3 Nuclear choride channel 1 (CLIC1)

CLIC1 is also known as nuclear chloride ion channel 27 or regulatory nuclear chloride channel protein. CLIC1 was 4.4-fold downregulated in N+C invasive vs. N+C non-invasive cells. CLIC1 is a member of the p64 protein family. This protein localizes principally to the cell nucleus and exhibits both nuclear and plasma membrane chloride ion channel activity. CLIC1 has been found over expressed in gastric cancer and hepatocellular carcinoma (Miyagi *et al.* 2003). The expression of CLIC1 has been found to be strongly correlated with lymph node metastasis, lymphatic invasion, perineural invasion, and pathological staging in gastric cancer (Chen *et al.* 2007). However its involvement in breast cancer and invasion is still not established.

4.1.1.2.4 RAD23B

RAD23B is one of two human homologs of the *Saccharomyces cerevisiae* nucleotide excision repair (NER) gene product RAD23 and a component of a protein complex that specifically complements the NER defect (Huang *et al.* 2004a). RAD23B was 2-fold up-regulated in N+C non-invasive vs. N+C invasive cells. This protein contains an N-terminal ubiquitin-like domain, which was reported to interact with 26S proteasome, and thus this protein may be involved in the ubiquitin-mediated proteolytic pathway in cells (Huang *et al.* 2004b). RAD23B was shown to interact with and elevate the nucleotide excision activity of 3-methyladenine-DNA glycosylase (MPG), which suggests a role in DNA damage recognition in base excision repair. Genetic variants in genes involved in the NER pathway may increase the risk to many types of cancers including lung, bladder and breast

(van Laar *et al.* 2002). However, a role for RAD23B in invasion has not been previously reported.

4.1.1.2.5 Summary

Thus this data suggests an important role of PSME1, CLIC1 and RAD23B in normal and cancer invasion of breast cells, however the exact role they play is unclear. Our study indicates that alterations in the expression levels of PSME1, CLIC1 and RAD23B could possibly contribute to the invasive phenotype.

4.1.1.3 Cancer invasive (BT20) vs. normal invasive +normal non-invasive +cancer-non-invasive (HMEC+MCF-10A+MCF-7)

This comparison enabled us to identify proteins that may have a role in cancer invasion. A total of 92 proteins were differentially regulated with 24 proteins up-regulated and 68 down-regulated in **group 1** (cancer invasive cell line (BT20)) compared to **group 2** (normal invasive+normal non-invasive+cancer-non-invasive cells (HMEC+MCF-10A+MCF-7)) as mentioned in Box 1. A total of 25 proteins were identified, of which 15 proteins were up-regulated and 10 were down-regulated in group 1 compared to group 2 (Table 3.1.7). This analysis identified a number of known proteins such as gelsolin as well as other proteins that have not been previously linked to breast cancer invasion such as TXNRD1, ERP29, 3-PGDH, TALDO1, CLP and QPRT. These proteins may have roles in the regulation of breast cancer invasion and therefore could be potential targets for further study (Table 3.1.7).

4.1.1.3.1 Gelsolin (GSN)

Gelsolin (GSN) is a calcium-binding protein which binds to and regulates actin filaments (Garcia-Pedrero *et al.* 2004, Shen *et al.* 2005, Mechanic *et al.* 2006, Chen and Madura 2006). In our study, GSN was found to be 4.2-fold down-regulated in group 1 compared to group 2. Decreased expression of cytoplasmic GSN has been shown in several types of human cancers, including bladder cancer (Kumar *et al.* 2004), non-small cell lung cancer (Tanaka *et al.* 2006), prostatic adenocarcinoma (Dosaka-Akita *et al.* 1998) and breast cancer (Lee *et al.* 1999) suggesting its possible role as a tumour suppressor. GSN expression suppresses the activation of phospholipases C (PLC)/protein kinase C (PKCs) that are involved in phospholipid signalling pathways, thus inhibiting cell proliferation and tumourigenicity (Winston *et al.* 2001). Severing and capping of actin filaments due to reduced GSN levels enhances the rate of cell migration (Sagawa *et al.* 2003) and invasion (Cunningham *et al.* 1991). Therefore, loss of GSN could possibly contribute to the development of the invasive phenotype in breast cancer.

4.1.1.3.2 Thioredoxin reductase 1 (TXNRD1)

TXNRD1 is a cytosolic enzyme that plays a central role in controlling cellular redox homeostasis and was 4.6-fold over-expressed in group 1 compared to group 2. Tumour

cells often show increased glycolytic metabolism associated with rapid cell division. It has been suggested that TXNRD1 may have a possible role in cellular defence against oxidative damage (Tanaka *et al.* 2006).

TXNRD1 has been investigated for its role in cell proliferation and therefore has been suggested as a potential anti-cancer molecular target. A few studies have shown that TXNRD1 enhances tumour proliferation (Hirota *et al.* 2000b, Hirota *et al.* 2000a, Wei *et al.* 2000, Karimpour *et al.* 2002). TXNRD1 has been observed elevated in various cancers including breast (Smart *et al.* 2004), colon cancer (Kirkpatrick *et al.* 1998, Choi *et al.* 2002), prostate (Gladyshev *et al.* 1998), hepatic (Gladyshev *et al.* 1998) and pancreatic cancer (Kirkpatrick *et al.* 1998, Choi *et al.* 2002). The inhibition of TXNRD1 expression has been shown to inhibit cell proliferation due to G_1 cell cycle arrest (Han *et al.* 1999). Moreover, TXNRD1 has also been observed to inhibit the activity of thioredoxin-dependent TIMPs and could increase invasion (Engman *et al.* 2003, Wipf *et al.* 2001). Over-expression of TXNRD1 has been also correlated with aggressive tumour growth, poorer prognosis and decreased patient survival (Farina *et al.* 2001).

4.1.1.3.3 Endoplasmic reticulum protein 29 (ERp29)

ERp29 was 10.4-fold down-regulated in group 1 compared to group 2. ERp29 is expressed ubiquitously and abundantly in metazoan cells, implying it has a general 'housekeeping' function. ERp29 is thought to facilitate the transport of secretory proteins in the early secretory pathway (Kakolyris *et al.* 2001, Raffel *et al.* 2003). Distinctively, ERp29 lacks classical chaperone, disulfide-editing, calcium-buffer, and stress-response properties (Cheretis *et al.* 2006). ERp29 has been found highly expressed in a variety of tumours and cancer cell lines including hepatocellular carcinoma (Demmer *et al.* 1997, Sargsyan *et al.* 2002), SK-N-SH (bone marrow neuroblastoma), HeLa (cervix carcinoma), MCF-7 (breast carcinoma), A-375 (malignant melanoma) & A549 (lung carcinoma) (Seow *et al.* 2000), ovarian tissue (Myung *et al.* 2004) and in basal-cell carcinoma of the skin (Bengtsson *et al.* 2007). On the other hand, ERp29 has been shown to be down-regulated in pancreatic adenocarcinoma tissue compared to normal pancreatic tissue (Cheretis *et al.* 2006). The roles of ERp29 in breast cancer and invasion are still unclear.

4.1.1.3.4 3-phosphoglycerate dehydrogenase (3-PGDH)

3-PGDH was 5.2-fold over-expressed in group 1 compared to group 2. 3-PGDH catalyzes the transition, using NAD+/NADH as a cofactor, of 3-phosphoglycerate into 3-phosphohydroxy pyruvate, which is the first and rate-limiting step in the serine biosynthesis pathway (phosphorylated) (Lu *et al.* 2004). The serine is not only required for protein synthesis; it also serves as a precursor for the biosynthesis of glycine, cysteine, tryptophan, and phospholipids. High expression of 3-PGDH, which contributes towards energy metabolism, has been noticed in developing cells (Jun *et al.* 2008). Expression of 3-PGDH was also reported to be increased in colon cancer (Yamasaki *et al.* 2001) implicating its associated with the increased glycolytic metabolism of the cancer cells. The up-regulation of 3-PGDH in the cancer invasive cell line suggests that 3-PGDH could be involved in breast cancer invasion.

4.1.1.3.5 Transaldolase (TALDO1)

TALDO1 regulates the balance of metabolites in the pentose-phosphate pathway. TALDO1 was identified at two different locations on the 2D-gel and was 4.1-fold and 2.7-fold over-expressed in group 1 compared to group 2. Up to 20 genetic variants of TALDO1 have been reported and a few variants have been positively associated with the regulation of mitochondrial homoeostasis and apoptosis (Snell *et al.* 1988) and with increased risk of squamous cell carcinoma of the head and neck (SCCHN) (Qian *et al.* 2008). TALDO1 has previously been identified at 7 spots in 2D-gels due to phosphorylation of this protein (Basta *et al.* 2008). The activity of TALDO1 has also been observed to be improved due to these PTMs in transformed Xeroderma Pigmentosum (XP) diploid fibroblasts and SV40-tranformed cells compared to normal cells as transcription of TALDO1 mRNA was unchanged in these cells (Lachaise *et al.* 2001). Therefore, the presence of TALDO1 at two locations on the 2D-gel in this investigation could be possibly due to either genetic variation of TALDO1 or PTMs in TALDO1 which may occur during the process of development of cancerous and invasive phenotype in breast cells. The expression of TALDO1 has been observed increased in breast carcinoma tissues (Lachaise *et al.* 2001). However, no specific role of TALDO1 in breast cancer invasion has yet been reported. The

up-regulation of TALDO1 in the cancer invasive cells suggests that TALDO1 could be involved in facilitating invasion of breast cancer.

4.1.1.3.6 Coactosin-Like Protein (CLP)

CLP was 5.1-fold over-expressed in group 1 compared to group 2. It is an actin-binding protein and is involved in the regulation of the actin cytoskeleton. CLP has been reported over-expressed in tumour tissue and cell lines as a tumour antigen and can be recognized by cellular and humoral immune systems (Jung *et al.* 2007). CLP is also reported as a human pancreatic cancer antigen (Li *et al.* 2004). CLP can up-regulate the expression of 5-lipoxygenase (5LO), which is the first committed enzyme in leukotriene biosynthesis and also modulate 5LO activity (Nakatsura *et al.* 2002). The increased expression of 5LO has been associated with improved survival of prostate cancer cells (Rakonjac *et al.* 2006). However, the involvement of CLP in breast cancer and invasion is still unreported.

4.1.1.3.7 Quinolinate phosphoribosyltransferase (QPRT)

QPRT was 5.2-fold over-expressed in group 1 compared to group 2. QPRT, also known as nicotinate-nucleotide pyrophosphorylase, is involved in the *de novo* synthesis of Nicotinamide adenine dinucleotide (NAD). NAD acts as a coenzyme in redox reactions and therefore has essential roles in cellular metabolism. Inhibition of NAD has been associated with induced apoptosis in cancer cells (Ghosh 2004). The observed increased expression of QPRT in this study is in keeping with the fact that cancer cells show increased metabolic activity compared to normal cells. The role of QPRT in the regulation of the invasive phenotype in cancer has not been previously reported.

4.1.1.3.8 Summary

Therefore the data presented here indicate that increased expression of TXNRD1, TALDO1, 3-PGDH, QPRT and CLP could be contributing towards enhancing invasion in breast cancer, whereas loss of ERp29 could contribute to the invasion by breast cells.

4.1.1.4 Identification of invasion-specific proteins by overlapping lists of differentially expressed proteins

The list of differentially expressed identified proteins for the comparison of the cancer non-invasive vs. cancer invasive cells (MCF-7 vs. BT20) was overlapped with the list of differentially expressed identified proteins for the comparison of the normal non-invasive vs. normal invasive cells (MCF-10A vs. HMEC) (supplementary data: appendix 1). This comparison enabled us to identify normal+cancer invasion-specific differentially expressed proteins.

Four proteins, ANXA1, SERPINB5, ARCN1 and a hypothetical protein, were found differentially expressed only in the normal cell comparison group (MCF10A vs. HMEC). Of these ANXA1 and SERPINB5 are well known to be involved in cancer and invasion.

- ANXA1 was up-regulated in the normal noninvasive cells (MCF-10A) compared to the normal invasive cells (HMEC) in this investigation (Figure 3.1.3). The role of this protein in cancer has been described in 4.1.1.1.1. The loss of ANXA1 could lead to the cancer phenotype. However, its role in the regulation of invasion in normal cells is unknown.
- SERPINB5 was up-regulated in the normal noninvasive cells (MCF-10A) compared to the normal invasive cells (HMEC) (Figure 3.1.3). The role of this protein in cancer has been described in 4.1.1.1.2. SERPINB5 could be involved in maintaining the normal phenotype in breast cells. However, its role in the regulation of invasion in these normal cells remains unclear.

A total of 27 proteins, including keratin 8 and 18 (KRT8 and KRT18) and heterogeneous ribonucleoprotein K (HNRPK), were specifically differentially expressed in the cancer cell comparison group (MCF-7 vs. BT20).

- KRT8 and KRT18 were down-regulated in MCF-7 compared to BT20 (Figure 3.1.3). KT8 and KT18 are the most common and characteristic members of the large intermediate filament gene family expressed in 'simple' or single layer epithelial tissues of the body. KT8 and KT18 have already been observed up-regulated in cancerous cell lines (Muruganandham *et al.* 2005). The higher expression of KT8 and KT18 has been associated with increased invasion (Oshima

et al. 1996, Kralovich *et al.* 1998, Putz *et al.* 1999). Therefore differential expression of KRT8 and KRT18 could be associated with the regulation of cancer and invasion in breast cells.

- HNRPK has been shown to be associated with cancer (Matthias *et al.* 2008, Hendrix *et al.* 1997) and is a member of the p53 MDM2 pathway. HNRPK has also been shown to be involved in cell migration (a process necessary for cancer metastasis) (Moumen *et al.* 2005). The over expression of HNRPK in breast cancer invasive cells significantly increased the expression of c-Myc protein and enhanced breast cancer cell proliferation (de Hoog *et al.* 2004). HNRPK was observed down-regulated in MCF-7 compared to BT20 in this study supporting its role in tumour growth and invasion in breast cells (Figure 3.1.3).

10 proteins including high-mobility group box 1 (HMGB1) were overlapped in the normal (MCF-10A vs. HMEC) and cancer (MCF-7 vs. BT20) comparison groups indicating that these proteins could be involved in both cancer and normal invasion as these proteins were differentially expressed between the normal non-invasive and the normal invasive cells (MCF-10A vs. HMEC) as well as between the cancer non-invasive cell line and cancer invasive cell line (MCF-7 vs. BT20) (Figure 3.1.3). This overlapping also identified invasion-associated differentially expressed proteins including some proteins that have not been previously reported to be associated with breast cancer invasion such as RAD23B, PSME1, CLIC1 and TALDO1 (section 4.1.1.2 & 4.1.1.3).

HMGB1 is a cytokine-like nuclear protein, which is an important mediator of the body's inflammation, ischemia, and injury. HMGB1 was observed abundantly expressed in breast cancer tissues compared to normal breast tissues (Mandal *et al.* 2001). However, the roles of HMGB1 in the regulation of invasion in breast cells still need to be explored.

4.1.1.5 Screening for invasion-specific proteins for siRNA functional analysis

A total of 9 invasion-associated protein targets were selected based on the expression patterns observed using 2D-DIGE and limited information available in the published literature for their potential role in the regulation of invasion in breast cells.

Cancer invasion-related proteins- TXNRD1, TALDO1, ERp29, 3-PGDH, QPRT and CLP
Normal+cancer invasion-related proteins- RAD23B, PSME1 and CLIC1

The expression of these 9 proteins was investigated using Western blotting in a panel of breast cell lines representing invasive and non-invasive phenotypes to further ensure the invasion-specific expression. Of the 6 cancer invasion-related proteins, only TXNRD1 and 3-PGDH showed a cancer-related invasion pattern across all cell lines, except UACC-812. Briefly in this investigation, TXNRD1 and 3-PGDH were observed to be up-regulated in the invasive cell lines (SKBR-3, MDA-MB-231, Hs578T, HCC1937 and BT20) compared to the normal cells (HMEC and MCF-10A) and cancerous non-invasive cell lines (T47D and MCF-7) (Figure 3.1.10A and 3.1.11B). However, in contrast to T47D and MCF-7 cells, both of these proteins were also expressed in a cancerous non-invasive cells line, UACC-812. The reason why the remaining four targets did not show a cancer invasion-related expression pattern could be that similar types of cell lines may also differ in the expression of certain proteins due to their genetic heterogeneity (Brezniceanu *et al.* 2003). For example, two breast cell lines, T47D and MCF-7, have been observed to express drastically differing levels of MUC1 protein (Lacroix and Leclercq 2004, Walsh *et al.* 1999), although both of these cell lines are similar in their origin (luminal type ductal carcinoma cells and were isolated from metastatic tumours) (Walsh *et al.* 1999). The possible explanations for UACC-812 as an outlier could be that although T47D, MCF-7 and UACC-812 are luminal type, UACC-812 could be different in the expression of certain proteins compared to T47D and MCF-7, as it is an ER and PR negative cell line that was isolated from a primary tumour (Lacroix and Leclercq 2004). T47D and MCF-7 are ER and PR positive cell lines and were isolated from metastatic tumours (Lacroix and Leclercq 2004).

Of the 3 normal+cancer invasion-related proteins, only RAD23B showed the normal+cancer-related invasion pattern across all cell lines as it was highly expressed in the non-invasive cells (MCF-10A, MCF-7, UACC-812 and T47D) compared to the invasive cells (MDA-MB-231, Hs578T, HCC1937, BT20, HMEC and SKBR-3) (Figure 3.1.10A and 3.1.12B).

Therefore 3 proteins, TXNRD1, 3-PGDH and RAD23B, were chosen to investigate their involvement in invasion and cell proliferation using siRNA knockdown technique.

4.1.1.6 Analysis of knock down of expression of protein targets using siRNAs

4.1.1.6.1 TXNRD1

Two independent siRNA molecules were transfected into the two invasive cell lines, i.e. MDA-MB 231 which is highly invasive and SKBR-3 which is low invasive, to inhibit the expression of TXNRD1 for investigating its potential effects on cell proliferation and invasion. Following the inhibition of TXNRD1 expression in both cell lines, MDA-MB 231 and SKBR-3, the invasion status of TXNRD1 siRNA treated cultures was decreased in both cell lines in comparison to the scrambled siRNA treated control cultures (Figure 3.1.16 & 3.1.17). However this decrease was greater in the case of SKBR-3 (3.1 fold) compared to MDA-MB 231 (1.4-fold). Therefore, these results suggest that TXNRD1 could have a role in the regulation of breast cancer invasion.

4.1.1.6.1.1 Possible role of TXNRD1 in breast cancer invasion

The gelatinases, MMP-2 and MMP-9 are over-expressed by many tumours and have been implicated in the induction and maintenance of the malignant phenotype, tumour invasion and tumour-associated angiogenesis (Lacroix and Leclercq 2004). MMPs are believed to be crucial to facilitate invasion by degrading extracellular matrix macro-molecules such as type IV collagen (Deryugina and Quigley 2006, Ray and Stetler-Stevenson 1994, Deryugina *et al.* 1997, Ballin *et al.* 1988). The MMPs activation and inhibition are regulated by members of the family of tissue inhibitors of MMPs (TIMPs). The MMP/TIMP balance is therefore a critical determinant of net proteolytic activity and its imbalance is generally considered a prerequisite for MMP involvement in tumour pathology (Tryggvason *et al.* 1993). TXNRD1 protein catalyses the reduction thioredoxins as well as other substrates that play a role in selenium metabolism and protection against oxidative stress (Liotta and Stetler-Stevenson 1991, Nagase 1997). Thioredoxins have been reported as inhibitors of TIMP-1, TIMP-2 and MMP-2 and can alter the MMP/TIMP balance in favour of MMP activity stimulating the SK-N-SH neuroblastoma cell invasion (Hirota *et al.* 2000a & b, Wei *et al.* 2000, Karimpour *et al.* 2002). TXNRD1 promotes alterations in the thioredoxin-dependent MMP/TIMP balance (Farina *et al.* 2001). Our study showed that knockdown of TXNRD1 in the invasive cells (SKBR-3 and MDA-MB 231) was able to reduce *in vitro* invasion of these low and highly invasive cell lines. It

could be possible that silencing of TXNRD1 protein could reduce thioredoxin dependent inhibition of TIMP-1, TIMP-2, MMP-2 and MMP-9 and finally suppressing the MMPs activity thus reducing invasion in breast cancer cells. However, further studies are required to confirm this hypothesis.

4.1.1.6.1.2 Possible role of TXNRD1 in cell proliferation

Proliferation assays of TXNRD1 siRNA transfected cells, MDA-MB 231 and SKBR-3 were performed after 3 days of transfection (Figure 3.1.18). No significant differences in the growth of TXNRD1 siRNA-treated cultures were observed in comparison to the control culture (Scrambled-treated culture) and this indicates that the loss of TXNRD1 does not affect cell growth in MDA-MB 231 and SKBR-3 cells. Thus the results indicate that TXNRD1 has no obvious effect on cell proliferation.

The data presented here showed that TXNRD1 is an excellent target to reduce the invasion potential in breast cancer. However, further functional studies such as siRNA knockdown in a panel of invasive cell lines and over-expression in low and non-invasive cells lines will be beneficial to confirm its involvement in the regulation of invasion in breast cells.

4.1.1.6.2 RAD23B

Three independent siRNA molecules were transfected into the normal non-invasive cells (MCF-10A) and cancer non-invasive cell line (MCF-7) to inhibit the expression of RAD23B in order to investigate its effects on cell proliferation and invasion in breast cells.
Following the down regulation of RAD23B expression in both non-invasive cells, MCF-10A and MCF-7, the invasion status of RAD23B siRNA-treated cultures was increased in both cell lines in comparison to the scrambled siRNA-treated control cultures (Figure 3.1.20 & 3.1.21). Cancerous non-invasive cells (MCF-7) and normal non-invasive cells (MCF-10A) showed a 4-fold and 2.9-fold increase in their invasion status, respectively. Therefore our results suggest that RAD23B could play a significant role in normal breast invasion and cancerous breast invasion.

4.1.1.6.2.1 Possible role of RAD23B in breast invasion

The role of RAD23B in invasion is unknown. RAD23B is involved in the nucleotide excision repair (NER) pathways of mammalian cells. NER is a particularly important mechanism by which the cell can prevent unwanted mutations by removing DNA damage (mostly in the form of thymine dimers and photoproducts). NER can be categorized into two classes- global genome NER (GG-NER) and transcription-coupled NER (TC-NER). RAD23B has roles in GC-NER where it forms a complex (XPC-RAD23B) with xeroderma pigmentosum group C protein (XPC) which is responsible for distortion recognition, open complex formation, and repair protein complex formation (Farina *et al.* 2001). XPC-RAD23B has been observed to be necessary to recruit the general transcription factor IIH (TFIIH) to the damaged DNA for global genome repair (Yokoi *et al.* 2000), however the exact functions of RAD23B remain unclear. Genetic variations in genes due to defects in NER pathway have been associated with increased risk of many types of cancer including lung, bladder and breast (Yokoi *et al.* 2000). The mutation in a homolog of RAD23B, RAD23, has been observed to result in reduced proficiency in excision repair in *Saccharomyces cerevisiae* (Garcia Pedrero *et al.* 2004, Shen *et al.* 2005, Mechanic *et al.* 2006, Chen and Madura 2006). RAD23B has also been observed to be involved in the regulation of ubiquitin-dependent proteolysis (Watkins *et al.* 1993). Moreover, RAD23B has been observed to be reduced due to over expression of miR-373 during hypoxic stress conditions, where genomic stability is reduced (Huang *et al.* 2004b). The increased invasion in both normal and cancerous non-invasive cells (MCF-10A and MCF-7) following siRNA-mediated down regulation of RAD23B expression suggests that RAD23B could be one of the proteins that can regulate invasion (normal and cancerous). Normal and cancer invasion use similar molecular mechanisms and the only difference them is that malignant invasion can persist (Crosby *et al.* 2009). Therefore, it is possible that RAD23B could be modulated by other unknown regulators to start/stop invasion in normal breast cells, whereas the tumour cells might have lost these unknown regulators during development of cancerous and/or invasive phenotypes in breast cells (Liotta and Stetler-Stevenson 1991). However, the exact mechanism needs to be elucidated through further studies. To our knowledge, this is the first study showing that RAD23B could possibly play a significant role in the invasion of normal breast cells and cancerous breast cells.

4.1.1.6.2.2 Possible role of RAD23B in cell proliferation

Proliferation assays of RAD23B siRNA transfected cells, MCF-10A and MCF-7, were performed after 3 days of transfection (Figure 3.1.22). No significant differences in the growth of RAD23B siRNA treated cultures were observed in comparison to the control culture (Scrambled-treated culture). This indicates that the loss of RAD23B does not affect cell growth in MCF-10A and MCF-7 cells.

4.1.1.6.3 3-PGDH

Increased expression of 3-PGDH was observed in the invasive cells (MDA-MB-231, Hs578T, HCC1937, BT20, HMEC and SKBR-3) compared to the non-invasive cells (MCF-10A, MCF-7 and T47D), except UACC-812 (Section 4.1.2.6) indicating its potential involvement in the invasion in breast cells. Following optimized transfection conditions, 30nM of three independent siRNA molecules for 3-PGDH was transfected into MDA-MB 231 and SKBR-3 cells. The Western blot analysis for the protein samples harvested after 72 hrs of transfection revealed no inhibition of 3-PGDH expression for both cell lines (Figure 3.1.23). However as expected a significant reduction in cellular growth for the positive control cultures (kinesin siRNA-treated) was observed in both cell lines for the same experiment (Figure 3.1.24).

Since cell lines can behave differently for particular siRNA molecules and protein targets, transfection was performed in another cell line (BT20). No effect on the expression of 3-PGDH in siRNA treated samples compared to controls was again observed in Western blotting, although the positive control worked as expected. Increased concentrations of 3-PGDH siRNA (50 and 125nM) were then used for SKBR-3 and MDA-MB 231. The samples collected at 96 hrs after transfection again showed no inhibition of 3-PGDH expression. These results indicated that the expression of 3-PGDH at the protein level was unaffected in all transfection conditions tested in this investigation. The possible explanation for this includes that 3-PGDH may have a slow turn over in cells and therefore investigation of knockdown at the mRNA level could help to understand the efficiency of transfection reactions since the proteins already translated and present in the cytoplasm can not be altered by siRNA molecules. Moreover the siRNA molecules used in this

investigation have not been validated by the supplier to date and we can not be sure about the efficiency of siRNA molecules. In this condition, the use of siRNAs designed using improved algorithms and/or validated siRNAs from other suppliers could inhibit the expression of 3-PGDH. The potential role of 3-PGDH in the regulation of cancer has been described in section 4.1.1.3.4.

4.1.2 Proteomic analysis of conditioned media from HMEC, MCF-10A, MCF-7 and BT20

The term "secretome" was first proposed by a genome-based global survey on secreted proteins of *Bacillus subtilis* (Liotta and Stetler-Stevenson 1991). In a broader sense, the secretome harbours proteins released by a cell, tissue or organism through classical and nonclassical secretion pathways (Tjalsma *et al.* 2000). These secreted proteins could be stimulatory growth factors, proteases and cytokines (Volmer *et al.* 2005) and could be essential in the processes of differentiation, invasion, metastasis and angiogenesis of cancers by regulating cell-to-cell and cell-to-extracellular matrix interactions (Ariztia *et al.* 2006). More importantly, these cancer-secreted proteins always enter body fluids such as blood or urine and can be measured by quantitative assays. Thus, the cancer secretome analysis is a promising tool supporting the identification of cancer biomarkers and drug targets.

Cells are commonly cultivated in rich media supplemented with fetal bovine serum (FBS) medium. The use of serum-free media is preferred in order to guarantee the successful analysis of the cancer secretome *in vitro*. The reason lies in the fact that the highly abundant serum proteins such as albumin may mask and dilute the secretome. Therefore, in this investigation cells were initially cultured in serum-supplemented medium to achieve growth and then medium was replaced with serum-free medium to perform proteomic analysis. This enabled us to maintain highly viable cells at the time of sample collection and reduced the risk of getting proteins from autolysis of cells. Similar methods have already been used to analyse secreted proteins (Kulasingam and Diamandis 2007).

In this investigation, protein profiles for serum-free conditioned media from four cell lines were investigated using 2D-DIGE coupled with mass-spectrometry to identify proteins that may be involved in cancer and invasion in breast cells. For this, data was analysed in 3 main groups (Box 1).

4.1.2.1 Cancer (MCF-7+BT20) vs. Normal (HMEC+MCF-10A)

This comparison revealed 81 secreted protein spots differentially expressed, of which 56 were up-regulated and 25 were down-regulated in the cancerous cells (BT20 +MCF-7) compared to the normal cells (HMEC+MCF-10A). Of the 81 differentially expressed protein spots, 43 proteins were successfully identified and from them 25 were up-regulated and 18 were down-regulated in the cancer cells. A number of well-known and other proteins which have very limited information for their role in breast cancer were identified in this investigation. These proteins includes-

- Serine protease inhibitors (SERPINA1, SERPINB1, SERPINB5, SERPINB7 and SERPINF1)
- Cathepsin proteases (CTSA, CTSB and CTSD)
- Other proteins, such as NUCB1, FHC and PDIA4

4.1.2.1.1 Protease inhibitors (Serine protease inhibitors)

Serine protease inhibitors, serpins, are a group of structurally similar proteins that can inhibit proteases in cells. About 36 serpins have been identified in humans to date and of which, approximately two thirds have been proven to perform extracellular roles (Kulasingam and Diamandis 2007, Mbeunkui *et al.* 2006). Five types of serpins, SERPINA1, SERPINB1, SERPINB5, SERPINB7 and SERPINF1, were observed to be down-regulated in this investigation, of which SERPINA1, SERPINB5 and SERPINF1 are well known (Box 2), and SERPINB1 and SERPINB7 are poorly known for their roles in breast cancer.

4.1.2.1.1.1 SERPINB1

SERPINB1, also known as monocyte/neutrophil elastase inhibitor, was identified at two different locations on the 2D-gel. It was observed to be up-regulated by 8.31 fold at one spot and 7.98 fold down-regulated at another spot in the cancer compared to the normal cells (Table 3.2.2). This protein has been discussed in section 4.1.1.1.5 Moreover two isoforms of SERPINB1 have been observed in the chronic myelogeneous leukemia (CML) patients, however the role of these isoforms is not known yet (Rawlings *et al.* 2004).

Box 2: Known serpins identified from 2D-DIGE study			
Serine Protease inhibitors	**Cancer vs. normal (Fold change)**	**Function**	**Regulation in cancer**
SERPINA1	-12.58	Negative regulator of cell growth (Grigoryev 2007)	Down in breast cancer (Yavelow *et al.* 1997), anaplastic large cell lymphoma (ALCL) (Yavelow *et al.* 1997, Wozniak *et al.* 2005) and pancreatic cancer (Duplantier *et al.* 2006)
SERPINB5	-41.75	Inhibits tumour growth and metastasis (Thakur *et al.* 2008), induces apoptosis (Shi *et al.* 2001)	Down in breast cancer (Toillon *et al.* 2007)
SERPINF1	-3.2	Apoptosis and anti-angiogenic apoptosis (Vecchi *et al.* 2008, Maass *et al.* 2001b, Toillon *et al.* 2007)	Down in breast cancer (Chen and Madura 2006, Volpert *et al.* 2002, Ho *et al.* 2007) and hepatocellular carcinoma (Cai *et al.* 2006)

4.1.2.1.1.2 SERPINB7

SERPINB7, also known as Megsin, was found to be 3.8-fold down-regulated in the cancer cells compared to the normal cells. Over expression of SERPINB7 has been observed to induce progressive mesangial cell proliferation (Barnstable and Tombran-Tink 2004, Matsumoto *et al.* 2004). It was over expressed in primary lung tumours compared to normal tissue (Miyata *et al.* 2002, Smith *et al.* 2003). The role of SERPINB7 in breast cancer is unclear.

4.1.2.1.2 Cathepsin proteases

Cathepsins are cysteine and aspartic proteases, a type of protein that breaks apart other proteins, found in cells. Most of the members become activated at the low pH found in lysosomes (Miyata *et al.* 2002, Smith *et al.* 2003). Thus, the activity of this family lies almost entirely within those organelles. In this investigation, we identified three *pro* forms of cathepsin (CTSA, CTSB and CTSD) down-regulated in the cancer cell lines compared to normal cells, and of these procathepsin B and procathepsin D are well known (Box 3) whereas procathepsin A is poorly known for its association with breast cancer.

Box-3: Known procathepsins identified from 2D-DIGE study

Cathepsins proteases	**Cancer vs. normal (Fold change)**	**Function**	**Regulation in cancer**
Procathepsin B	-31.23	Metastasis and invasion (Erickson 1989)	Up in breast (Mai *et al.* 2000), squamous cell carcinomas (Sloane *et al.* 1981) and prostrate tumours (Kawada *et al.* 1997)
Procathepsin D	-2.41	Tumour growth, metastasis and invasion (Sinha *et al.* 1998)	Up in breast cancer (Ohri *et al.* 2008, Shi *et al.* 2001), nongynecological solid tumours (Ohri *et al.* 2008, Briozzo *et al.* 1988, Vetvicka *et al.* 2002) and ovarian cancer (Leto *et al.* 2004)

Procathepsin A (CTSA)

Procathepsin A, also known as lyososomal carboxypeptidase A or lysosomal protective protein precursor, was found to be 2.1-fold down-regulated in the cancer vs. normal comparison. Cathepsin A is a multifunctional enzyme that expresses deaminidase and esterase activities at neutral pH and carboxypeptidase activity at acidic pH. The role of cathepsin A in breast cancer is still not known. Activity of lysosomal enzymes has been observed to be increased in patients with gynecologic cancers compared to normal (Bazzett

et al. 1999). The down regulation of procathepsin A in cancer cell lines could be associated with the abrogated conversion of procathepsin A into cathepsin A and as a result we observed reduction in procathepsin A levels. The observed reduction in procathepsin A in this study suggests that it could be involved in breast cancer.

4.1.2.1.3 Other proteins such as NUCB1, PDIA4 and FHC

4.1.2.1.3.1 Nucleobindin 1 precursor (NUCB1)

NUCB1 was identified at 3 different spots and was found to be 14, 12.55 and 12.56-fold up-regulated at all three spots in the cancer vs. normal cells. Exogenous administration of recombinant NUCB1 to MRL/n mice has been found to induce an autoimmune phenomena and thymic apoptosis, indicating that NUCB1 plays an important role in inducing autoimmunity and apoptosis (Beratis *et al.* 2005). NUCB1 can be cleaved by caspase activity which could be involved in the initiation of apoptosis (Kanai *et al.* 1995). Accumulating evidence suggests that NUCB1 performs multiple functions through its Ca2+-binding, DNA-binding and EF-hand-mediated interactions with other proteins (Valencia *et al.* 2008). NUCB1 has up to 10 potential sites for phosphorylation including three protein kinase C sites which may well impact on its conformation and activity (Miura *et al.* 1992, Lin *et al.* 1998) and suggest it as an important signal transduction molecule. Higher expression of NUCB1 was observed in colon, gastric and lymphatic cancers (Wendel *et al.* 1995). A study on gastric adenocarcinoma has demonstrated that in 50 gastric adenocarcinomas with lymph node metastasis, 56% of cases showed an increased level of NUCB1 (Chen *et al.* 2007b, Wang *et al.* 1993, Kubota *et al.* 1998). The subcellular distribution analysis of NUCB1 examined by subcellular fractionation and immunofluorescence revealed that NUCB1 is a membrane-associated protein and mostly distributed in Golgi (Wang *et al.* 1993). The role of NUCB1 in breast cancer is unclear.

4.1.2.1.3.2 Protein disulfide isomerase family A, member 4 (PDIA4)

Protein disulfide isomerase family A, member 4 (PDIA4), a member of the PDI family, was found to be 37-fold up-regulated in the cancer vs. normal cells. PDIA4 is responsible for forming disulfide bonds between polypeptide(s) during protein folding and ensures the proper folding that is required to perform defined biological functions in cells (Chen *et al.*

2007b). Increased levels of PDIA4 have been observed in differentiating mouse F9 teratocarcinoma cells (Bulleid and Freedman 1988). Over-expression of PDIA4 was also reported in hepatocellular carcinoma cell lines and in metastasized HCC patient's sera (Miyaishi *et al.* 1998). Although, the exact function of PDIA4 is not known yet; it is one of the critical factors for a normal growth rate as well as for calcium homeostasis (Chen *et al.* 2008a).

4.1.2.1.3.3 Ferritin heavy chain (FHC)

Ferritin is a ubiquitous and highly conserved iron binding protein and has two chains, ferritin light chain (FLC) and ferritin heavy chain (FHC). FHC was found to be 3.6-fold down-regulated in the cancer vs. normal cells. Ferritin is localized predominantly intracellular; however it is also actively secreted by cells (Li and Lee 1991). A study based on 42 breast cancer patients showed that high levels of FHC mRNA was directly related to lymph-node status, presence of metastatic disease, and clinical stage (Addison *et al.* 1972). FHC mRNA was also found to be high in the immortalized human breast epithelial cell line MCF-10F but not in the mortal line S-130, from which MCF-10F cells were derived (Yang *et al.* 2002). An *in vitro* study showed that antisense FHC oligodeoxynucleotides specifically inhibited the growth of MCF-7 cells (Higgy *et al.* 1997). Ferritin promotes programmed cell death with low ratios of Bcl-2 to Bax mRNA and protein expression providing evidence that they specifically inhibited MCF-7 cell growth through increased apoptosis (Torti *et al.* 1988). However FHC was observed down-regulated in the cancer cells in our study and the reason remains unclear.

4.1.2.1.4 Summary

This analysis identified a number of interesting secreted protein targets that have not been previously associated with breast cancer and could be associated with breast cancer. These include PDIA4, NUCB1, CTSA, SERPINB1, SERPINB7, and FHC. Investigation of these targets in the serum or plasma samples from breast cancer patients and normal people may enable us to identify potential biomarkers for early diagnosis and prognosis of breast cancer.

4.1.2.2 Invasive (HMEC+BT20) vs. Non-invasive (MCF-10A+MCF-7)

A total of 33 protein spots were observed differentially expressed in this comparison group, of which 29 were up-regulated and 4 were down-regulated in the nornal+cancer (N+C) invasive cells (HMEC+BT20) in comparison to N+C non-invasive cells (MCF-10A+MCF-7) (Table 3.2.1 and 3.2.4). A total of 15 secreted proteins were identified, out of which 14 proteins were up-regulated and 1 protein was down-regulated in the N+C invasive cells. This analysis identified some secreted proteins that are already known to be involved in the development of cancerous and invasive phenotypes in breast cells. For example,

- Disulfide isomerase family A, member 3 precursor (PDIA3)
- Heat shock 90 (HSP 90)

Some secreted proteins which are still unknown to be involved in the development of cancerous and invasive phenotypes in breast cells were also identified. For example,

- Transaldolase-1 (TALDO1)
- Glutathione synthetase (GSS)

4.1.2.2.1 Disulfide isomerase family A, member 3 precursor (PDIA3)

PDIA3 is a soluble ER luminal protein that belongs to the protein disulfide isomerase family. PDIA3 was identified at four different locations on 2D-gels and was 2.1, 1.8, 2.0 and 2.2-fold up-regulated in the N+C invasive cells compared to N+C non-invasive cells. PDIA3 has also been found in the cytosol and nucleus and has been predicted to be secreted by cells (Torti *et al.* 1988). Three phosphorylated variants of PDIA3 have already been identified on the 2D-gel containing protein samples from human coronary artery smooth muscle cells (SMCs) (Hirano *et al.* 1995).

PDIA3 has been observed to be up-regulated in hepatocarcinoma (Tokutomi *et al.* 2007). The over-expression of PDIA3 was reported in the more aggressive phenotype of a melanoma cell line (Chen *et al.* 2008a). The loss of PDIA3 was positively correlated with invasion, advanced stage of disease, and poor survival of gastric cancer (Dowling *et al.* 2007b). However, PDIA3 has also been reported expressed in the larvae of *Schistosoma mansoni*, a parasite of humans, where it facilitates the penetration and migration processes

by the parasite (Leys *et al.* 2007). This suggests that the protein could be involved in the regulation of cell migration and the invasive phenotype of breast cells. Moreover investigation of potential PTMs in PDIA3 could improve our understanding of invasion in breast cells and could help to identify potential drug targets and/or biomarkers for breast cancer.

4.1.2.2.2 Heat shock 90 (HSP 90)

HSP90, a member of heat shock protein family, was found to be 3.1-fold up-regulated in the N+C invasive vs. N+C non-invasive. HSP90 was initially identified as a tumour-specific transplantation antigen in mice (Wippersteg *et al.* 2002). Recently, HSP90 expression was reported to be dramatically up-regulated in malignant melanoma cells when compared to benign melanocytic lesions, and HSP90 was expressed on the surface of seven of eight melanoma metastases (Ullrich *et al.* 1986). In many cases, HSP90 client proteins are mutated or activated in cancer cells (Becker *et al.* 2004). For example, HSP90 had been found in complex with the oncogenic tyrosine kinase v-Src, the mutated oncogene Bcr/Abl, and the serine/threonine kinase Raf-1 (Neckers 2002). HSP90 is constitutively expressed at 2-10-fold higher levels in tumour cells compared with their normal counterparts suggesting that it could be crucially important for the growth and/or survival of tumour cells (Neckers 2002). Indeed, HSP90 over-expression in breast cancer cells has been correlated with acquired resistance to some forms of chemotherapy (Ferrarini *et al.* 1992). This suggests that the chaperone activity of Hsp90 may be crucial for signalling proteins that contribute to invasion in breast cells.

4.1.2.2.3 Transaldolase-1 (TALDO1)

Transaldolase-1 (TALDO1), a key enzyme of the pentose phosphate pathway, was found to be 2-fold up-regulated N+C invasive cells compared to N+C non-invasive cells. The potential involvement of intracellular TALDO1 in cancer has been discussed in section 4.1.1.3.5, however the role of secreted TALDO1 in the invasion of breast cells is not clear.

4.1.2.2.4 Glutathione synthetase (GSS)

Glutathione synthetase (GSS), a member of the glutathione synthetase ATP binding domain-like superfamily, was found to be 1.8-fold up-regulated in the N+C invasive vs. N+C non-invasive cells. GSS catalyzes the second step in the biosynthesis of glutathione (tripeptide of l-glutamine, l-cysteine and glycine) (Dinescu *et al.* 2004). Glutathione protects cells from damage caused by unstable oxygen-containing molecules, which are byproducts of energy production. High levels of glutathione in cancer cells may detoxify endogenous and exogenous toxic compounds, which are thought to contribute to resistance of cancer cells to chemotherapeutic agents (Dinescu *et al.* 2004). The increased level of glutathione synthetase has been observed in a human hepatoma cell line (HepG2) compared to a normal hepatic cell line (Chang) (Burg and Mulder 2002). The increased expression of mRNA for GSS was also shown in cancerous liver tissue from patients compared to normal controls (Lee *et al.* 2002). This suggests that the defense-related enzymes are largely modulated in tumour cells, which might be linked to their uncontrolled growth and maintenance. GSS expression has been observed to positively correlate with survival in early stage adenocarcinoma of the lung (Huang *et al.* 2001). The role of GSS in invasion of breast cells is unclear.

4.1.2.2.5 Summary

This analysis identified some proteins that are well known such as PDIA3 and HSP90 and some other proteins that are poorly known, such as TALDO1 and GSS, for their involvement in regulation of invasion in breast cells. The investigation of these proteins in the secreted fractions (plasma, serum etc.,) from breast cancer patients compared to normal individuals could enable us to identify potential biomarkers as well as drug targets for early detection and treatment of breast cancer disease.

4.1.2.3 Cancer-invasive (BT20) vs. normal invasive +normal non-invasive +cancer-non-invasive (HMEC+MCF-10A+MCF-7)

This comparison revealed 52 proteins differentially regulated in the cancer invasion-specific manner with 35 proteins up-regulated and 17 down-regulated in **group 1** (cancer invasive cell line (BT20)) compared to **group 2** (normal and cancer-non-invasive cells (HMEC+MCF-10A+MCF-7)). A total of 28 proteins were identified, of which 19 proteins were up-regulated and 9 were down-regulated in group 1 (Table 3.2.5).

These include some secreted proteins that are already known to be involved in regulation of invasion in breast cells. For example,

- Disulfide isomerase family A, precursor (PDIA3)
- Plastin-2 (LCP1)

A number of interesting secreted proteins were also found to be differentially expressed and could possibly have a role in breast cancer invasion. These include -

- Dimethylarginine dimethylaminohydrolase 1 (DDAH1)
- Fragment of Heparan sulfate proteoglycan 2 (HSPD2)
- Nucleobindin 2 precursor (NUCB2)

4.1.2.3.1 Disulfide isomerase family A, precursors (PDIA3)

PDIA3 is involved in protein folding and was observed to be 1.8-fold up-regulated in group 1 compared to group 2. The involvement of PDIA3 in cancer and invasion has been discussed in section 4.1.2.2.1.

4.1.2.3.2 Plastin-2 (LCP1)

LCP1 was identified at two different locations on the 2D-gels possibly due to post-translational modification and was found to be 9.8-fold and 8-fold up-regulated in group 1 compared to group 2. LCP1 is an actin-binding protein and has been proposed to be involved in the control of cell adhesion and motility leading to invasion and metastasis in cancer (Allen *et al.* 2007). LCP1 was reported to be up-regulated in many types of cancer including breast (Jones *et al.* 1998, Foran *et al.* 2006, Samstag and Klemke 2007, Lapillonne *et al.* 2000), prostate (Ostergaard *et al.* 1997) and colorectal cancers (Zheng *et*

al. 1997). In breast carcinoma, LCP1 was only observed in malignant epithelial cells in around 14% of the tumours analyzed (Otsuka *et al.* 2001, Lapillonne *et al.* 2000). LCP1 could be phosphorylated in melanoma cells, either by signaling events from the tumour cell environment, or by constant activation of signal transduction pathways in the course of transformation into cancerous or a cancer invasive (Ostergaard *et al.* 1997). The phosphorylation of LCP1 was observed to facilitate metastasis and invasion cells (Samstag and Klemke 2007). Thus LCP1 and its variants could be facilitating the invasive property of breast cancer cell lines.

4.1.2.3.4 Dimethylarginine dimethylaminohydrolase 1 (DDAH1)

DDAH1 plays a role in nitric oxide generation by regulating cellular concentrations of methylarginines, which in turn inhibit nitric oxide (NO) synthase activity. DDAH1 was observed 2.9-fold up-regulated in group 1 compared to group 2. Inhibition of DDAH1 activity results in the accumulation of asymmetric dimethylarginine (ADMA), which reduces the production of NO by inhibiting NO synthases (NOS). NO is involved in a diverse range of physiological processes including motility and invasion (Samstag and Klemke 2007). Increased DDAH1 activity could lead to significantly increased cell motility and invasion (Cartwright *et al.* 1999, Ayling *et al.* 2006). The role of DDAH1 in invasion in breast cancer is still unknown.

4.1.2.3.5 Nucleobindin 2 precursor (NUCB2)

Nucleobindin 2 precursor (NUCB2) was found to be 5.2-fold up-regulated in group 1 compared to group 2. NUCB2 contains a golgi retention motif at the N-terminal leucine/isoleucine-rich region (Ayling *et al.* 2006). The intracellular localization of NUCB2 is variable and cell type-specific (Nesselhut *et al.* 2001). For example, in KM3 cells, NUCB2 can be localized on the plasma membrane, in the cytosol and as a secreted protein (Barnikol-Watanabe *et al.* 1994, Taniguchi *et al.* 2000, Morel-Huaux *et al.* 2002). Like NUCB1, NUCB2 is also a substrate for caspases and could be involved in the regulation of apoptosis (Barnikol-Watanabe *et al.* 1994, Taniguchi *et al.* 2000, Morel-Huaux *et al.* 2002). However the role of NUCB2 in breast cancer is still unknown.

4.1.2.3.6 Fragment of Heparan sulfate proteoglycan 2 (HSPG2)

Fragment of Heparan sulfate proteoglycan 2 (HSPG2), a basement protein involved in cell adhesion, was found to be 5.3-fold down-regulated in group 1 compared to group 2. Heparan sulfate proteoglycan 2 (HSPG2), also known as Perlecan, is a part of the extracellular matrix in organisms from worms to humans (Valencia *et al.* 2008). HSPD2 can be either an intrinsic components of the basement membrane, the thin layer of connective tissue separating epithelial from mesenchymal cells, or in close association with the cell surfaces (Iozzo 2005). It has been reported to be involved in promoting the growth and invasion of tumour cells (Iozzo 1984). In human melanoma, HSPG2 acts as a cell surface marker for a more aggressive phenotype (Folkman *et al.* 1988), and levels of mRNA and protein were found to be increased in metastatic melanomas (Timar *et al.* 1992). However in contrast, reduction of HSPD2 expression has been shown to enhance *in vitro* migration, invasion, and adhesion to type IV collagen substrate in fibrosarcoma cells (Cohen *et al.* 1994). Degradation of endothelial-derived HSPD2 by MMP-1, MMP-3, plasmin and heparanase, has also been shown to release bound basic fibroblast growth factor that promote or maintain the angiogenic phenotype and tumour invasion (Mathiak *et al.* 1997). This suggests that reduced expression of HSPG2 could possibly be due to its cleavage by proteolytic enzymes that could facilitate breast cancer invasion.

4.1.2.3.7 Summary

This analysis identified a number of secreted proteins (PDIA3 and LCP1) that are known and other proteins (DDAH1, NUCB2 and fragment of HSPG2) that are unknown for their role in breast cancer invasion. Further investigation of the expression of DDAH1, NUCB2 and fragment of HSPG2 in serum/plasma samples from breast cancer patients versus normals could enable us to identify potential biomarkers for breast cancer invasion.

4.1.2.4 Identification of invasion-specific proteins by overlapping lists of differentially expressed proteins

The list of differentially expressed proteins between the cancerous cell lines (MCF-7 vs. BT20) was overlapped with the list of differentially expressed proteins between the normal cells (MCF-10A vs. HMEC) (Supplementary data: appendix 2). This comparison further enabled us to identify alterations in proteins level that are specifically differentially expressed in normal and cancer cells with invasive phenotypes and those that are required for invasion in both normal and cancer (Figure 3.2.3).

Eight proteins were specifically differentially regulated in the normal cells comparison group (MCF-10A vs. HMEC), of which 2 proteins (PRDX6 and SOD1) were up-regulated in MCF-10A. PRDX6 (peroxiredoxin 6) protects against oxidative injury (cell death) (Stetler-Stevenson 1999, Whitelock *et al.* 1996). The over expression of PRDX6 has been observed to contribute to a more invasive phenotype and metastatic potential in human breast cancer (Moon *et al.* 2005). The role of SOD1 in cancer and invasion has been described in section 4.1.1.1.6. A total of 6 proteins, including SERPINA1, were down-regulated in MCF-10A. The role of SERPINA1 in cancer has been described in Box 2. The specific roles of these proteins in the regulation of the invasive status of normal breast cells are unclear. However, these results suggest that the modulation of these proteins, i.e. up-regulation of PRDX6 and SOD1 and/or down regulation of SERPINA1, in normal-noninvasive cells could possibly lead to the development of normal-invasive phenotype in breast cells. A total of 23 proteins were only differentially regulated in the cancer cell lines comparison group (MCF-7 vs. BT20). Of these 23 proteins, 6 proteins including GSN and CTSD were up-regulated in the cancerous-non-invasive cells (MCF-7) compared to the cancerous invasive cell lines (BT20). The roles of GSN and CTSD have been described in section 4.1.1.3.1 and Box 3, respectively. Whereas, 17 proteins, including PDIA3, DDAH1 and NUCB2, were down-regulated in the cancerous-non-invasive cells (MCF-7). The role of PDIA3, DDAH1 and NUCB2 in cancer and invasion has been described in section 4.1.2.2.1 and 4.1.2.3.4-5. However, the involvement of these proteins in the regulation of the invasive status of normal breast cells is unknown. Our results suggest that the modulation of these proteins, i.e. up regulation of GSN and CTSD and/or down regulation

of DDAH1, PDIA3 and NUCB2, in cancer-non-invasive cell lines could lead to the development of invasive phenotype in breast cancer cells.

Five proteins were common in both lists of differentially expressed proteins (MCF-10A vs. HMEC and MCF-7 vs. BT20). Two of these, LAMC2 and PDIA3/BAT1/FKBP4 (multiple identifications (PDIA3, BAT1 and FKBP4) were observed for single spots by LC-MS/MS), were down-regulated in both the normal (MCF-10A vs. HMEC) and the cancer (MCF-7 vs. BT20) cell lines. Over expression of LAMC2 has been correlated with the cancer and invasion status of human glioma (Chang *et al.* 2007). This suggests that down regulation of these proteins could contribute to inhibit the development of invasive phenotypes in breast cells. The remaining 3 proteins include HSP60, CTSB and SERPINB1. HSP60 was up-regulated in the cancer-invasive cell line (BT20) compared to the cancer-non-invasive cell line (MCF-7) and was down-regulated in the normal invasive cells (HMEC) compared to the normal non-invasive cells (MCF-10A). This indicates that HSP60 could be involved in invasion but needs to be specifically down-regulated for normal invasion and up-regulated for cancer invasion. CTSB and SERPINB1 were down-regulated in the cancer invasive cells (BT20) compared to cancer non-invasive cells (MCF-7) but were up-regulated in the normal invasive cells (HMEC) in comparison to the normal non-invasive cells (MCF-10A). The roles of CTSB and SERPINB1 have been described in Box 3 and section 4.1.1.1.5 respectively. Therefore CTSB and SERPINB1 have to be possibly down-regulated to result in cancer invasion while up-regulated for normal invasion in breast cells, however the role of these proteins in invasion specifically in normal breast cells needs to be elucidated.

4.1.3 Proteomic analysis of isolated membrane fractions from HMEC, MCF-10A, MCF-7 and BT20 cells

Membrane proteins account for approximately one third of all predicted proteomes (Guo *et al.* 2005) and are part of cell boundaries as well as boundaries of cell organelles like mitochondria, nucleus, plastids, etc. Membrane proteins are involved in the regulation of a variety of cellular functions by serving as transporters and receptors. For example, cell surface membrane proteins play important roles in cell signalling, adaptation to the environment and cell–matrix interactions, and all of these phenotypes have been considered critical for the regulation of growth, motility, invasive and metastatic properties of tumour cells (Wallin and von Heijne 1998, Stevens and Arkin 2000). Identification of differentially expressed membrane and membrane-associated proteins between cell lines representing normal, non-invasive and invasive phenotypes could therefore enable us to identify potential drug targets and diagnostic markers.

In this investigation, profiles for membrane and membrane-associated proteins from four cell lines were investigated using 2D-DIGE coupled with mass-spectrometry to identify proteins that may be involved in the development of the cancerous and invasive phenotype in breast cells. For this, data was analysed in 3 main groups (Box-1).

4.1.3.1 Cancer (MCF-7+BT20) vs. normal (HMEC+MCF-10A)

The comparison of profiles for membrane and membrane-associated proteins from cancer cells (MCF-7+BT20) with normal cells (HMEC+MCF-10A) enabled us to identify cancer-associated differential expression of proteins in cellular membranes. A total of 95 protein spots were observed to be differentially expressed in this comparison. A total of 41 differentially expressed protein spots were identified, of which 11 were up-regulated and 30 were down-regulated in the cancerous cell lines (Table 3.3.2). The data suggests that mitochondrial membrane and membrane-associated proteins, members of the S100 protein family, FKBP5 and ASNAP1 among other proteins may have important roles in breast cancer, and include-

- Mitochondrial membrane and membrane-associated proteins (RHOT2, IMMT and HSPA9)
- Plasma membrane associated S100 proteins (S100A2, S100A6, S100A9, S100A14 and S100A16)
- FK506 binding protein 5 (FKBP5)
- Alpha-soluble NSF attachement protein (ASNAP1)

4.1.3.1.1 Mitochondrial membrane and membrane-associated proteins

Mitochondria are dynamic organelles that play a central role in cellular metabolism. The primary metabolic function of mitochondria is oxidative phosphorylation to synthesise energy in the form of ATP for cellular activities. However, mitochondria are also sites for several additional key metabolic processes such as the oxidative decarboxylation of pyruvate, the tricarboxylic acid cycle, and fatty acid oxidation. In addition, mitochondria are one of the major orchestrators of metabolism and apoptosis (Dowling *et al.* 2008) and therefore proteins encoded by the mitochondrial genome could potentially regulate development of cancer (Kroemer 1999, Green and Reed 1998). A number of mitochondrial membrane and membrane-associated proteins were observed to be differentially regulated in this investigation. These include known proteins (HSPA9, PHB and DIABLO) (Box 4) and others potentially interesting proteins (RHOT2, IMMT, ATP5B and UQCRC1) which have limited information in the literature for their role in cancer.

4.1.3.1.1.1 Mitochondrial Rho 2 (RHOT2)

Mitochondrial Rho 2 (RHOT2) is a GTPase enzyme and was observed to be 4.2 fold down-regulated in the cancer vs. normal cells. The Mitochondrial Rho GTPases (also called Miro) are additional members of the Rho GTPase family as they are structurally different from the classical Rho GTPases (Sanchez-Pino *et al.* 2007, Modica-Napolitano *et al.* 2007, Modica-Napolitano and Singh 2004). Another feature that distinguishes Miro from the classical Rho GTPases is the apparent absence of a role in regulating actin dynamics. Instead, the Miro are permanently localised to the mitochondria (Aspenstrom *et al.* 2004, Wennerberg and Der 2004). However the exact role of RHOT2 is not known to date. It has been reported to be involved in the regulation of mitochondrial homeostasis by regulating the mitochondrial trafficking apparatus (Fransson *et al.* 2003 & 2006). The over expression of RHTO2 has been observed to increase the aggregation of mitochondria in COS-7 cells and it was expected to function as a link between the mitochondria and the microtubules (Fransson *et al.* 2006). Therefore, down regulation of RHOT2 should abrogate the mitochondrial functions and should induce apoptosis in culture (Fransson *et al.* 2006). In contrast, the expression of RHOT2 was reduced in the cancerous cells compared to the normal cells in this investigation which indicates that RHOT2 may have other unknown functions rather than regulating homeostasis in the breast cancer cells. Further investigations of RHOT2 could clarify its involvement in the process of tumour development.

Box-4: Known mitochondrial membrane and membrane-associated proteins			
proteins	**Cancer vs. normal (Fold change)**	**Function**	**Regulation in cancer**
Stress-70 protein, mitochondrial precursor (HSP9)	-2.03 and -1.8	Proliferation, stress response and inactivation of p53 function (Fransson *et al.* 2006)	Up in breast (Wadhwa *et al.* 1993, 2002 & 2006) and hepatocellular carcinoma (Wadhwa *et al.* 2006)
Prohibitin (PHB)	2.76	Cell growth, transcription and molecular chaperones (Yi *et al.* 2008)	Up in breast cancer (Gamble *et al.* 2004, Nijtmans *et al.* 2001 & 2002), bladder cancer (Jupe *et al.* 1996) and gastric cancer (Wu *et al.* 2007)
Diablo homolog, mitochondrial precursor (DIABLO)	2.36	Promotes cell death (Ren *et al.* 2006)	Up in cervical cancer (Kashkar *et al.* 2003 & 2006, Du *et al.* 2000)

4.1.3.1.1.2 Mitofilin (IMMT)

Mitofilin (IMMT), an inner membrane protein, is one of the most abundant mitochondrial proteins. IMMT was observed to be 4.2 fold down-regulated in the cancer cells compared to the normal cells. Little is known to date about the role of IMMT in cells. IMMT has been shown to play an important role in the maintenance of cristae morphology (Espinosa *et al.* 2004, Arellano-Llamas *et al.* 2006). Cristae formation is critical for achieving a high surface-to-volume ratio of the inner membrane. Down-regulation of IMMT using siRNAs

resulted in a drastic change in the organization of the inner membrane in HeLa cells (John *et al.* 2005). Rather than organizing into tubular cristae, the inner membrane formed concentric layers that interconnected at numerous sites following inhibition of IMMT. This resulted in reduced cell growth and increased apoptosis in culture. However, IMMT was observed to be down-regulated in the cancerous cells compared to the normal cells in our investigation. Cancerous cells show increased growth and reduced apoptosis in culture. However, the role of IMMT is unknown in cancer; it has been observed to be over expressed in a human hepatocarcinoma cell line (John *et al.* 2005). Therefore the role of IMMT in the regulation of growth and apoptosis in cancer could be cell or tissue (i.e. breast or liver, etc) specific and which remains unclear. Investigation of IMMT could further improve our understanding of growth and apoptosis in cancer cells and may enable us to identify potential mechanisms of breast cancer.

4.1.3.1.1.3 ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor (ATP5B)

ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor (ATP5B) was observed to be 1.67 and 1.77 fold down-regulated in the cancer vs. normal cells. ATP5B transduces the energy contained in electrochemical proton gradients of the membranes into the energy required for synthesis of high-energy phosphate bonds. Tumours often cope by ramping up an alternative energy production strategy (Feng *et al.* 2007). For most of their energy needs, normal cells rely on respiration, which consumes oxygen and glucose to make energy-storing molecules of adenosine triphosphate (ATP). However, cancer cells typically depend more on glycolysis, the anaerobic breakdown of glucose into ATP (Garber 2006). The decreased expression of ATP5B has been linked to an increased expression of several markers of the glycolytic pathway in many cancer (Cuezva *et al.* 2004). For example, ATP5B has been observed to be reduced in 97.3% of samples from 90 lung adenocarcinomas (Garber 2006, Cuezva *et al.* 2002 & 2004). Moreover, inhibition of ATP5B using a chemical inhibitor has been shown to reduce apoptosis in yeast cells indicating the involvement of ATP5B in the regulation of cell death (Cuezva *et al.* 2004). Therefore down regulation of ATP5B in the cancerous cell lines could be an indication of altered energy generation and reduced apoptosis in tumour cells.

4.1.3.1.1.4 Ubiquinol-cytochrome c reductase core protein I (UQCRC1)

Ubiquinol-cytochrome c reductase core protein I (UQCRC1) is a nuclear-encoded protein localized to the inner mitochondrial membrane. UQCRC1 was found to be 10.49 fold down-regulated in the cancer vs. normal cells. This protein has been discussed in section 4.1.1.1.4. We also observed the expression of UQCRC1 up-regulated in the cancer vs. normal cells in the cell lysate study (table 3.1.5), which is in agreement with the published results (Matsuyama *et al.* 1998). In contrast, the expression of UQCRC1 in the membrane fraction from cancerous cells compared to normal cells was reduced which possibly complicates its potential role in breast cancer. However, it is possible that the UQCRC1 we observed in the membrane fraction study could be a post-translationaly modified form of UQCRC1, which needs to be investigated.

4.1.3.1.2 Plasma membrane associated S100 proteins

S100 proteins belong to the Ca^{2+}- binding EF-hand motif superfamily and have the ability to form homodimers, heterodimers and oligomers. S100 proteins can be found in a soluble and a membrane-bound form (Kulawiec *et al.* 2006). S100 proteins have been involved in several activities including cell communication, cell growth, cell structure, energy metabolism, contraction and intracellular signal transduction (Zimmer *et al.* 1995), and all of these process plays critical roles in the development of cancer. In this study, five members of the S100 protein family, S100A2, S100A6, S100A9, S100A14 and S100A16 were observed to be differentially regulated in the cancer vs. normal cell comparison. These include some proteins that are already known such as S100A2 and S100A6, and some other members that are poorly known such as S100A14 for its involvement in the regulation of breast cancer (Box 5). S100A14 has been observed to be up-regulated in breast cancer (Zimmer *et al.* 1995, Donato 1986) and down regulated in oral squamous cell carcinoma (Pietas *et al.* 2002), esophageal squamous cell carcinoma (Sapkota *et al.* 2008) and bladder cancers (Ji *et al.* 2004) (Box 5). Although the role of S100A14 in cancer is unclear, the data suggests that its expression may be cell line or origin specific.

Box-5: Known S100 proteins identified from 2D-DIGE study			
S100 proteins	**Cancer vs. normal (Fold change)**	**Function**	**Regulation in cancer**
S100A2	-182.99	Proliferation (Yao *et al.* 2007)	Down in prostate cancer (Tsai *et al.* 2006), lung cancer (Gupta *et al.* 2003), oral cancer (Feng *et al.* 2001) and breast cancers (Suzuki *et al.* 2005)
S100A6	-18.04	Apoptotic (Liu *et al.* 2000a, Lee *et al.* 1992)	Down in prostrate cancer (Slomnicki *et al.* 2008) and breast cancer (Rehman *et al.* 2004)
S100A9	-18.04	Metastasis (Sanders *et al.* 2008, Carlsson *et al.* 2005) and motility (Rafii and Lyden 2006)	Down in esophageal squamous cell carcinoma (Hiratsuka *et al.* 2006), breast cancer (Luo *et al.* 2004) and bladder cancer (Rhee *et al.* 2008)
S100A14	-3.37	Not known	Down in oral squamous cell carcinoma (Yao *et al.* 2007), esophageal squamous cell carcinoma (Sapkota *et al.* 2008), bladder cancer (Ji *et al.* 2004) and kidney, rectum & colon tumours (Yao *et al.* 2007). Up in ovary, breast and uterus tumours (Pietas *et al.* 2002).
S100A16	-2.24	Not known, possibly invasion suppressor (Pietas *et al.* 2002)	Down in breast cancer (Bianchi *et al.* 2005)

4.1.3.1.3 FK506 binding protein 5 (FKBP5)

FK506 binding protein 5 (FKBP5) is a 51-kDa FK506 binding protein with peptidyl-prolyl isomerase (PPIase) activity, also named as FKBP51. FKBP5 was identified at two different spots on the 2D-gel and was observed to be 4.22-fold and 2.3-fold down regulated in the cancer vs. normal cells. FKBP5 can form a complex with HSP90 and can modulate the translocation of steroid receptors, such as progesterone and glucocorticoid receptors to the nucleus (Bianchi *et al.* 2005). FKBP5 was found to be associated with mitogen-activated protein pathways which could regulate cell proliferation and death (Wochnik *et al.* 2005). However its role in breast cancer needs to be elucidated. FKBP5 has been observed to facilitate cell survival and proliferation in melanoma cell line compared to normal cells (Bouwmeester *et al.* 2004). It was also observed to regulate the growth of glioma (Romano *et al.* 2004). In contrast, FKBP5 was down regulated in the breast cancerous cell lines in our study and this suggests that the effect of FKBP5 could be cell line or origin specific (i.e. breast and melanoma, etc).

4.1.4.1.5 Alpha-soluble NSF attachement protein (ASNAP1)

Alpha-soluble NSF attachement protein (ASNAP1) has widespread involvement in vesicular transport pathways leading to exocytosis. ASNAP1 was observed to be 1.85 fold up regulated in the cancer vs. normal cells. ASNAP1 has been shown to facilitate the secretion from lung cells and its inhibition using siRNA knockdown technique was observed to reduce the secretion of proteins (Jiang *et al.* 2008). Similarly the inhibition of ASNAP1 in β-pancreatic cells was observed to reduce insulin secretion (Abonyo *et al.* 2003). The role of ASNAP1 protein in breast cancer is still unknown.

4.1.4.1.6 Summary

Taken together, a number of interesting membrane and membrane-associated proteins (RHOT2, IMMT, UQCRC1, S100A14, FKBP5 and ASNAP1) which possibly have roles in the regulation of tumour development were observed to be differentially regulated in this investigation. Further investigation of these proteins could potentially help to understand the molecular mechanisms whereby a normal cell transforms into a cancerous cell.

4.1.3.2 Invasive (HMEC+BT20) vs. non-invasive (MCF-10A+MCF-7)

A total of 85 protein spots were observed to be differentially expressed in this comparison group (N+C invasive cells (HMEC+ BT20) vs. N+C non-invasive cells (MCF-10A+MCF-7)), of which 22 were up-regulated and 63 were down-regulated in the N+C invasive cells (Table 3.1.3). From these, 27 proteins were identified (21 up-regulated and 6 down-regulated in the N+C invasive cells (Table 3.2.4). In this analysis, a number of mitochondrial membrane and membrane-associated proteins were observed to be differentially regulated. These proteins include some proteins that are already known, such as HSPD1, and some other proteins that are poorly known, such as NDUFS1, for their involvement in regulation of breast cancer invasion. Two known S100 proteins (S100A9 and S100A16) were identified. Some other known proteins such as NME1, as well as other interesting proteins such as GANAB and WDR1 were also identified in this study.

4.1.3.2.1 NADH dehydrogenase (ubiquinone) Fe-S protein 1, precursor (NDUFS1)

NDUFS1 was observed to be 1.61 fold down-regulated in the N+C invasive vs. N+C non-invasive cells. NDUFS1 belongs to the complex-I 75kDa subunit family. It locates at the mitochondrial inner membrane. NDUFS1 has NADH dehydrogenase activity and oxidoreductase activity. It transfers electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. This protein is the largest subunit of complex I and it is a component of the iron-sulfur (IP) fragment of the enzyme. It may form part of the active site crevice where NADH is oxidized. Ablations in this gene could disrupt complex I deficiency and hence the electron transport leading to production of ROS, loss of ATP production, mitochondrial damage and ultimately cell death (Nagamatsu *et al.* 1999). Limited information is available for the role of NDUFS1 protein in cancer and invasion. Recently, the expression of NDUFS1 has been observed reduced in a patient with metastatic melanoma when treated with indisulam drug (Ricci *et al.* 2004).

4.1.3.2.2 60 kDa heat shock protein 1, mitochondrial precursor (HSPD1)

HSPD1 was observed to be 2.27 fold increased in the N+C invasive vs. N+C non-invasive cells. HSPD1 is a member of the Hsp60 family of heat shock proteins that are expressed

both constitutively and under conditions of stress (Baur *et al.* 2007). It forms heptamers and tetradecamers in association with Hsp10 and ATP and is an important part of the mitochondrial chaperone system (Bukau and Horwich 1998) and involved in apoptosis (Fink 1999, Bukau and Horwich 1998). HSPD1 has been found over expressed in many cancer including breast (Lin *et al.* 2001), colorectal (Desmetz *et al.* 2008) and ovarian cancer (Cappello *et al.* 2003) and this is in agreement with our observations in this study.

4.1.3.2.3 S100 proteins

S10016 were observed to be 2.32 fold down-regulated whereas S100A9 was observed to be 1.8 fold up-regulated in the N+C invasive vs. N+C non-invasive cell lines. The involvement of S100A9 and S100A16 proteins in cancer and invasion has been described in Box 5.

4.1.3.2.4 Non-metastatic protein 1 (NME1)

NME1 was observed to be 2.6-fold down-regulated in the N+C invasive vs. N+C non-invasive. As described in section 4.1.1.3.1, the reduced expression of NME1 has been correlated with high tumour metastatic potential in several human breast carcinomas (Schneider *et al.* 1999).

4.1.3.2.5 Alpha glucosidase II alpha subunit isoform 2 (GANAB)

Alpha glucosidase II alpha subunit isoform 2 (GANAB), also known as Glucosidase II, is an endoplasmic reticulum-localized enzyme that has an important function in the folding and maturation of glycoproteins. GANAB was identified at two different locations on the 2D-gel and was observed to be 2.37 and 2.69-fold up-regulated in the N+C invasive vs. N+C non-invasive cells. GANAB trims two α-1, 3-linked Glc residues from the glycoprotein oligosaccharide Glc2Man9GlcNAc2 to give Glc1Man9GlcNAc2 and Man9GlcNAc2 in the endoplasmic reticulum. Monoglucosylated glycans generated by this process play a key role in glycoprotein quality control in the ER, as they are primary ligands for the lectin chaperones calnexin (CNX) and calreticulin (CRT) that are involved in ensuring glycoprotein quality prior to recruiting nascent proteins for their biological function (Stahl *et al.* 1991, Heimann *et al.* 1998). GANAB has been observed in precursor and mature forms as it contains 7 sites for glycosylation and several sites for

phosphorylation (Totani *et al.* 2006). Inappropriate post-translational modifications of GANAB could result in functionally altered or inactive forms of GANAB (Reuser *et al.* 1985, Hermans *et al.* 1991, Hasilik and Neufeld 1980). GANAB was identified at two different locations on the 2D-gel in this study possibly due to post-translational modifications (glycosylation and/or phosphorylation). Although, the role of GANAB and its post-translationally modified variants in breast cancer and invasion is not known, the increased expression of GANAB in the N+C invasive cells suggests that it could be associated with ensuring the quality of increased expression of invasion-associated proteins and/or compromised quality of invasion-suppressor proteins.

4.1.3.2.6 WD repeat-containing protein 1 (WDR1)

WD repeat-containing protein 1 (WDR1) was observed to be 2.4 fold up-regulated in the N+C invasive vs. N+C non-invasive cells. WD repeats are approximately 30- to 40-amino acid domains containing several conserved residues, mostly including a trp-asp at the C-terminal end. WD domains are involved in protein-protein interactions and may help induce the disassembly of actin filaments. Inhalation of mixtures of insoluble and soluble nickel compounds by humans has been found associated with excess lung and nasal sinus cancers. Nickel-transformed cell lines have shown up-regulation of the WDR1 gene (Reuser *et al.* 1985, Hermans *et al.* 1991). The role of this protein in breast cancer and invasion is still not known.

4.1.3.2.7 RuvB-like 2 (RUVBL2)

RuvB-like 2 (RUVBL2) was observed to be 4.0-fold up-regulated in the N+C invasive vs. N+C non-invasive cells. RUVBL2 is a DNA helicase essential for homologous recombination and DNA double-strand break repair (Verma *et al.* 2004). Functional analysis showed that this gene product has both ATPase and DNA helicase activities. Over expression of RuvB-like 2 (RUVBL2) has been reported in hepatocellular carcinoma (HCC) and was correlated with the aggressiveness of tumours (Bal *et al.* 2005). It was also suggested that RUVBL2 is required for tumour cell viability. However, its roles in breast cancer are still unknown.

4.1.3.2.8 Adipocyte plasma membrane-associated protein (C20ORF3)

Adipocyte plasma membrane-associated protein (C20ORF3) was observed to be 1.9-fold down-regulated in the N+C invasive vs. N+C non-invasive cells. C20ORF3 resides on chromosome 20, open reading frame 3 and is a member of the lactonohydrolase super family. The molecular functions of C20ORF3 are unknown. C20ORF3 has been suggested potentially to be involved in enzymatic processes and differentiation of adipocytes. The observed down regulation of C20ORF3 in regulated in the invasive cells in comparison to the non-invasive cells suggests that it could be associated with invasion of breast cells.

4.1.3.2.9 Summary

Taken together, the data presented here indicates that a number of proteins, NDUFS1, GANAB, RUVBL2, WDR1 and C20ORF3 which have not been previously linked with invasion in breast cells, were differentially regulated in the invasive vs. the non-invasive cells comparison. Therefore investigation of these membrane and membrane-associated proteins could help us to identify potential drug targets or biomarkers for breast cancer.

4.1.3.3 Cancer-invasive (BT20) vs. normal invasive +normal non-invasive +cancer-non-invasive (HMEC+MCF-10A+MCF-7)

This comparison revealed 109 proteins differentially regulated in a cancer invasion-specific manner with 87 proteins up-regulated and 22 proteins down-regulated in **group 1** (cancer invasive cell line (BT20)) compared to **group 2** (normal and cancer-non-invasive cells (HMEC+MCF-10A+MCF-7)). A total of 37 proteins were identified, of which 27 proteins were up-regulated and 10 were down-regulated in group 1 (Table 3.3.5). In this analysis, a number of mitochondrial membrane and membrane-associated proteins were observed to be differentially regulated. These proteins include some known in literature such as PHB and some other proteins which have very limited information for their role in breast cancer invasion such as ATP5B. From the S100 protein family, some members that are well known, such as S100A2, and some other members that are poorly known such as S100A14 for their involvement in the regulation of breast cancer invasion were again found differentially expressed in our study. A number of other interesting proteins that have not been linked previously with breast cancer invasion were also identified, such as CES1, AHCY, TALDO1 and RUVBL2.

4.1.3.3.1 Prohibitin (PHB) and ATP5B

Prohibitin (PHB) and ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor (ATP5B) were found to be 1.85 and 2.3-fold up-regulated in group 1 vs. group 2, respectively. PHB has also been observed over expressed in breast cancer (Rousseau *et al.* 2007), bladder cancer (Jupe *et al.* 1996) and gastric cancer (Wu *et al.* 2007) and possibly involved in apoptosis and/or cell migration (Ren *et al.* 2006) which is an agreement with literature. The role of PHB and ATP5B in cancer has been discussed in box 3 and section 4.1.3.1.1.3 respectively.

4.1.3.3.2 S100 proteins

S100A2 and S100A14 were found to be 155.42 and 3.53 fold down-regulated respectively in group 1 vs. group 2. The involvements of these proteins in the cancer and invasion have been described in box 5.

4.1.3.3.3 RuvB-like 2 (RUVBL2)

RUVBL2 was observed to be up-regulated (5.57 and 2.0-fold) at two different locations on the gel, in group 1 vs. group 2. The involvement of RUVBL2 protein in cancer and invasion has been described in sections 4.1.4.3.7. However, its role in breast cancer is still unknown. Therefore up regulation of RUVBL2 in the cancer invasive cell line could be associated with acquiring the changes in genome that favours the development of an invasive phenotype in breast cancer cells.

4.1.3.3.4 Liver carboxylesterase 1 precursor (CES1)

CES1 which is involved in detoxification of compounds in cells was found to be 45.2-fold over-expressed in group 1 vs. group 2. CES1 is involved in the hydrolysis of aromatic and aliphatic esters & thioesters and amide-bond-containing molecules and is necessary for cellular cholesterol esterification and homeostasis (Zhu *et al.* 2006). The cholesterol homeostasis in cancer patients has already been reported to be modulated (Satoh and Hosokawa 1998, Zhao *et al.* 2005). It may also play a role in detoxification in the lung and/or protection of the central nervous system from ester or amide compounds (Dessi *et al.* 1994 & 1997). Fibrosarcoma (HT1080) and colon carcinoma cell lines (SW480) with increased levels of CES1 were observed significantly more sensitive to irinotecan (an anticancer drug) treatment as compared to the parental cells (Satoh and Hosokawa 1998). CES1 has also been observed to be a positive predictor of prostate cancer recurrence (Kojima *et al.* 1998, Matzow *et al.* 2007). The roles of CES1 in breast cancer and invasion are still not known.

4.1.3.3.5 Adenosylhomocysteinase (AHCY)

AHCY was found to be 2.7-fold up-regulated in group 1 vs. group 2. AHCY converts S-adenosylhomocysteine to homocysteine and regulates the intracellular S-adenosylhomocysteine (SAH) concentration facilitating the sustained flux of methionine sulfur toward cysteine and controlling the transmethylation reactions (Glinsky *et al.* 2005). DNA methylation and histone modification have already been associated with various diseases including cancer (Leal *et al.* 2008). The role of AHCY in cancer is still unclear. For example, AHCY has been observed to be down regulated in colon cancer (Esteller

2007, Widschwendter *et al.* 2004) but on the other hand, inhibition of AHCY has been observed to induce efficient apoptotic cell death in breast cancer (MCF-7), colorectal cancer (HCT116) and leukemia (HL-60 and U-937) cells possibly due to mitochondria dysfunction and caspase activation (Leal *et al.* 2008).

4.1.4.3.6 TALDO1

Transaldolase-1 (TALDO1) was found to be 4.2-fold up-regulated in group 1 vs. group 2. The involvement of TALDO1 protein in cancer and invasion has been described in sections 4.1.1.3.5

4.1.3.3.7 Summary

Therefore, the presented data indicate that a number of membrane and membrane-associated proteins that have not been linked previously with breast cancer invasion such as ATP5B, S100A14, CES1, AHCY, TALDO1 and RUVBL2 were altered in the cancer invasive cells and could have roles in the regulation of the breast cancer invasive phenotype. Further investigation of these proteins could enable us to identify potential drug targets and/or biomarkers for breast cancer disease.

4.1.3.4 Identification of invasion-specific proteins by overlapping lists of differentially expressed proteins

The list of differentially expressed membrane and membrane-associated proteins within cancerous cells (MCF-7 vs. BT20) was overlapped with the list of differentially expressed proteins within normal cells (MCF-10A vs. HMEC) (Supplementary data: appendix 3). This comparison further enabled us to identify alterations in proteins level that are specifically differentially expressed in normal and cancer cell lines with invasive phenotypes and those that are required for invasion in both (Figure 3.3.2).

29 proteins were specifically altered between the two normal cell lines (MCF-10A vs. HMEC). These proteins include ITGA3, LAMB1, KRT8 and UQCRC1. ITGA3, LAMB1 and KRT8 were up-regulated and UQCRC1 was down-regulated in the normal non-invasive cells (MCF-10A) compared to the normal invasive cells (HMEC). Although, the specific role in the regulation of the invasion process in normal cells is unknown, based on the information from cancerous cell line models, these proteins have been associated with the regulation of invasion. ITGA3, a member of the integrin family, controls cell attachment to the extracellular matrix (ECM) and has been observed to play an essential role in the invasion process by degradation of the ECM (Tan *et al.* 2007, Kim *et al.* 2000). LAMB1, extracellular matrix glycoprotein, is the major non-collagenous constituent of basement membranes and its expression has been found to positively correlate with an increased invasive potential of many cancers including breast (Kurokawa *et al.* 2008, Felding-Habermann 2003). KRT8, an intermediate filament protein, may be associated with tumour progression. The role of UQCRC1 in cancer and invasion has been described in sections 4.1.1.1.4 and 4.1.3.1.1.4. This suggested that the modulation (up- or down regulation) of these proteins is associated with the development of the invasive phenotype in normal breast cells.

A total of 28 unique proteins were identified to be altered between the cancer non-invasive (MCF-7) and the cancer invasive (BT20) cell lines. These include PNPO, HSPD1 and PDIA3. PNPO was up-regulated and HSPD1 and PDIA3 were down-regulated in the cancer non-invasive cell line (MCF-7) compared to cancer invasive cell lines (BT20). PNPO catalyzes the rate-limiting step in the synthesis of vitamin B6 and defects in this could lead to various diseases including cancer (Bair *et al.* 2005). The roles of HSPD1 and PDIA3 have been discussed in sections 4.1.3.2.2 and 4.1.2.2.1, respectively. This suggests

that the modulation of these proteins, i.e. up regulation of PNPO, and down regulation of HSPD1 and PDIA3, in the cancer-non-invasive cells could lead to the development of the invasive phenotype in breast cancer cells.

The overlapping revealed seven proteins, including GANAB, RUVBL2, C20ORF and S100A16, that could be involved in the regulation of normal and cancer invasion. For example, GANAB and RUVBL2 proteins were down-regulated in both comparisons. The role of GANAB and RUVBL2 in invasion is unknown (sections 4.1.3.2.5 and 4.1.3.2.7, respectively), however down regulation of these proteins in non-invasive cells suggests their potential role in development of invasive phenotype of breast cells. C20ORF3 and S100A16 were up-regulated in both comparisons. The role of C20ORF in cancer invasion is unknown (section 4.1.3.2.8) and S100A16 is possibly an invasion suppressor (Box 5) (Nutter *et al.* 1983). The increased expression of these proteins in non-invasive cells could possibly be associated with reduced invasion in breast cells. Therefore, alterations in the expression of GANAB, RUVBL2, C20ORF and S100A16 could lead to the invasive phenotype of breast cell lines.

4.1.4 Enrichment of protein in three different fractions

The differentially expressed identified proteins from all three fractions (total cell lysate, conditioned media and membrane fractions) for all comparison groups (cancer vs. normal, N+C invasive vs. N+C non-invasive and cancer-invasive vs. normal-invasive +normal-non-invasive +cancer non-invasive) were overlapped to identify common as well as unique proteins in each fraction. Box-6 shows the proteins which are present only in the cell lysate fraction, conditioned media fraction and membrane fraction and proteins which are commonly identified in all of these fractions.

A large number of proteins were identified that were unique to each fraction, i.e. lysate, conditioned media or membrane. A total of 24 unique differentially expressed proteins, such as TXNRD1, CLP, QPRT and RAD23B, were identified from the cell lysate fraction (Box 6). 37 unique differentially expressed proteins, including SERPINB7, SERPINF1, FHC and NUCB1, were identified from the conditioned media. 33 unique differentially expressed proteins, such as IMMT, S100A16 and GANAB, were identified from the membrane fraction. This suggests that each fraction could reveal different and valuable information and could be linked together in many functions such as regulatory pathways or could have their own function to facilitate cancerous and/or invasive phenotypes in breast cells.

Only five proteins, ACTB, ANXA3, PDIA3, SERPINB5 and TALDO1, were observed in all three fractions as shown in Box-6. The majority of these proteins have already been reported to be cytosolic, membranous, and extracellular in localization such as SERPINB5, ATCB and PDIA3.

- SERPINB5 has been reported to localize primarily to the cytoplasm (Bianchi *et al.* 2005), but can also localize to the extracellular (Khalkhali-Ellis *et al.* 2008) and the cell surface (Toillon *et al.* 2007). SERPINB5 has pro-apoptotic, anti-angiogenic, and anti-metastatic functions (Pemberton *et al.* 1997).
- ATCB also has been previously observed to be in present in cell lysates (Maass *et al.* 2001a, Shi *et al.* 2001, Lockett *et al.* 2006), isolated membrane fraction (Wong *et al.* 2008) and in conditioned media (Dowling *et al.* 2007b). ACTB is a highly

conserved protein and is involved in cell motility, structure and integrity (Walsh *et al.* 2008).

- PDIA3 has been reported in conditioned media (Peckham *et al.* 2001, Ng *et al.* 1985), membrane (McClelland and Gullick 2007) and cytosolic (Dowling *et al.* 2007b) fractions. PDIA3 has been shown to interact with the lectin-like ER chaperones calreticulin (CRT) and calnexin to mediate disulfide bond rearrangements in nascent glycoproteins bearing monoglucosylated N-linked glycans (Celli and Jaiswal 2003).

Taken together, the data indicates that we have identified a unique pattern of differentially expressed proteins in each fraction, i.e. serpins (SERPINB7 and SERPINF1) in conditioned media, and mitochondrial and S100 proteins in the membrane fraction. This ensures the efficiency of the fractionation method for proteomic investigation and therefore the identified differentially expressed proteins from different fractions are likely to be similar in localization in other cancer cells as we have observed. Therefore, investigation of different cellular fractions to gain insights into breast cancer and identify potential drug targets and/or biomarkers is an efficient method and could be helpful to the diagnosis and prognosis of breast cancer. This also proved the concept that each fraction from the cells has different protein information and could be linked together in many functions such as regulatory pathways or could have their own function to facilitate cancerous and/or invasive phenotypes in breast.

Box 6: Distribution of identified proteins in all three fraction						
Proteins only identified in cell lysates fraction		**Proteins only identified in conditioned media fraction**		**Proteins only identified in membrane fraction**		**Proteins identified common in 3 fractions**
3-PGDH	ME3	APOE	GSS	A2M	FKBP5	ACTB
AKR1A1	NDRG1	BAT1	HSPG2	ACTA1	GANAB	ANXA3
ANXA1	NUDC	BF	INHBA	ANXA5	GCN1L1	PDIA3
ARCN1	Psma6	C1R	KRT10	ATP5B	HSPD1	SERPINB5
BTRC	QPRT	CAPZA	LAMC2	C20ORF3	IMMT	TALDO1
CLP	RAD23B	CAPZB	LCP1	CCT6A	JUP	
DCI	TXNRD1	CPA4	NP	CES1	LOC224626	
DYNC1LI2	CCT-beta	CTSB	NUCB1	CLIC4	ASNAP1	
HNRPF		DDAH1	NUCB2	DIABLO	NDUFS1	
HNRPK		EIF4A	PA2G4	PHB	NPM1	
KRT18		FHC	PDIA4	PSME2	NT5E	
KRT19		FKBP4	PGM2	RHOT2	TCP1	
LGALS1		GDI2	PLS3	RUVBL2	WDR1	
LMNA		GLUL	PPGB	S100A14	YWHAB	
		SERPINF1	PRDX6	S100A16	SAR1B	
		TPTE2	RDX	S100A2		
		TSP	RNPEP	S100A6		
		SERPINB7	VIL2	S100A9		
			CCT8			

4.1.5 Analysis of distribution of identified differentially expressed proteins in biological functions

The identified differentially expressed proteins from all three fractions (total cell lysate, conditioned media and membrane fractions) for each comparison group (cancer vs. normal, N+C invasive vs. N+C non-invasive or cancer-invasive vs. normal-invasive +normal-non-invasive +cancer non-invasive) were analysed to gain ontology information as the knowledge of significantly affected biological pathways could enable us to identify potential regulators of these complex biological pathways and may help to identify potential drug targets and biomarkers for breast cancer disease.

4.1.5.1 Cancer (MCF-7+BT20) vs. normal (MCF-10A+BT20)

The identified differentially expressed protein for the comparison of cancer (MCF-7+BT20) vs. normal (HMEC+MCF-10A) group from cell lysate, conditioned media and membrane fractions were grouped together to investigate which biological functions are being affected in the process of transformation of a normal cell into a cancer cell. Of the identified differentially expressed proteins from the cancer vs. normal comparison group, the majority of proteins were observed to be involved in the regulation of cell structure (16%) and proteolysis (14%) followed by cell proliferation and differentiation (7%) (Figure 4.1.5.1).

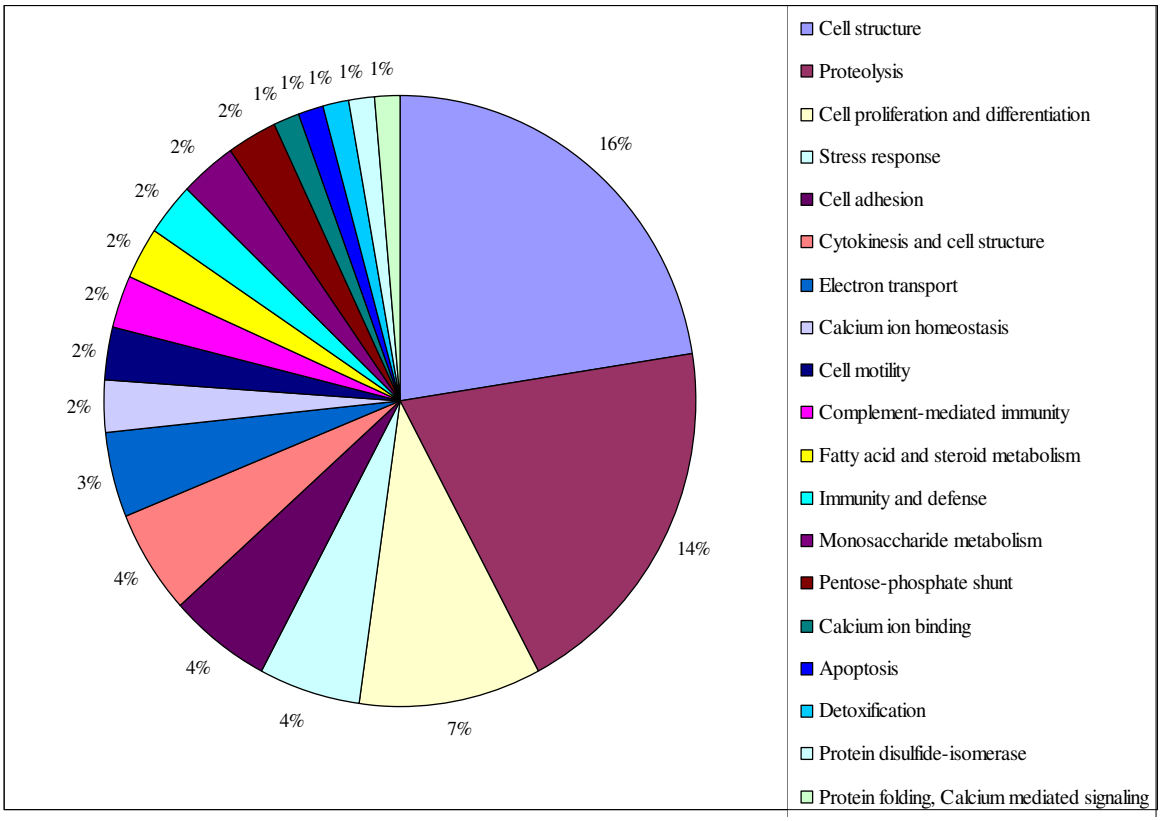


Figure 4.1.5.1. A pie chart demonstrating the percentage of diferentially expressed proteins in each biological function identified in the cancer (MCF-7+BT20) vs. normal (HMEC+MCF-10A) comparison group from 2D-DIGE study of cell lysate, conditioned media and membrane fractions. The information for the biological function was obtained from the NCBI database.

4.1.5.2 Normal+cancer-related invasion (HMEC+BT20 vs. MCF-10A+MCF-7)

The identified differentially expressed protein for the comparison of normal+cancer invasive (HMEC+BT20) vs. normal+cancer non-invasive (MCF-10A+MCF-7) group from cell lysate, conditioned media and membrane fraction were also analysed to investigate the biological functions significantly affected in the process of transformation of a non-invasive cell into an invasive cell. The majority of proteins identified in this comparison group were observed to be associated with the regulation of cell structure, protein disulfide isomerase reaction and in stress management in the cell, and account for 18.9%, 14.4%, and 7.6% respectively (Figure 4.1.5.2).

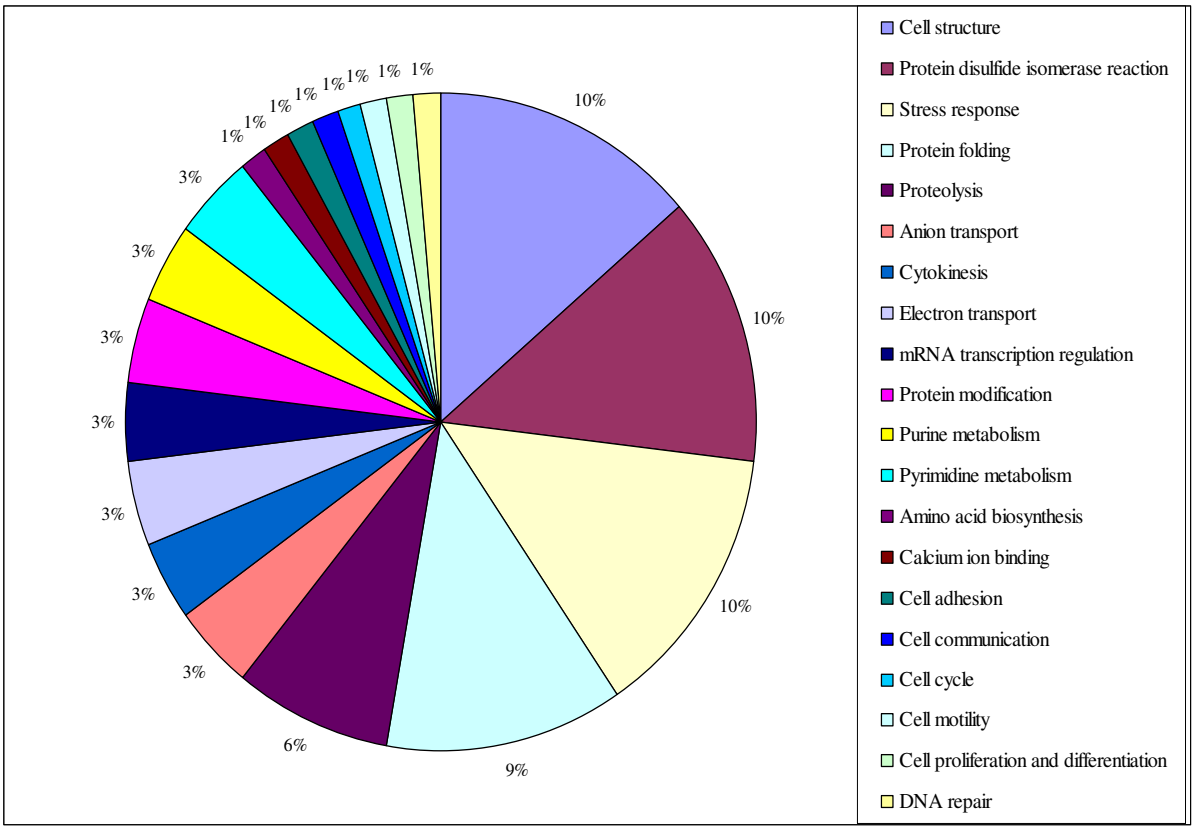


Figure 4.1.5.2. A pie chart demonstrating the percentage of diferentially expressed proteins in each biological function identified in the non-invasive (MCF-10A+MCF-7) vs. invasive (HMEC+BT20) comparison group from 2D-DIGE study of cell lysate, conditioned media and membrane fractions. The information for the biological function was obtained from NCBI database.

4.1.5.3 Cancer-related invasion (BT20 vs. MCF-10A+HMEC+MCF-7)

The identified differentially expressed protein from the comparison of cancer invasive (BT20) vs. normal-invasive+normal non-invasive+cancer non-invasive (HMEC+MCF-10A) group from the cell lysate, conditioned media and membrane fractions were grouped together to investigate the biological functions that are mainly affected in the process of transformation of a normal cell into a cancer invasive cell. Of the identified differentially expressed proteins from this comparison group, the majority of proteins were observed to be involved in the regulation of cell structure (13%) and proteolysis (10%) (Figure 4.1.5.3).

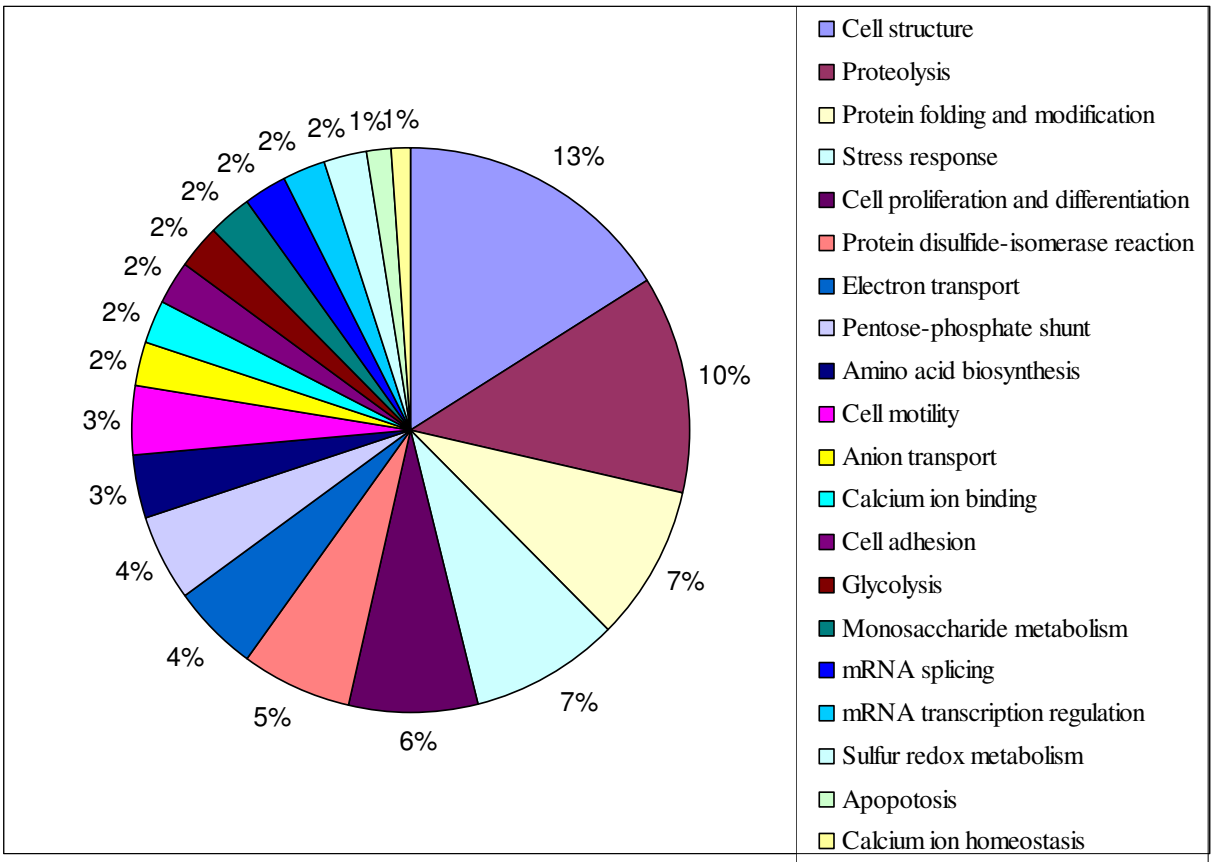


Figure 4.1.5.2. A pie chart demonstrating the percentage of proteins in each biological function identified in the cancer invasive (BT20) vs. normal invasive+normal non-invasive+cancer non-invasive (HMEC+MCF-10A+MCF-7) comparison group from 2D-DIGE study of cell lysate, conditioned media and membrane fractions. The information for the biological function was obtained from the NCBI database.

Our data indicates that the development of the cancerous and invasive phenotypes in breast cells mainly affect the proteins that are involved in the regulation of cell structure, motility and protein metabolism and modification in cells. However, the number of proteins identified here is insufficient for a comprehensive analysis of the main biological functions affected in this system, as we may be observing a bias towards the identification of the more highly expressed metabolic and structural proteins within the cell such as ACTB. However, the data presented is in agreement with the previous published literature as the cell structure and motility, protein metabolism and modification biological functions have already been shown to be the dominantly affected biological function in tumour cells (Zapun *et al.* 1998).

4.1.6 Summary of identified potential targets using proteomics tools

Box-7 summaries the high priority cellular, secreted and membranal protein targets identified in this study. Little information on the role of these protein targets in breast cancer and invasion is available from the literature and these protein targets could possibly be involved in the regulation of cancerous and/or invasive phenotypes in breast cells. The targets identified from the cell lysates and membranal fraction could be further investigated using immunohistochemistry whereas the targets identified from the conditioned media fraction are potential targets to be screened using breast cancer patients and normal sera. The inhibition or over expression studies for these targets in breast cancer cell lines as well as in normal breast cells could further improve the understanding of the process of transformation of a normal cell in to a cancerous as well as invasive phenotype in breast cells and offers potential to identify potential drug targets and/or biomarkers for breast cancer disease.

Box-7: High priority interesting target from 2D-DIGE study			
2D-DIGE study	**Cancer associated**	**Normal+cancer invasion associated**	**Cancer invasion associated**
Cell lysates	EIF4A3, UQCRC11, DCI, SOD1 and SERPINB1	RAD23B	TXNRD1 and 3-PGDH
Conditioned media	PDIA4, SERPINB1, SERPINB7, NUCB1, CTSA and FHC	TALDO1 and GSS	NUCB2 and DDAH1
Membrane fraction	IMMT, FKBP5, UQCRC1 and S100A14	WDR1, RUVBL2 C20ORF3 and GANAB	ATP5B, TALDO1, CES1, RUVBL2 and AHCY

4.2 SELDI-ToF MS analysis of conditioned media from human cell lines

The incidence of melanoma is increasing dramatically in the developed world. However the molecular markers that are being used for melanoma, i.e. MIA, S100β and LDH, are still not satisfactory for detecting melanoma at an early stage of its development. Therefore the discovery of new markers for melanoma diagnosis could lead to the establishment of novel prognosis and diagnosis methods. Melanoma develops from a series of architectural and phenotypically distinct stages and becomes progressively aggressive culminating in metastasis. The development of invasive and metastatic behaviour during cancer progression requires the concerted action of multiple genes and cellular functions, including cytoskeleton remodeling, acquisition of migratory phenotypes, ability to invade the tumour-surrounding tissues, and survival of disseminated tumour cells. One aspect of these complicated processes is that invasion and metastasis may sometimes be related to the multidrug resistance (MDR) phenotype (Winston *et al.* 2001, Thompson *et al.* 2007, Koukourakis *et al.* 2007, Olson and Sahai 2008). A relationship between these two phenotypes has been demonstrated by two types of observation. Firstly, some tumour cells selected for resistance to drugs are more invasive/metastatic relative to non-resistant parental cells (Liang *et al.* 2002). Secondly, in some cases, secondary (more metastatic) tumours are more resistant to chemotherapeutic drugs than their primary counterparts (Liang *et al.* 2002, Liang *et al.* 2001, Haga *et al.* 1997). A better understanding of any relationship between drug resistance and cancer invasion could lead to a more effective melanoma invasion treatment. Therefore, proteomic analysis of conditioned media from melanoma cell line MDA-MB-435S-F and its paclitaxel-resistant (MDA-MB-435S-F/Taxol10p4pSI), doxorubicin-resistant (MDA-MB-435S-F/Adr-10p10p) cell lines were performed using SELDI-ToF MS to find proteins/peptides changes that may be associated with melanoma invasion.

4.2.1 MDA-MB-435 cells are from melanoma origin

MDA-MB-435 has been used for decades as a model of metastatic human breast cancer. This cell line was derived by R. Cailleau in 1976 from a pleural effusion from a 31-year old woman with a history of breast cancer (Cillo *et al.* 1987). In a gene expression analysis of MDA-MB-435 cells in conjunction with 60 other cell lines, it was revealed that the pattern of gene expression for MDA-MB-435 more closely resembled that of melanoma cell lines

than of other breast lines (Cailleau *et al.* 1978). Another expression study showed breast-specific genes were not detectably expressed in MDA-MB-435, however, they expressed melanocyte-specific genes such as RXRG, TYR, ACP5, and DCP (Ross *et al.* 2000). Additionally, MDA-MB-435 cells were found to express breast differentiation-specific proteins and secrete milk lipids as are other well-established breast cancer cell lines, as well as expressing melanocyte-specific proteins, tyrosinase and melan-A (Ellison *et al.* 2002). MDA-MB-435 cells are in fact derived from the melanoma cell line M14 revealed by single-nucleotide polymorphism (SNP) arrays analysis (Sellappan *et al.* 2004). Therefore, MDA-MB-435 are now considered a melanoma cell line.

Previously in NICB, paclitaxel (MDA-MB-435S-F/Taxol10p4pSI) and doxorubicin (MDA-MB-435S-F/Adr-10p10p) selected variants of an *in vitro* invasive clonal population of the human melanoma cancer cell line, MDA-MB-435S-F, were established by pulse selection (Rae *et al.* 2007). MDA-MB-435S-F cells are invasive and the resistance variants, MDA-MB-435S-F/Taxol-10p4p and MDA-MB-435S-F/Adr-10p, were found to be highly invasive.

4.2.1.1 SELDI ToF MS analysis of conditioned media from MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI and MDA-MB-435S-F/Adr-10p10pSI

The secretome of cells and tissues may reflect a broad variety of pathological conditions and thus represents a rich source of information. Large proteins are only present in the conditioned media intact if they are actively secreted by cells. Degradation and cleavage can generate fragments small enough to be easily secreted by cells, producing diagnostic traces. Thus the low-molecular-weight (LMW) region is a mixture of small intact proteins plus fragments of large proteins. This treasure trove of diagnostic information has largely been ignored until now. SELDI-ToF MS has been a promising technology to identify differential expression of low molecular weight proteins/peptides using a very small amount of sample and has been quite successful. It has been used for the analysis of serum, plasma, urine, cell lysates and cellular secretions focusing on the discovery and identification of potential biomarkers for various diseases (Glynn *et al.* 2004). SELDI-ToF MS utilizes the different chromatographic properties of ProteinChip® arrays to bind different subsets of peptides and proteins for subsequent analysis (Ward *et al.* 2008,

Nakamura *et al.* 2006, Caspersen *et al.* 2007, Gourin *et al.* 2007, Furuta *et al.* 2004). Recent reports employing SELDI-ToF MS-based approach have demonstrated that protein profiles are useful for the early detection of breast, prostate and colon cancers (Merchant and Weinberger 2000). Distinct protein profiles of many carcinoma cell lines and associated variants are now established using this technology (Chen and Madura 2006, Li *et al.* 2002, Petricoin *et al.* 2002, Chen *et al.* 2005).

In order to investigate changes in the secretome that might be associated with mechanisms involved in drug resistance and invasion, or for establishing biomarkers associated with the melanoma phenotype, conditioned media from the parental cell line, MDA-MB-435S-F and the drug-selected superinvasive population, MDA-MB-435S-F/Taxol-10p4pSI were subjected to biochemical and proteomic analysis. Initial analysis carried out using the IMAC30-Cu^{2+} ProteinChip arrays demonstrated that the expression level of a 7.6kDa peak in MDA-MB-435S-F/Taxol-10p4pSI cells was increased when compared with MDA-MB-435S-F cells. The 7.6kDa protein was found to be 4-fold greater in the taxol-resistant cells with a statistically significant p-value of < 0.04 (Figure 3.4.2). A time course study showed that the levels of the 7.6kDa protein detectable in conditioned media from MDA-MB-435S-F/Taxol-10p4pSI cells taken at 24hrs, 48hrs and 72hrs respectively, slowly decreases (Figure 3.4.3).

Further analysis performed on the conditioned media from the doxorubicin-resistant variant MDA-MB-435S-F/Adr-10p10pSI cells was used to confirm if the increased expression of 7.6kDa peak is related to taxol-drug resistant specifically or invasion. The 7.6kDa protein/peptide was also found to have a 2.6-fold greater intensity in the doxorubicin-resistant variant MDA-MB-435S-F/Adr-10p10pSI compared to the parental cell line (Figure 3.4.4). This result indicates that the 7.6kDa protein/peptide peak was possibly associated with either invasion or drug-resistance phenotype.

4.2.1.2 Purification and identification of the 7.6kDa peak as a possible fragment of bovine transferrin

Purification and identification of the 7.6kDa peak was performed using conditioned media from the MDA-MB-435S-F/Taxol-10p4pSI population. Firstly the conditioned media was fractionated using BioSepra IMAC-Cu^{2+} HyperCel spin columns, with the 250mM

Imidazole fraction showing the highest concentration of the 7.6kDa protein along with the least amount of contaminating peaks (Figure 3.4.5 A). Secondly the 250mM fraction was run on a 1D gel under reducing conditions. Silver staining of the gel allowed a band at 7.6kDa to be excised. Tryptic digests of the protein was subject to MALDI ToF/ToF analysis and identified as a possible 7.6kDa protein fragment of bovine transferrin (Figure 3.4.5.B).

The transferrins are comprised of a family of large non-heme iron-binding glycoproteins (~77kDa) that are believed to originate with the evolutionary emergence of vertebrates or pre-vertebrates. Serum transferrin, ovotransferrin and lactoferrin are three major types of transferrins that have been characterized. Serum transferrin occurs in blood and other mammalian fluids including bile, amniotic fluid, cerebrospinal fluid, lymph, colostrom, and milk in the body. Serum transferrin has the role of carrying iron from the sites of intake into the systemic circulation to the cells and tissues (Chen *et al.* 2005, Shiwa *et al.* 2003). It is also likely to be involved in the transportation of a wide range of other metal ions other than iron, including therapeutic metal ions, radio diagnostic metal ions, and some toxic metal ions (Morgan 1964). Ovotransferrin (oTf) is found in avian and reptilian oviduct secretions and in avian egg white (Savigni and Morgan 1998), and lactoferrin (Lf) is found in milk, tear, saliva, and other secretions (Jeltsch and Chambon 1982, Williams *et al.* 1982). Transferrin is mainly synthesized by hepatocytes, with a concentration of 2.5 mg/ml and 30% occupied with iron in blood plasma (Baggiolini *et al.* 1970). Another member of the transferrin family called melanotransferrin (also called p97) was identified as an integral membrane protein in human malignant melanoma cells and in some fetal tissues (Leibman and Aisen 1979), although its function is not clear yet.

4.2.1.3 Investigation of protease activity in conditioned media

To further investigate the proteases involve in the truncation of transferrin, gelatin zymography of conditioned media from MDA-MB-435S-F and its variant MDA-MB-435S-F/Taxol10p4pSI was performed. The inhibition of MMPs activity by the use of MMPs inhibitors, EDTA and 1,10 Phenatheoline confirmed the increased protease activity of the MMPs in the highly invasive MDA-MB-435S-F/Taxol10p4pSI cell lines compared to its parent population MDA-MB-435S-F (Figure 3.4.7 A). The two secreted bands observed on

the zymogram were bands corresponding to MMP-2 (approximately 62 kDa) and MMP-9 (approximately 92 kDa). This indicates that melanoma cells are possible active secretors of MMP 2 and MMP 9 proteases which could be involved in invasion, metastasis and tumourigenicity. However this enzymatic cleavage of transferrin may be due to the extracellular proteases secreted into the medium by cells or due to the uptake of bovine transferrin from the culture media followed by intracellular cleavage and secretion of transferrin fragments by cells. The most likely explanation of our finding could be that tumour cells require a higher level of iron intake than normal cells and proteolytic cleavage of bovine transferrin in the presence of proteases enhances rapid release of iron and the ability of melanoma cells to obtain iron from iron chelates. Many reports in the literature on transferrin production relates to the secretion of transferrin by primary or established cell lines (Rose *et al.* 1986). However there is little information on the intracellular level of transferrin protein (Onoda *et al.* 1990, Väisänen *et al.* 2008, Vandewalle *et al.* 1989).

The presence of the fragment of transferrin in conditioned media suggests that transferrin protein is being cleaved due to increased proteolytic activity, possibly due to increased levels of secreted MMP2 and MMP9 in the culture media of melanoma cells. Therefore we were interested to identify possible cleavage sites for MMP 2 and MMP 9 in the full transferrin protein sequence. The analysis of transferrin protein for the presence of potential cleavage motifs for MMP2 and MMP9 using *in-silico* tools indicated that transferrin does not contain any cleavage site for these MMPs. It is likely that these MMPs might be cleaving transferrin using an amino acid signature for cleavage which is not known yet. However based on the molecular weight, we can suspect that these MMPs might be cleaving at 69 residues from the C-terminus of the protein and this could result in the cleaved transferrin fragment with a molecular weight of 7.6kDa (Figure 3.4.9).

4.2.1.4 Role of MMP 2 and MMP 9 in melanoma and invasion

The MMPs belong to a large family of secreted or transmembrane proteolytic enzymes known as the metzincin superfamily. MMPs can process or degrade all kinds of bioactive molecules including extracellular matrix proteins. MMPs can cleave cell surface receptors, release of apoptotic ligands (such as the FAS ligand) and chemokine in/activation, and therefore play a major role in cell behaviours such as cell proliferation, migration

(adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defense. The importance of MMPs during tumour metastasis may be related to their proteolytic activity against type IV collagen, which is a major component of epithelial basement membranes. Most of the MMPs are produced in the form of biologically inactive proenzymes and need to become activated by another enzyme (s) (Anderson *et al.* 1986).

MMP-2 and MMP-9 (gelatinase A and gelatinase B) are involved in the systemic dissemination of tumours. MMP2 has been reported to be over expressed in human melanocytic tumours (Chambers and Matrisian 1997, Nelson *et al.* 2000) and in more invasive melanomas (Hofmann *et al.* 2000, Vaisanen *et al.* 1996 & 1998). The over expression of MMP-2 has been linked to hematogenous metastasis and, thus, to impaired survival in male melanoma patients, suggesting a difference according to sex (Simonetti *et al.* 2002). The over expression MMP-9 strongly correlates with the metastatic capacity of tumours (Vaisanen *et al.* 1998). The level of MMP-9 expression has been shown to be elevated in melanomas and associated with a significantly higher incidence of metastatic disease (O'Grady *et al.* 2007). So far, the role of MMP-2 and MMP-9 as a prognostic factor in melanoma has not been confirmed.

In this study the use of MMP 2 and MMP 9 inhibitors in the culture of MDA-MB-435S-F/Taxol10p4pSI resulted in a decreased mean intensity of the m/z 7653 peak in MMP 9 and MMP2 inhibitor-treated samples in comparison to untreated control samples. This confirmed the increased activity of MMP 2 and MMP 9 in MDA-MB-435S-F/Taxol10p4pSI cells. Although the effect of MMP 2 inhibitor was more intense in inhibiting the cleavage of transferrin compared to MMP 9 inhibitor, both MMPs were found to cleave transferrin protein in culture (Figure 3.4.8). Thus MMP 2 and MMP 9 inhibition indicates that bovine transferrin is possibly being cleaved by extracellular proteases secreted by MDA-MB-435S-F/Taxol10p4pSI into the media.

4.2.1.5 Screening of bovine transferrin fragment (7.6kDa) as a potential biomarker for melanoma

The presence of truncated transferrin in the conditioned medium from the highly invasive cell line indicates the potential involvement of proteases in breaking down the transferrin protein into fragments which could be related to the development of the invasive phenotype

in the cell (El-Shabrawi *et al.* 2001). Therefore the expression of truncated transferrin was further investigated for its specificity in a panel of melanoma, breast or lung cancer cells and for possible mechanisms of proteolytic cleavage of the transferrin protein. The conditioned media from melanocytes (NHEM), normal mammary cells (HMEC) and melanoma (MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI, MDA-MB-435S-F/Adr-10p10pSI, M14, HT144, SKMEL-28, SKMEL-5, Malme-3M and Lox-IMVI), breast (MCF-7 and BT20), and lung (DLKP and H1299) cancer cell lines were profiled using SELDI-ToF MS and mass spectrometry to further investigate the expression of truncated transferrin as a potential biomarker for cancer. The truncated transferrin was observed to be expressed in all melanoma cell lines except HT144 but not expressed in the conditioned media from melanocytes, normal mammary cells and breast, and lung cancer cell lines (Figure 3.4.10).

The source of bovine truncated transferrin could be suspected to be derived from the cultivation of cell lines as the cells were grown in 10% FCS supplemented culture medium. The method used for the generation of samples in this study is a common procedure and has been used by other research laboratories for generating serum-free conditioned media for SELDI-ToF MS analysis (Dowling *et al.* 2007a). The sample preparation for all cell lines was also the same, even though the 7.6kDa peak was observed only in the melanoma cell lines (Figure 3.4.10.1). Moreover in another independent study in this laboratory, SELDI-ToF MS profiles were generated for 0.5% FCS supplemented cell-free culture media at 37^0C over the period of 6 days in CHO cells and no peak at m/z 7653 was observed in these culture conditions (Ito *et al.* 2007, Sato *et al.* 2001).

To see if there was any correlation between the levels of the 7.6kDa protein fragment with invasion status of the melanoma cell lines, *in vitro* 48 hours invasion assay were performed on melanocytes (NHEM) and the melanoma cell lines MDA-MB-435S-F, MDA-MB-435S-F/Taxol10p4pSI, MDA-MB-435S-F/Adr-10p10pSI, M14, HT144, SKMEL-28, SKMEL-5, Malme-3M and Lox-IMVI. The levels of expression of the 7.6kDa bovine transferrin fragment in melanoma cell lines and their invasion status does not shown any correlation. SKMEL-5 is a non-invasive cell line and a high level of the 7.6kDa bovine transferrin fragment was observed in SELDI-ToF analysis of the conditioned media from the cell lines. Malme 3M is invasive but was found to express low levels of 7.6kDa peak in

the conditioned media. Also melanocytes are invasive in nature and there was no expression of the 7.6kDa peak in the conditioned media (Firgure 3.4.11).

4.2.1.6 Expression of transferrin receptor in melanocytes and melanoma cell lines

Western blot analysis of whole cell lysates from both melanocytes and MDA-MB-435S-F/Taxol10p4pSI phenotypes using an antibody against the transferrin receptor showed a marked increase in the abundance levels of the receptor in MDA-MB-435S-F/Taxol10p4pSI cells. The transferrin receptor was found to be approximately 35.9-fold increased in the melanoma cell line MDA-MB-435S-F/Taxol10p4pSI compared to the parental cell line (Figures 3.4.12A). This 35.9-fold increase in the receptor possibly corresponds to a 34.2-fold increase in the transferrin fragment identified by SELDI-ToF MS analysis found in the MDA-MB-435S-F/Taxol10p4pSI conditioned media compared to melanocytes (Figure 3.4.12B). The transferrin receptor was also found to be approximately 3.5-fold increased in the MDA-MB-435S-F/Taxol10p4pSI compared to the parental cell line MDA-MB-435S-F. This 3.5-fold increase in the receptor possibly corresponds to a 4-fold increase in the transferrin fragment identified by SELDI analysis found in the MDA-MB-435S-F/Taxol10p4pSI conditioned media (Figure 3.4.13C).

4.2.1.7 Potential mechanism of transferrin receptor-mediated cellular iron uptake in melanoma cells

Transferrin receptor (TfR1) is a carrier protein for transferrin. It is a transmembrane homodimer that consists of two identical monomers with a molecular mass of approximately 90 kDa; each monomer is joined by two disulfide bonds (Kumar *et al.* 2008). Acquisition of iron in higher organisms is mainly by the receptor mediated transferrin-bound iron (Jing and Trowbridge 1987). After binding to its receptor on the cell surface, transferrin (Tf) is rapidly internalized by invagination with the formation of endocytic vesicles (Figure 4.4.4A). Briefly, the process is triggered by the binding of Fe-Tf to a specific cell-surface TfR1 (Andrews 1999). After endocytosis, which bud from the plasma membrane as membrane bound vesicles or endosomes, the Fe-Tf·TfR1 complex is routed into the endosomal compartment. Upon maturation, the endosome becomes competent to pump protons in a process energized by ATPase, and the endosomal lumen is rapidly

acidified to a pH of about 5.5 (Morgan 1964, Morgan and Appleton 1969). At this pH, the binding of iron to Tf is weakened, leading to iron release from the protein. The free Fe^{3+} released to endosomes and reduced to Fe^{2+} on the *cis*-side of the endosomal membrane probably mediated by oxidoreductase (Dautry-Varsat *et al.* 1983, Paterson *et al.* 1984). Fe^{2+} is subsequently transported out of the Tf cycle endosome by the divalent metal transporter DMT1, i.e., from the endosomal membrane to the cytosol (Neuhoff *et al.* 2004). After release of iron into the endosome, the resultant apo-Tf·TfR1 complex is then recruited through exocytic vesicles back to the cell surface. At extracellular physiological pH, apo-Tf dissociates from its receptor due to its low affinity at pH 7.4, is released into the circulation, and reutilized (Fleming *et al.* 1998, Tabuchi *et al.* 2000). ATP-mediated energy is necessary for sustaining TfR-mediated endocytosis and recycling (Morgan 1996 & 2001, Qian *et al.* 1997 & 2002).

The present study indicates that the transferrin protein (704 a.a long protein) is possibly being cleaved by secreted MMPs (MMP2 and MMP9) as a 7.6kDa fragment of transferrin and was observed to be increased in the melanoma cell lines. This suggest that MMPs secreted by melanoma cells could potentially be cleaving transferrin at the ~635 a.a. position and releasing a fragment of ~69 a.a. (7.6kDa) from the C-terminus of the transferrin from the Fe-Tf·TfR1 complex (Figure 4.4.4B). The 7.6kDa fragment was observed in serum-free conditions because the cells were initially cultured in serum-supplemented medium from where the transferrin could have bound to the TfR1. Cells may retain Fe-Tf.TfR1 complex on the cell surface and therefore the presence of bovine transferrin in serum-free culture condition is not unexpected. The transfer of cells to serum-free conditions possibly resulted in the release of the 7.6kDa fragment into serum-free medium where this fragment was detected by SELDI-ToF MS due to enrichment of cell secreted proteins as well as reduced hindrance from serum proteins in the culture. However the possible reason for cleavage of transferrin fragment from the Fe-Tf·TfR1 complex is not clear from this present study. It could be expected to facilitate the efficient internalization of the Fe-Tf·TfR1 complex and/or release of iron into the melanoma cells. The increased expression of transferrin recepotors with transferrin fragment in melanoma cell lines strengthens this hypothesis. Once in the cytosol, iron is utilized as a cofactor for aconitase, the cytochromes, RNA reductase, and heme, or stored as ferritin.

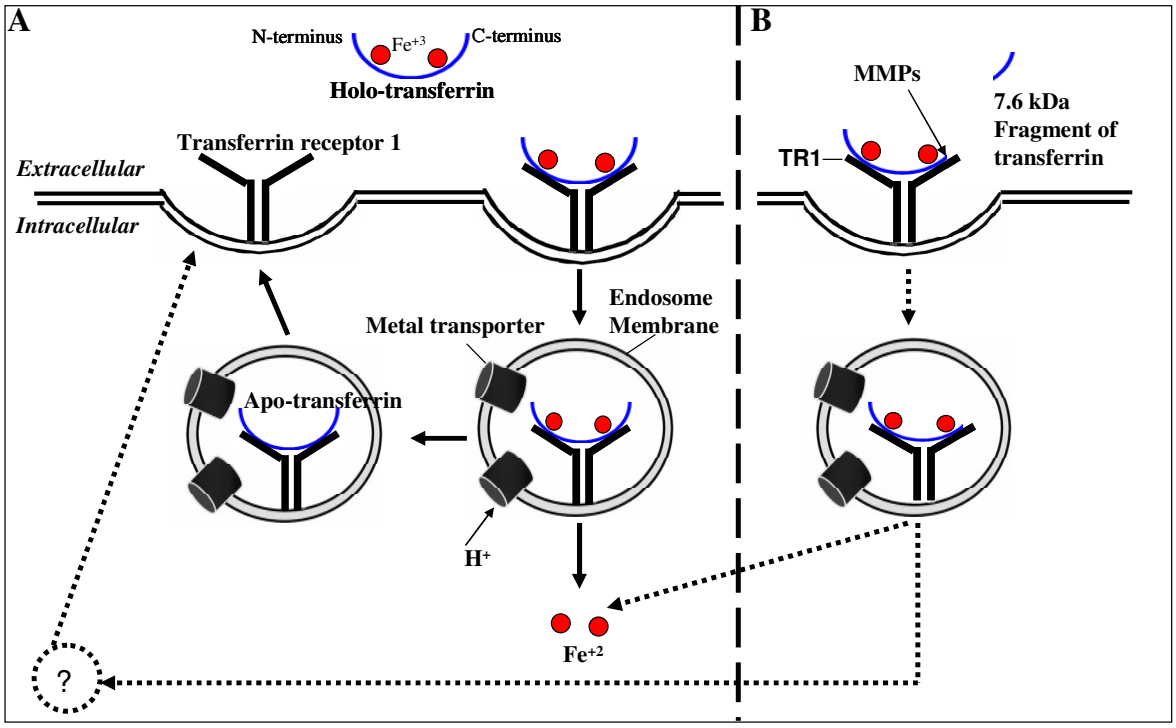


Figure 4.4.4 The cycle of transferrin and transferrin receptor 1-mediated cellular iron uptake. **A. Classical Pathway:** Differic transferrin (holo-transferrin) binds to transferrin receptor on the cell surface. The resulting complex is internalized and acidified through the action of a proton pump. Iron is subsequently released from transferrin and transported out of endosomes via the divalent metal transporter DMT1. Apo-transferrin (transferrin without iron) and transferrin receptor 1 both returned to the cell surface, where they dissociate at neutral pH, and both participate in another round cycle of iron uptake. Intracellular iron is either incorporated into heme or stored in ferritins (Podbilewicz and Mellman 1990). **B. Hypothesis for transferrin cleavage:** Once holo-transferrin binds to the transferrin receptor 1 in melanoma, MMPs (MMP2 and MMP9) possibly cleave holo-transferrin at the c-terminus releasing a fragment of 7.6kDa in to the culture medium. However the biological significance of transferrin cleavage has not been established, it could be associated with more efficient internalization or release of iron into the melanoma cells.

Transferrins facilitate extracellular iron storage and transport in culture medium. Almost all cells require transferrin for growth and therefore human cells cultured in fetal calf serum supplemented culture medium could acquire iron, and other trace elements that may be carried by transferrin from the culture medium. However few studies have suggested that bovine transferrin does not bind to the human transferrin receptor and therefore fails to deliver iron to human cells (Qian *et al.* 2002). Others have shown, using competition studies, that bovine transferrin probably does bind to the human transferrin receptor, but only weakly (Sephton and Kraft 1978). Since almost all cells require transferrin for growth (Tsavaler *et al.* 1986), the absence of binding of bovine transferrin would imply that human cells cultured in fetal-bovine serum may be able to acquire iron and other trace elements that may be carried by transferrin, by a mechanism other than the normal receptor-mediated endocytosis of transferrin. The inability of bovine transferrin to bind to human cells could be due to a simple species difference in affinity for the receptor, but an alternative explanation could be that the internal proteolytic cleavage, which can occur in bovine transferrin (Barnes and Sato 1980), could decrease the binding affinity. Transferrins are important extracellular antioxidants and they bind iron so tightly under physiological conditions that virtually no free iron exists to catalyze the production of free radicals.

In summary, the most likely explanation of our finding from this study, could be that tumour cells acquire a higher level of iron intake than normal cells and proteolytic cleavage of transferrin in the presence of proteases enhances rapid release of iron and the ability of melanoma cells to obtain iron from these iron chelates. Increased expression levels of transferrin receptors could explain the increased abundance of the truncated bovine transferrin in melanoma cell lines compared to melanocytes. However future studies would need to check the specificity of the 7.6kDa bovine transferrin fragment in a wide range of cell lines from different tissue types and validation of its expression in sera from melanoma patients and normal controls.

4.1.2 SELDI-ToF MS of conditioned media from melanocytes and melanoma cell lines

Melanoma is a malignant tumour arising from melanin producing cells known as melanocytes. Identification of differentially expressed proteins/peptides could be helpful to understand the process of transformation of melanocytes into melanoma. This is the first

study of profiling of conditioned media from melanocytes and melanoma using SELDI-ToF MS. There is no published literature on conditioned media from melanocytes and melanoma cell lines using SELDI-ToF MS to the best of our knowledge. However limited studies have been mentioned in literature on melanoma serum samples using SELDI-ToF MS which as been discussed in section 1.8 (Maeda *et al.* 1980).

4.1.2.1 Expression differential mapping

To investigate the differentially expressed proteins/peptide between normal melanocytes and melanoma cell lines, serum-free conditioned media from melanoma melanocytes (NHEM) and melanoma (M14, HT144, SKMEL-28, SKMEL-5, Malme-3M and Lox-IMVI) were profiled using SELDI-ToF MS on the IMAC-Cu^{++} surface. Four differentially expressed proteins/peptides at m/z 7406, m/z 8584, m/z 11959 and m/z 12081 were observed which were significantly differentially expressed in melanocytes and melanoma cell lines (section 3.5.1.1, 3.5.1.2 and 3.5.1.3).

The m/z 7406, m/z 8584, m/z 11959 and m/z 12081 peaks were dominantly expressed in all melanoma cell lines (HT144, Lox-IMVI, M14, Malme-3M, SKMEL-5 and SKMEL-28) compared to melanocytes, although the expression levels of each peak in each melanoma cell lines were different. There was no correlation seen in the expression levels of all four differentially expressed peaks and the invasion status of the cell lines. Therefore the data indicates that these proteins/peptides could be involved in the transformation of melanocytes to melanoma. We successfully identified the m/z 8584 peak as ubiquitin protein but we were not successful in identifying the 3 remaining peaks (m/z 7406, 11959 and 12081).

4.1.2.2 Ubiquitin

Ubiquitin, a 76-amino-acid-residue protein, is present in the cytoplasm and nucleus of eukaryotic cells and is one of the most highly evolutionarily conserved proteins. Ubiquitin acts through the ubiquitin/proteasome pathway by labeling a wide variety of proteins by a covalent ligation leading the resulting ubiquitinated proteins to rapid degradation. Ubiquitin-mediated proteolysis, a major pathway for selective protein degradation, plays a variety of regulatory roles in cellular processes, including cell cycle, apoptosis,

transcription, protein trafficking, signaling, DNA replication and repair, and angiogenesis (Shen *et al.* 2002). The complete ligation mechanism requires the sequential action of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) (Mani and Gelmann 2005, Chen *et al.* 2006, Hoeller *et al.* 2006). The resulting covalent ubiquitin ligations form polyubiquitinated conjugates that are rapidly detected and degraded by the 26S proteasome complex. Then the substrates are hydrolyzed to produce small peptides, and ubiquitin is spared from degradation through its release from the substrates by deubiquitinating enzymes.

Aberrations in the mechanisms of ubiquitin-mediated proteolysis mechanisms in cancer cells include inhibition of ubiquitination and proteolysis of proteins that promote cell growth and survival as well as accelerated ubiquitination and degradation of proteins that suppress tumour growth and induce apoptosis. Despite the approval of a first drug of this class (bortezomib) by the US Food and Drug Administration for the treatment of relapsed or refractory multiple myeloma and its current testing in clinical trials for the treatment of a wide variety of malignancies (Hershko and Ciechanover 1998), other specific targets of the ubiquitin system remain to be identified and tested.

Ubiquitin, which can conjugate with cellular proteins, is classified into two forms: free ubiquitin and multiubiquitin chains. Ubiquitin exists *in vivo* as free ubiquitin prior to ubiquitination and as multiubiquitin chains after functional modification or degradation. Higher level of multiubiquitin chains and free ubiquitin has been found in the serum of rheumatoid arthritis and hemodialysis patients compared to healthy subjects (Adams 2002). A few reports have mentioned higher levels of cellular ubiquitin in various cancers (Takada *et al.* 1997, Ishibashi *et al.* 2004), however the form of ubiquitin was not measured. The immunoassays to detect free and/or multiubiquitin chains has recently been developed which could allow quantification of free ubiquitin and multiubiquitin form of ubiquitin in serum (Ishibashi *et al.* 2004, Kanayama *et al.* 1991, Osada *et al.* 1997). Both forms of ubiquitin (free ubiquitin and multiubiquitin chains) have been observed highly expressed in colon cancer tissue compared to normal tissue (Takada *et al.* 1997). The expression of secreted ubiquitin was observed to be increased in all of melanoma cell lines compared to melanocytes in this investigation. However, no correlation was observed between the invasion status of the cell lines and the expression pattern of ubiquitin. The peak for

ubiquitin in this study was observed at m/z 8584 (8.5kDa) which is the molecular weight of single ubiquitin suggesting that it could be the free form of ubiquitin secreted by melanoma cell lines in conditioned media. Over-expression of secreted ubiquitin in conditioned media of melanoma cells could indicate possibly increased ubiquitin synthesis and ubiquitin-dependent proteolysis which could possibly be related to the metabolism and catabolism of proteins that are involved in cellular proliferation.

Section 5

Summary and conclusions

5.1 Proteomic analysis of HMEC, MCF-10A, MCF-7 and BT20 cells

Comparative protein profiles for cellular, secreted and membrane & membrane-associated proteins from invasive normal mammary epithelial cells (HMEC), non-invasive and non-tumourigenic immortalized mammary epithelial cells (MCF-10A), non-invasive breast cancer cells (MCF-7) and invasive breast cancer cells (BT20) were generated using 2D-DIGE. These protein profiles were compared in the following three groups in order to identify proteins up-regulated or down-regulated with the cancerous and invasive status of the cell lines.

1. Cancer (MCF-7+BT20) vs. normal (MCF-10A+HMEC)
2. Invasive (HMEC+BT20) vs. non-invasive (MCF-10A+MCF-7) **(Normal+ cancer-related invasion)**
3. Cancer invasive (BT20) vs. normal invasive, normal non-invasive and cancer non-invasive (HMEC+ MCF-10A+MCF-7) **(Cancer-related invasion)**

5.1.1 Differential protein profiling of cell lysates

5.1.1.1 Cancer vs. normal (MCF-7+BT20 vs. HMEC+MCF-10A)

- A total of 44 proteins were identified, of which 27 were up-regulated and 17 down-regulated in cancer cell lines.
- A number of proteins that are already known to be associated with cancer such as annexin 1 (ANXA1) and maspin (SERPINB5) were observed to be down regulated in cancer cell lines and are in agreement with the literature.
- Five cellular proteins, EIF4A3, UQCRC1, DCI, SOD1 and SERPINB1, which have limited information on their possible involvement in breast cancer, were also observed to be differentially regulated in this comparison. EIF4A3, UQCRC1, DCI and SOD1 were up-regulated whereas SERPINB1 was down-regulated in cancer cell lines.

5.1.1.2 Normal+ cancer-related invasion (HMEC+BT20 vs. MCF-10A+MCF-7)

- A total of 15 differentially expressed proteins (15 up-regulated and 8 down-regulated in the N+C invasive cells) were identified from this comparison group. This enabled us to

identify proteins which may be involved in the regulation of invasion in cancer as well as normal cells.

- This comparison identified a number of proteins that are well known to be involved in the regulation of the cancer invasion process. For example, non-metastatic cells 1 (NME1) and keratins 8 (KRT8) and 18 (KRT18). NME1 was up-regulated whereas KRT8 and KRT18 were down-regulated in N+C invasive cells.
- A number of proteins that have not been reported previously as being invasion-associated, such as proteasome activator subunit 1 isoform 2 (PSME1), nuclear chloride channel 1 (CLIC1) and RAD23B, were also identified in this comparison and were found up-regulated in N+C non-invasive cells.

5.1.1.3 Cancer-related invasion (BT20 vs. HMEC+ MCF-10A+MCF-7)

- A total of 25 differentially regulated proteins, 16 up-regulated and 9 down-regulated in BT20, were identified from this comparison. This enabled us to identify proteins which may be involved in the regulation of the invasion process specifically in cancer cells.
- A number of proteins were identified that have been already shown to be involved in the regulation of invasion in cancer cells, for example, gelsolin (GSN) and heterogeneous nuclear ribonucleoprotein (HNRPK). Both were found down-regulated in BT20.
- This comparison revealed a number of differentially expressed proteins for which limited information is available in regard of their role in the regulation of cancer-related invasion. These include thioredoxin reductase 1 (TXNRD1), 3-phosphoglycerate dehydrogenase (3-PGDH), endoplasmic reticulum protein 29 precursor (ERp29), transaldolase (TALDO1), coactosin-like protein (CLP) and quinolinate phosphoribosyltransferase (QPRT). TXNRD1, 3-PGDH, TALDO1, CLP and QPRT were up-regulated whereas ERp29 was down-regulated in BT20.

5.1.1.4 Functional validation

- Western blot analysis was carried out on a panel of 6 selected cancer invasion associated proteins (TXNRD1, 3-PGDH, ERp29, TALDO1, CLP and QPRT) and 3 normal+cancer invasion-associated proteins (PSME1, CLIC1 and RAD23B) using a panel of invasive and non-invasive breast cancer cell lines. From the analysis, TXNRD1 and 3-PGDH

were observed to up-regulated in the cancer-invasive cell lines compared to the normal-non-invasive, normal-invasive and cancerous-non-invasive cell lines except UACC-812. RAD23B was observed to be up-regulated in the non-invasive cell lines compared to the invasive cell lines. This suggests that TXNRD1, 3-PGDH and RAD23B may have roles in the regulation of invasion process in breast cells and therefore were selected as candidates for further functional investigations using siRNA knockdown.

- TXNRD1 was observed to be 4.6-fold over-expressed in the cancer invasive cell lines (BT20) in comparison to normal invasive+normal non-invasive+cancer non-invasive (HMEC+MCF-10A+MCF-7) cells. TXNRD1 has been reported to be involved in the regulation of a variety of biological processes including tumourigenesis, invasion, metastasis and resistance to therapy in cancer. However, the mechanisms are still poorly understood. A significant reduction in the invasion status of SKBR-3 and MDA-MB 231 was observed following reduction of TXNRD1 protein levels. This suggests that TXNRD1 is actively involved in the regulation of the invasion process in breast cancer cells and therefore could be a potential therapeutic target for breast cancer.

- RAD23B is involved in the nucleotide excision repair (NER) process. RAD23B was 2-fold up-regulated in the normal and cancer invasive cell lines (HMEC+BT20) compared to normal and cancer non-invasive cells (MCF-10A+MCF-7). siRNA knockdown studies were performed using one normal and one cancer non-invasive cell line (MCF-10A and MCF-7). The inhibition of RAD23B increased the invasion status of both cell lines and established RAD23B as a potential target to regulate the process of invasion in breast normal and cancer cells.

5.1.2 Differential protein profiling of conditioned media

5.1.2.1 Cancer vs. normal (MCF-7+BT20 vs. HMEC+MCF-10A)

- A total of 42 differentially expressed secreted proteins were identified in this comparison, of which 25 up-regulated and 18 down-regulated in cancer cells.
- Five members of the SERPIN family (SERPINA1, SERPINB1, SERPINB5, SERPINF1 and SERPINB7) were found down-regulated in the cancer cell lines. Of these,

SERPINA1, SERPINF1 and SERPINB5 are well known, whereas SERPINB1 and SERPINB7 are still unexplored for their potential role in the breast cancer.

- Three members of the cathepsin family, pro-cathepsin A (CTSA), pro-cathepsin B (CTSB) and pro-cathepsin D (CTSD) were found down-regulated in cancer cell lines. CSTB and CSTD are already known to be involved in the regulation of cancer, however a role for CTSA in breast cancer is not known.
- Some other protein that have not been previously linked with cancer disease, such as Nucleobindins-1 (NUCB1), Nucleobindins-2 (NUCB2), Ferritin heavy chain polypeptide (FHC) and Protein disulfide isomerase family A4 (PDIA4), were found up-regulated in cancer cell lines.

5.1.2.2 Normal+ cancer related invasion (HMEC+BT20 vs. MCF-10A+MCF-7)

- A total of 15 differentially expressed secreted proteins, 14 up-regulated and 1 down-regulated in the normal+cancer invasive compared to the N+C non-invasive, were identified.
- Some of the identified proteins, such as Heat shock protein 90 (HSP90) and Heat shock protein 5 (HSP5), are well known to be involved in cancer invasion from the literature, were found up-regulated in N+C invasive cells.
- Two proteins which are not previously reported as associated with invasion, Transaldolase-1 (TALDO1) and Glutathione synthetase (GSS), were found to be up-regulated in the normal and cancer invasive cell lines.

5.1.2.3 Cancer related invasion (BT20 vs. HMEC+ MCF-10A+MCF-7)

- A total of 27 differentially expressed secreted proteins, 19 up-regulated and 8 down-regulated in BT20, were identified.
- Some of proteins, such as Protein disulfide-isomerase A3 (PDIA3) and Plastin 2 (LCP1), have been previously reported to be involved in cancer invasion in the literature. PDIA3 and LCP1 were found up-regulated in cancer invasive cell line (BT20).
- Two secreted proteins, which have not been previously reported to be associated with invasion, such as Dimethylarginine dimethylaminohydrolase 1 (DDAH1) and

Nucleobindin 2 (NCUB2), were found to up-regulated in the cancer invasive cell line (BT20).

5.1.3 Differential protein profiling of membrane fraction

5.1.3.1 Cancer vs. normal (MCF-7+BT20 vs. HMEC+MCF-10A)

- A total of 41 differentially expressed membrane and membrane-associated proteins (11 up-regulated and 30 down-regulated) were identified in the comparison of cancerous vs. normal cell lines.
- A total of 7 mitochondrial membrane and membrane-associated proteins; Mitochondrial Rho GTPase 2 (RHOT2), mitofilin (IMMT), stress-70 protein, mitochondrial precursor (HSP9), ATP synthase subunit beta, mitochondrial precursor (ATP5B), ubiquinol-cytochrome c reductase core protein I (UQCRC1), prohibitin (PHB) and diablo homolog, mitochondrial precursor (DIABLO), were observed to be differentially regulated in this comparison.
- Of these, HSPA9, PHB and DIABLO are well known for their involvement in the cancer, whereas RHOT2, IMMT, ATP5B, and UQCRC1 are still unexplored for their potential role in breast cancer. RHOT2, IMMT, HSPA9, ATP5B and UQCRC1 down-regulated whereas PHB and DIABLO were up-regulated in cancer cell lines
- Five plasma membrane and membrane-associated S100 proteins, S100A2, S100A6, S100A9, S100A14 and S100A16 were found down-regulated in the cancer cell lines. Member of the S100 protein family have been reported to be differentially expressed in breast cancer, however very limited information is available for S100A14 for its potential involvement in the regulation of breast cancer and invasion.
- FK506 binding protein 5 (FKBP5), a FK506 binding protein with peptidyl-prolyl isomerase (PPIase) activity which have not been previously reported to be associated with breast cancer, was observed down-regulated in the cancer cell lines.

5.1.3.2 Normal+ cancer-related invasion (HMEC+BT20 vs. MCF-10A+MCF-7)

- A total of 27 differentially expressed membrane and membrane-associated proteins (21 up-regulated and 6 down regulated in normal+cancer invasive cells) were identified.

- Some of mitochondrial membrane and membrane-associated proteins were observed to be differentially regulated. These proteins include some proteins that are already known, such as 60 kDa heat shock protein 1, mitochondrial precursor (HSPD1), and some other proteins that are poorly known, such as NADH dehydrogenase (ubiquinone) Fe-S protein 1, precursor (NDUFS1), for their involvement in the regulation of breast cancer invasion. NDUFS1 was down-regulated and HSPD1 was up-regulated in N+C invasive cells.
- Two members of the S100 family, S100A6 and S100A16, which are known to be involved in the regulation of cancer and invasion, were observed to be differentially regulated. S100A16 was observed to be down-regulated whereas S100A9 was observed to be up-regulated in the N+C invasive cells.
- A number of other proteins, such as Alpha glucosidase II alpha subunit isoform 2 (GANAB), RuvB-like-2 (RUVBL2), WD repeat-containing protein 1 (WDR1) and Adipocyte plasma membrane-associated protein (C20ORF3), which have not been previously linked with invasion, were observed to be differentially regulated in this comparison. GANAB, WDR1 and RUVBL2 were found to be up-regulated whereas C20ORF3 was down-regulated in the N+C invasive cells.

5.1.3.3 Cancer-related invasion (BT20 vs. HMEC+ MCF-10A+MCF-7)

- A total of 35 membrane and membrane-associated proteins were identified, of which 25 proteins were up-regulated and 10 were down regulated in the cancer invasive cell line (BT20).
- A number of mitochondrial proteins were also observed to be differentially regulated. These include some proteins that are known such as PHB and some other proteins that are not known such as ATP5B to be involved in breast cancer invasion in the literature. PHB and ATP5B were observed to be up-regulated in the cancer invasive cell line (BT20).
- From the S100 protein family, some members that are well known, such as S100A2, and some other members that are poorly known such as S100A14 for their involvement in the regulation of breast cancer invasion were differentially expressed. S100A14 and S100A2 were observed to be up-regulated in the cancer invasive cell line (BT20).

- A number of other proteins which have not been previously linked with invasion, such as RuvB-like-2 (RUVBL2), Liver carboxylesterase 1 precursor (CES1), Transaldolase-1 (TALDO1) and Adenosylhomocysteinase (AHCY), were up-regulated in the cancer invasive cell line (BT20).

5.2 Identification of truncated transferrin and ubiquitin as potential biomarkers for melanoma using SELDI-ToF MS

- Conditioned media from drug resistant and highly invasive variants (paclitaxel-resistant cell lines MDA-MB-435S-F/ Taxol10p4pSI and doxorubicin-resistant cell line MDA-MB-435S-F/Adr-10p10pSI) of the melanoma cell line MDA-MB-435S-F were profiled using SELDI-ToF MS. A protein/peptide species at m/z 7653 was found to be 4-fold up-regulated in MDA-MB-435S-F/ Taxol10p4pSI and 2.6 fold up-regulated in MDA-MB-435S-F/Adr-10p10pSI compared to the parent cell line, MDA-MB-435S-F, suggesting that it may be associated with drug resistance and/or the invasive potential of these cells. This protein was identified by MALDI-ToF/ToF as a fragment of bovine transferrin.
- Gelatin zymography analysis of conditioned media from MDA-MB-435S-F and MDA-MB-435S-F/Taxol10p4pSI revealed increased activity of Matrix metalloproteinases (MMPs) in MDA-MB-435S-F/Taxol10p4pSI. Further investigation in MDA-MB-435S-F/Taxol10p4pSI confirmed that MMP 2 and MMP 9 secreted by MDA-MB-435S-F/Taxol10p4pSI were cleaving the transferrin protein in culture medium. Although both MMPs were involved in the cleavage of transferrin, MMP 2 was more intense in activity than MMP 9.
- The transferrin fragment was also investigated for its specificity as a melanoma biomarker in conditioned media from melanocytes, melanoma, normal epithelial, breast and lung cancer cell lines with invasive and non-invasive phenotype using SELDI-ToF MS. This protein fragment was specifically expressed in melanoma cell lines (SKMEL-28, SKMEL-5 Malme-3M, LOX IVI and M14), except HT144. No correlation was observed for the transferrin fragment in respect of the invasive phenotype of the melanoma cells. Our study suggests that the cleavage of transferrin could possibly serve as a potential biomarker for melanoma.

- Based on a SELDI ToF-MS screening, four other peaks (7.4, 8.5, 11.9 and 12 kDa) were also observed differentially expressed in the conditioned media of the melanoma cell lines compared to melanocytes. An 8.5kDa peak was identified as ubiquitin using MALDI-ToF MS. Ubiquitin is the central component of the ubiquitin-proteasome protein degradation pathway which has emerged as an important mechanism in regulating cell growth and survival.

Future work

- TXNRD1 has been shown to reduce the invasion of invasive breast cancer cell lines, SKBR3 and MDA-MB 231. Over-expression of TXNRD1 (stably or transiently) in non-invasive or low invasive breast cancer cell lines would lead to a further understanding of the involvement of TXNRD1 in the process of invasion in breast cancer.

- RAD23B induced invasion of normal and cancer non-invasive breast cells (MCF-7 and MCF-10A) in our study. The investigations mainly focused on cell invasion and other functional analyses such as adhesion, anoikis, motility and colony formation in soft agar could be carried out to determine functional involvement of RAD23B in invasion of breast cells. Over-expression (stably or transiently) of the RAD23B protein in invasive breast cell lines would further confirm its role in the regulation of invasion in breast cells.

- The analysis of differential expression of proteins between the cells expressing (stably) TXNRD1 and RAD23B with their respective controls using 2D-DIGE could enable the identification of proteins that are associated with TXNRD1 and/or RAD23B in the process of altering invasion in breast cells.

- The ultimate aim of this research is to contribute towards the identification of biomarkers and drug targets for breast cancer treatment. Therefore investigation of the expression of TXNRD1 and RAD23B in patients' tissue from different stages of breast cancer could disclose the prognostic value and involvement of these targets in invasion in breast cancer.

- A number of protein targets from the cell lysate and membrane study were narrowed down on the basis of their expression pattern observed using 2D-DIGE and the published literature and these targets could be important for early detection of breast cancer and therapeutic development. It will be useful to determine the expression of

these protein targets in breast cancer patient tissue sections in comparison to the normal tissue using immunohistochemistry. These targets include

1) Lysates study- EIF4A3, UQCRC1, DCI, SOD1 and SERPINB1

2) Membrane study- IMMT, UQCRC1, S100A14 and FKBP5

- Some protein targets from the conditioned media and membrane studies, which have very limited information for their role in breast cancer, were observed to be differentially regulated in the comparison of the invasive cells (HMEC+BT20) with the non-invasive cells (MCF-10A+MCF-7). It would be interesting to see if these targets are capable of inducing/reducing invasion following siRNA knockdown and/or cDNA over-expression studies in normal and cancer cell lines. These proteins include

  1) Conditioned media study- TALDO1 and GSS

  2) Membrane study- WDR1, RUVBL2, C20PRF3 and GANAB

- A number of protein targets from the conditioned media and membrane studies which have very limited information for their role in breast cancer invasion were observed to be differentially regulated in the comparison of the cancer invasive cell line (BT20) with invasive normal+ non-invasive normal+ non-invasive cancerous cells (HMEC+MCF-10A+MCF-7). Therefore further functional studies (siRNA knockdown and over-expression) in cancer cell lines can confirm the involvement of these proteins in breast cancer.

  1) Conditioned media study- DDAH1 and NUCB2

  2) Membrane study- ATP5B, RUVBL2, TALDO1, CES1 and AHCY

- From the conditioned media study a number of targets were identified as potentially of great interest on the basis of their expression pattern observed using 2D-DIGE and the published literature. Investigation of the expression of PDIA4, SERPINB1, SERPINF1, CTSA, NUCB1 and FHC proteins changes in serum samples from breast cancer patient and healthy controls and TALDO1, GSS, DDAH1 and NUCB2 of serum samples from non-metastatic breast cancer patients and metastatic breast cancer using western blotting could establish their value as potential biomarkers for breast cancer and invasion.

- A few proteins such as PDIA3, TALDO1, NUCB1, FKBP5 and RUVBL2 protein were identified from more than one spot on the 2D gel and this could be due to post-translational modification. Therefore further identification of these proteins using advance mass-spectrometry could enable to confirm the type of modification and potential involvement of these proteins in signalling pathways.

- The list of proteins of interest narrowed down using over expression, inhibition and/or immunohistochemistry studies could be investigated for their potential cellular localization and/or biological function using fluorescent protein fusion tagging based microscopy methods.

- A truncated transferrin was observed secreted from the melanoma cells using SELDI-ToF MS analysis of a panel of normal and cancerous cell lines. The inhibition of protease inhibitor activity in culture suggested that the transferrin protein was being cleaved by MMP 2 and MMP 9 in melanoma cell lines. Further validation of the transferrin fragment using melanoma patient serum will establish the clinical relevance of secreted truncated-transferrin in the discovery of potential biomarkers as well as drug targets for breast cancer treatment.

- Transferrin receptor were found to be differentially expressed in melanocytes and two melanoma cell lines and correlated with the expression levels of 7.6kDa species. Therefore further analysis of expression of transferrin receptor in a panel of melanoma cells lines could be helpful to establish the relationship between the expression of transferrin fragment and transferrin receptor.

- The 7.4, 11.9 and 12 kDa proteins/peptides were found differentially expressed in conditioned media from melanocytes and melanoma cell lines using SELDI-ToF MS. The identification of these proteins/peptides will improve our knowledge of cancer and invasion in melanoma and have potential to provide valuable biomarker(s) for early detection in melanoma. Further validation of these targets using melanoma patients will confirm clinical relevance of these targets with melanoma.

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Supplementary Data

Appendix -1

Table 1: Proteins identified from the cell lysates for the comparison of normal non-invasive (MCF-10A) vs. normal invasive (HMEC) groups MALDI TOF MS and/or LC-MS/MS

2D-DIGE No	Gene Symbol	Protein Identification	MCF 10A/ HMEC	Biological function
1019	PDIA3	Protein disulfide-isomeraseA3 precursor	-2.35	Protein disulfide isomerase reaction
1079	ARCN1	Coatomer subunit delta (Delta-coat protein)	-2.21	Constitutive exocytosis
1211	gi|31874087	Hypothetical protein	2.73	Unclassified
1230	UQCRC1	Ubiquinol-cytochrome-c reductase complex core protein 1, mitochondrial precursor	1.82	Electron transport
1279	NDRG1	N-myc downstream-regulated gene 1 protein	-2.61	Cell proliferation and differentiation
1378	RAD23B	RAD23B protein	1.96	DNA repair
1411	SERPINB5	Tumour Suppressing Serpin	2.26	Proteolysis
1450	TSSC1	Tumor-suppressing subchromosomal transferable fragment candidate gene 1 protein	1.92	Protein binding
1468	ANXA1	Annexin I	1.95	Cell motility
1469	ANXA1	Annexin I	2.45	Cell motility
1488	TALDO1	Transaldolase	1.92	Pentose-phosphate shunt
1527	TALDO1	Transaldolase	2.24	Pentose-phosphate shunt

1732	PSME1	Proteasome alpha 3 subunit, isoform 1	4.18	Proteolysis
1743	CLIC1	Chloride intracellular channel protein 1	6.96	Anion transport
1781	TIP1	Chain B, Human Triosephosphate Isomerase Of New Crystal Form	-2.0	Glycolysis
1832	HMGB1	HMGB1 high-mobility group box 1	3.59	Nucleic acid metabolism

Table 2: Proteins identified from the cell lysates for the comparison of cancerous non-invasive (MCF-7) vs. cancerous invasive (BT20) group using MALDI TOF MS and/or LC-MS/MS.

2D-DIGE No	**Gene Symbol**	**Protein Identification**	**MCF7/ BT20 Fold change**	**Biological function**
611	GSN	Gelsolin precursor	3.47	Cell structure
614	ANXA3	Annexin A3	2.5	Signal transduction
773	LMNA	Lamin-A/C	-3.66	Cell structure
897	HNRPK	Heterogeneous nuclear ribonucleoprotein K	-17.93	mRNA splicing
	HSP60	60 kDa heat shock protein		Stress response
901	HNRPK	heterogeneous nuclear ribonucleoprotein K	-18.32	mRNA splicing
	TUBA6	Tubulin alpha-1C chain		Stress response
907	KT18	Keratin, type I cytoskeletal 18	-16.77	Cell structure
	KT8	Keratin, type II cytoskeletal 8		Cell structure
958	ME3	Chain C, Crystal Structure Of A Human Malic Enzyme	-1.81	Cell adhesion
996	STIP1	STIP1 protein	2.1	Stress response
998	CBS	Cystathionine beta-synthase	-1.6	Amino acid biosynthesis
1019	PDIA3	Protein disulfide-isomeraseA3 precursor	-8.5	Protein disulfide isomerase reaction
1050	G6PD	Glucose-6-phosphate 1-dehydrogenase (G6PD)	-13.62	Monosacharide Metabolism
	CCT-beta	T-complex protein 1 subunit beta		Protein folding
1076	TXNRD1	Thioredoxin reductase 1, cytoplasmic precursor	-3.87	Electron transport

1098	3-PGDH	3-phosphoglycerate dehydrogenase	-7.6	Amino acid biosynthesis
1105	DYNC1LI2	Cytoplasmic dynein 1 light intermediate chain 2	1.95	Cell cycle and cell structure
1146	KRT8	Keratin, type IIcytoskeletal 8	2.83	Cell structure
1230	UQCRC1	Ubiquinol-cytochrome-c reductase complex core protein 1, mitochondrial precursor	1.77	Electron transport
1279	NDRG1	N-myc downstream-regulated gene 1 protein	2.12	Cell proliferation and differentiation
1292	NUDC	Nuclear migration protein	2.3	Cell proliferation and differentiation
1311	KRT19	Keratin, type I cytoskeletal 19	3.37	Cell structure
1316	KRT19	Keratin, type I cytoskeletal 19	1.65	Cell structure
1378	RAD23B	RAD23B protein	2.12	DNA repair
1405	CAPG	Chain A, Ca2+-Binding mimicry In The Crystal Structure Of The Eu3+-Bound Mutant Human Macrophage Capping Protein Cap G	2.14	Cell proliferation and differentiation
1411	SERPINB5	Tumour Suppressing Serpin	2.74	Proteolysis
1450	TSSC1	Tumor-suppressing subchromosomal transferable fragment candidate gene 1 protein	3.59	Protein binding
1488	TALDO1	Transaldolase	-2.24	Pentose-phosphate shunt
1527	TALDO1	Transaldolase	-3.78	Pentose-phosphate shunt
1502	AKR1A1	Chain A, Apo R268a Human Aldose Reductase	-8.3	Oxidoreductase activity

1552	QPRT	Qinolinate phosphoribosyl transferase	-4.47	NAD pyrophosphorylase activity
1732	PSME1	Proteasome alpha 3 subunit, isoform 1	2.51	Proteolysis
1743	CLIC1	Chloride intracellular channel protein 1	3.4	Anion transport
1752	ERP29	Endoplasmic reticulum protein ERp29 precursor	7.04	Constitutive exocytosis
1781	TPI1	Chain B, Human Triosephosphate Isomerase Of New Crystal Form	-2.19	Glycolysis
1798	Hsp27	Heat shock protein 27	4.43	Stress response
1800	Psma6	Proteasome subunit alpha type 6	3.07	Proteolysis
	HSPB1	Heat-shock protein beta-1		Stress response
1832	HMGB1	HMGB1 high-mobility group box 1	1.76	Nucleic acid metabolism
2139	NME1	Nm23 protein	3.02	Pyrimidine metabolism
2424	CLP	Chain A, Three Crystal Structures Of Human Coactosin-Like Protein	-8.19	Cell adhesion
2477	LGALS1	Galectin-1	-3.93	Cell adhesion

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Table 3: Proteins identified from the conditioned media for the comparison of normal non-invasive (MCF-10A) vs. normal invasive (HMEC) groups using MALDI TOF MS and/or LC-MS/MS.

DIGE No.	Gene Symbol	Identification	MCF10/HMEC Fold change	Biological function
231	LAMC2	Laminin, gamma 2 isoform a precursor	-2.6	Cell adhesion
513	HSP60	60 kDa heat shock protein	6.09	Stress response
557	PDIA3	Protein disulfide-isomerase A3 precursor	-2.05	Protein disulfide-isomerase reaction
	BAT1	Spliceosome RNA helicase BAT1		Nucleic acid metabolism
	FKBP4	FK506-binding protein 4		Protein folding
687	ACTR3	ARP3 actin-related protein 3 homolog	-3.04	Cell structure
763	CPSB	Chain A, Recombinant Procathepsin B	-5.95	Proteolysis Other oncogenesis
796	VIM	Vimentin	-3.82	Cell structure
818	VIM	Vimentin	-4.3	Cell structure
854	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade B, member 1	-2.13	Proteolysis
1014	THBS1	Thrombospondin 1 precursor	-10.74	Developmental process
1060	ANXA3	Annexin A3	-4.09	Lipid, fatty acid and steroid metabolism
1191	HC8	Proteasome alpha 3 subunit isoform 1	-1.77	Protein binding

1252	PRDX6	Peroxiredoxin 6	1.8	Antioxidation and free radical removal
1318	SERPINA1	Alpha-1-antitrypsin precursor	-3.48	Proteolysis
1580	SOD1	Superoxide dismutase	2.03	Immunity and defense

Table 4: Proteins identified from the conditioned media for the comparison of cancerous non-invasive (MCF-7) vs. cancerous invasive (BT20) group using MALDI TOF MS and/or LC-MS/MS.

DIGE No.	**Gene symbol**	**Identification**	**MCF-7/BT20 Fold change**	**Biological function**
231	LAMC2	Laminin, gamma 2 isoform a precursor	-7.04	Cell adhesion
267	GSN	Gelsolin isoform a precursor	5.47	Cell structure
380	HSPA5	Heat shock 70kDa protein 5	-3.92	Stress response
	HSPA8	Heat shock cognate 71 kDa protein		Stress response
384	HSP90	Heat shock protein HSP 90-beta	-9.15	Stress response
411	HSPA8	Heat shock 70kDa protein 8 isoform 1	-3.22	Stress response
429	HSPA1B	Heat shock 70kDa protein 1B	-3.45	Stress response
440	LCP1	Plastin-2	-15.64	Cell structure
509	CCT8	T-complex protein 1 subunit theta	-4.2	Protein folding
513	HSP60	60 kDa heat shock protein	-2.08	Stress response
557	PDIA3	Protein disulfide-isomerase A3 precursor	-2.39	Protein disulfide-isomerase reaction
	BAT1	Spliceosome RNA helicase BAT1		Nucleic acid metabolism
	FKBP4	FK506-binding protein 4		Protein folding
558	PDIA3	Protein disulfide isomerase-associated 3 precursor	-2.79	Protein disulfide-isomerase reaction
561	PDIA3	Protein disulfide isomerase-associated 3 precursor	-2.21	Protein disulfide-isomerase reaction

563	PDIA3	Protein disulfide isomerase-associated 3 precursor	-2.69	Protein disulfide-isomerase reaction
590	PDIA3	Protein disulfide-isomerase A3 precursor	-2.87	Protein disulfide-isomerase reaction
629	NUCB2	Nucleobindin-2 precursor	-4.27	Calcium ion homeostasis
677	GSS	Glutathione synthetase	-1.93	Sulfur redox metabolism
705	CTSD	Cathepsin D preproprotein	2.55	Proteolysis
711	GDI2	Rab GDP dissociation inhibitor beta	-3.06	Intracellular signaling
	SSB	Lupus La protein		tRNA metabolism
	AHCY	Adenosylhomocysteinase		Purine metabolism
726	PA2G4	Proliferation-associated protein 2G4	-10.57	Cell proliferation and differentiation
731	SERPINB7	Serpin B7	-4.98	Proteolysis
736	HSPA5	Heat shock 70kDa protein 5	-3.56	Stress response
	EIF4A	Eukaryotic initiation factor 4A-I		Translational regulation
763	CPSB	Chain A, Recombinant Procathepsin B	5.69	Proteolysis
790	ACTB	Beta actin	2.27	Cytokinesis, Cell structure
791	ACTB	Beta actin	1.88	Cytokinesis, Cell structure
854	SERPINB1	Serine (or cysteine) proteinase inhibitor, clade B, member 1	2.43	Proteolysis
867	CANT1	Soluble calcium-activated nucleotidase 1	4.76	Nucleic acid metabolism
916	TALDO1	Transaldolase 1	-2.81	Pentose-phosphate shunt
930	DDAH1	DDAH1 protein	-2.16	Cell proliferation and

				differentiation
969	CAPZA	F-actin capping protein alpha-1 subunit	-1.72	Cell structure
1131	TPI1	Triosephosphate isomerase	-9.74	Glycolysis
1140	CLIC1	Chloride intracellular channel 1	-1.89	Anion transport
1328	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein precursor (fragment)	1.81	Cell adhesion

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Table5: Proteins identified from the isolated membrane fractions for the comparison of normal non-invasive (MCF-10A) vs. normal invasive (HMEC) group using MALDI TOF MS and/or LC-MS/MS.

DIGE No	Gene symbol	Identification	MCF-10A/HMEC Fold change	Biological function
177	GANAB	Neutral alpha-glucosidase AB precursor (Glucosidase II subunit alpha)	-2.28	Protein modification
178	GANAB	KIAA0088	1.55	Protein modification
218	ITGA3	Integrin alpha-3 precursor	5.27	Cell adhesion
219	ABCB3	Novel protein similar to human transporter 2, ATP-binding cassette, sub-family B	7.71	Extracellular transport and import
229	RHOT2	Mitochondrial Rho GTPase 2	3.58	Apoptosis, Cell adhesion
351	NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa precursor	1.82	Electron transport
367	ATP5B	ATP synthase subunit beta, mitochondrial precursor	1.99	Electron transport
386	HSPA9	Stress-70 protein, mitochondrial precursor	2.07	Stress response
393	HSPA9	Stress-70 protein, mitochondrial precursor	2.9	Stress response
431	LMNB1	Lamin-B1	5.53	Cell structure
	HSPA8	Heat shock 70kDa protein 8 isoform 1		Stress response
434	RUVBL2	RuvB-like 2	-1.76	Cell motility
436	LMNB1	Lamin-B1	3.91	Cell structure
649	KRT8	keratin 8	4.02	Cell structure

695	ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor		Electron transport
726	C20ORF3	Adipocyte plasma membrane-associated protein	1.54	Other carbohydrate metabolism
812	UQCRC1	Ubiquinol-cytochrome c reductase core protein I	-3.54	Electron transport
867	ACTA1	Actin, alpha skeletal muscle (Alpha-actin-1)	1.76	Cytokinesis, Cell structure
876	ACTB	Actin, gamma 1 propeptide	-2.29	Cytokinesis, Cell structure
877	ACTB	Beta actin	-1.78	Cytokinesis, Cell structure
928	STOML2	stomatin (EPB72)-like 2	1.86	Cell structure and motility
929	CAPG	Macrophage-capping protein	2.72	Cell proliferation and differentiation
950	SERPINB5	Serpin B5 precursor	4.08	Proteolysis
955	SERPINB5	Serpin B5 precursor	2.8	Proteolysis
1029	NPM1	Nucleophosmin	2.34	rRNA metabolism
1085	LDHB	L-lactate dehydrogenase B chain	1.54	Glycolysis
1153	ANXA3	Annexin A3	-3.05	Fatty acid and steroid metabolism
1181	NAPA	Alpha-soluble NSF attachment protein	4.95	Intracellular protein traffic
1212	A2M	Alpha-2-macroglobulin precursor	4.34	Ligand-mediated signaling
1277	PHB	Prohibitin	3.43	Cell proliferation and differentiation
1303	PSME1	Proteasome activator complex subunit 1	2.89	Proteolysis
1359	HSPB1	Heat shock protein beta-1	-27.66	Stress response
1366	HSPB1	Heat shock protein beta-1	-6.8	Stress response
1386	HSPB1	Heat shock protein beta-1	-6.76	Stress response
1469	GSTP1	Glutathione S-Transferase P1- 1[v104] Complexed With	-1.64	Detoxification

		(9r,10r)-9-(S-Glutathionyl)-10- Hydroxy-9,10-Dihydrophenanthrene		
1714	DIABLO	Diablo homolog, mitochondrial precursor	3.32	Cell communication
1786	NME1	NME1 protein	4.85	Pyrimidine metabolism
2482	S100A16	S100-A16	2.23	Calcium ion binding
2509	S100A14	S100-A14	1.84	Calcium ion binding

Table 6: Proteins identified from the isolated membrane fractions for the comparison of cancerous non-invasive (MCF-7) vs. cancerous invasive (BT20) group using MALDI TOF MS and/or LC-MS/MS.

DIGE no	Gene symbol	Identification	MCF-7 vs. BT20 Fold change	Biological function
83	GANAB	Neutral alpha-glucosidase AB precursor	-2.53	Protein modification
84	GANAB	Alpha glucosidase II alpha subunit isoform 2	-4.74	Protein modification
149	GANAB	GANAB protein	-2	Protein modification
177	GANAB	Neutral alpha-glucosidase AB precursor	-3.25	Protein modification
209	HSPD1	60 kDa heat shock protein, mitochondrial precursor	-3.59	Stress response
346	ATP7A	Copper-transporting ATPase 1	-1.91	Cation transport
416	LMNB2	Lamin B2	-1.69	Cell structure
434	RUVBL2	RuvB-like 2	-10.28	Cell motility
441	WDR1	WD repeat-containing protein 1	-3.16	Cell motility
481	CCT6A	T-complex protein 1 subunit zeta	-3.7	Protein folding
499	TCP1	T-complex protein 1 subunit alpha	-3.52	Protein folding
527	CES1	Liver Carboxylesterase 1 precursor	-31	Detoxification
546	HSPD1	Chaperonin	-2.26	Stress response
550	HSPD1	Chaperonin	-2.51	Stress response
558	PDIA3	Protein disulfide-isomerase A3 precursor	-4.16	Protein disulfide isomerase reaction
600	PDIA3	Protein disulfide-isomerase A3 precursor	-4.04	Protein disulfide isomerase reaction

606	G6PD	Structure Of A Deletion Variant Of Human Glucose 6-Phosphate Dehydrogenase Complexed With Structural And Coenzyme Nadp	-2.3	Carbohydrate metabolism
671	RUVB1	RuvB1	-3.04	mRNA transcription regulation
688	RUVBL2	RuvB-like 2	-2.8	Cell motility
695	ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor	1.55	Electron transport
726	C20ORF3	Adipocyte plasma membrane-associated protein	1.94	Other carbohydrate metabolism
746	GCN1L1	Translational activator GCN1	-1.94	Protein biosynthesis
827	AHCY	Adenosylhomocysteinase	-3.29	Purine metabolism
1050	TALDO1	Transaldolase	-4.76	Pentose-phosphate shunt
1225	PSME2	Proteasome activator complex subunit 2	-4.13	Proteolysis
1270	CLIC4	Chloride intracellular channel protein 4	-1.97	Anion transport
1277	PHB	Prohibitin	-1.57	Cell proliferation and differentiation
1359	HSPB1	Heat shock protein beta-1	1.98	Stress response
1363	PNPO	Pyridoxine 5'-phosphate oxidase	3.37	Fatty acid metabolism
1366	HSPB1	Heat shock protein beta-1	3.61	Stress response
1386	HSPB1	Heat shock protein beta-1	5.73	Stress response
1406	gil116875831	Regulator of microtubule dynamics 1	2.9	Unknown
1408	HSPB1	Heat shock protein beta-1	5.85	Stress response

1417	HSPB1	Heat shock protein beta-1	8.7	Stress response
1714	DIABLO	Diablo homolog, mitochondrial precursor	-2.31	Cell communication
2353	S100A9	S100-A9	-5.21	Cell communication
2482	S100A16	S100-A16	2.55	Calcium ion binding
2659	S100A6	S100-A6	-2.74	Cell proliferation and differentiation

Appendix 4

Table 7: Detailed information (gene symbol, accession number, E-value, % of coverage of peptide for identified proteins, pI, molecular weight and number of peptide matched for protein identification) for all identified proteins from the cell lysates 2D-DIGE study using MALDI TOF MS and/or LC-MS/MS. * p-value of identified protein if protein identified using LC-MS/MS and expectation value (confidence in the database match) if protein identified using MALDI TOF MS.

DIGE no	Gene Symbol	Accession No	Identification	p-value/E-value*	Coverage	MW	pI	No of peptides Matched	ID by
611	GSN	gil121116	Gelsolin Precursor	3.1E-8	11.13	5.8	85.6	10	LC-MS/MS
641	EIF4A3	gil496902	Translation initiation factor	0.008	15.1	6.1	47.1	6	MALDI-ToF MS
773	LAMA	gil125962	Lamin-A/C	1.8E-14	44.73	6.5	74.0	25	LC-MS/MS
789	LMNB1	gil576840	Lamin B1	0.007	12.6	5.1	66.6	7	MALDI-ToF MS
818	G6PD	gil66361518	Chain B, X-Ray Structure Of Human Glucose-6-Phosphate Dehydrogenase (Deletion Variant) Complexed With Glucose-6-Phosphate	0	24.7	6.7	56.7	9	MALDI-ToF MS
829	LMNA	gil386856	Lamin A protein	0.001	12	6.4	57.7	5	MALDI-ToF MS
884	HSPA	gil6729803	Chain A, Heat-Shock 70kd Protein 42kd Atpase N-Terminal Domain	0	36.6	6.7	41.9	13	MALDI-ToF MS

897	HNRPK	gil62088704	Heterogeneous nuclear ribonucleoprotein K	0	28.3	5.5	49.0	10	MALDI-ToF MS
901	HNRPK	gil55958547	Heterogeneous nuclear ribonucleoprotein K	0	20.1	5.4	42.0	6	MALDI-ToF MS
	HNRPK	gil48429097	Heterogeneous nuclear ribonucleoprotein K	4.1E-9	27.21	5.2	50.9	11	LC-MS/MS
		gil20455322	Tubulin alpha-1C chain	1.8E-8	19.38	4.8	49.8	7	LC-MS/MS
907	KRT8	gil33875698	KRT8 protein	0	32.7	5.6	55.8	17	MALDI-ToF MS
	KRT18	gil125083	Keratin-18	7.6E-13	27.91	5.2	52.1	9	LC-MS/MS
958	ME3	gil78101466	Chain C, Crystal Structure Of A Human Malic Enzyme	0.005	16.3	5.6	60.8	8	MALDI-ToF MS
996	STIP1	gil73909112	STIP1 protein	0	21.4	8.1	68.7	21	MALDI-ToF MS
998	CSB	gil23200451	Chain F, Cystathionine-Beta Synthase: Reduced Vicinal Thiols	0.002	17.4	6.2	40.3	5	MALDI-ToF MS
1019	PDIA3	gil2245365	Protein disulfide-isomeraseA3 precursor	0.001	23.6	5.9	57.1	8	MALDI-ToF MS
	PDIA3	gil2507461	Protein disulfide-isomeraseA3 precursor	1.2E-10	36.2	5.9	56.7	16	LC-MS/MS
1050	G6PD	gil50403780	Glucose-6-phosphate 1-dehydrogenase	2.3E-12	26.9	6.4	59.2	12	LC-MS/MS
	CCT-beta	gil6094436	T-complex protein 1 subunit beta	1.6E-11	23.5	6.0	57.4	10	LC-MS/MS
1076	TXNRD1	gil50403780	Thioredoxin reductase 1, cytoplasmic	4.7E-10	14.8	6.0	54.6	6	LC-MS/MS

			precursor						
1079	ARCN1	gil1351970	Coatomer subunit delta	7.0E-6	12.9	5.2	57.0	7	LC-MS/MS
1098	3-PGDH	gil2674062	3-phosphoglycerate dehydrogenase	0	29.8	6.3	57.3	12	MALDI-ToF MS
1105	DYNC1LI2	gil3287825	Cytoplasmic dynein 1 light intermediate chain 2	6.9E-8	8.94	5.9	54.0	3	LC-MS/MS
1126	3-PGDH	gil74762146	Tubulin-specific chaperone E	3.0E-8	9.87	6.3	59.3	5	LC-MS/MS
1146	KRT8	gil33875698	KRT8 protein	0	29.8	5.6	55.8	11	MALDI-ToF MS
	KRT8	gil90110027	KRT8 protein	1.0E-8	42.65	5.3	53.6	21	LC-MS/MS
1174	ACTC1	gil4885049	Cardiac muscle alpha actin proprotein	0.005	16.2	5.2	42.3	7	MALDI-ToF MS
1191	VIM	gil37852	Vimentin	0	30.7	5.1	53.7	12	MALDI-ToF MS
	VIM	gil55977767	Vimentin	9.0E-10	60.09	4.9	53.6	26	LC-MS/MS
1211	gil31874087	gil31874087	Hypothetical protein	0.004	14.8	6.0	33.1	4	MALDI-ToF MS
1221	KRT18	gil12653819	Keratin 18	0.005	18.6	5.4	48.0	8	MALDI-ToF MS
1222	ACTB	gil46397316	Alpha-actin-1	7.8E-8	20.42	5.1	42.0	8	LC-MS/MS
	HNRPF	gil1710628	Heterogeneous nuclear ribonucleoprotein F	5.4E-13	9.4	5.2	45.6	4	LC-MS/MS

	UQCRC1	gil731047	Ubiquinol-cytochrome-c reductase complex core protein I, mitochondrial precursor	0.001	24.8	5.9	53.2	9	MALDI-ToF MS
1230	UQCRC1	gil92090651	Ubiquinol-cytochrome-c reductase complex core protein 1, mitochondrial precursor	3.9E-8	10.21	5.9	52.9	4	LC-MS/MS
1279	NDRG1	gil6166568	N-myc downstream-regulated gene 1 protein		15.48	5.4	42.8	5	LC-MS/MS
1292	NUDC	gil62287	Nuclear migration protein NudC	3.0E-7	15.71	5.2	38.2	5	LC-MS/MS
1311	KRT19	gil90111766	Keratin, type I cytoskeletal 19	1.5E-12	55.75	4.9	44.0	17	LC-MS/MS
	gil34039	gil34039	Keratin, type I cytoskeletal 19	0	35.5	5	44.09	14	MALDI-ToF MS
1316	KRT19	gil90111766	Keratin, type I cytoskeletal 19	1.1E-12	25.0	4.9	44.0	12	LC-MS/MS
	SERPINB1	gil62898301	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	0.001	32.7	5.9	42.8	11	MALDI-ToF MS
1341	SERPINB1	gil266344	Leukocyte elastase inhibitor (Serpin B1)	3.6E-10	24.01	5.8	42.7	10	LC-MS/MS
1374	BTRC	gil4519417	WD-40 repeat protein	0.004	16.9	4.9	38.8	4	MALDI-ToF MS
1378		gil18089249	RAD23B protein	0	14.7	4.8	43.2	5	MALDI-ToF MS
1397	SERPINB5	gil62738526	Chain B, The 2.8 A Structure Of A Tumour Suppressing Serpin	0	28.2	5.7	43.0		MALDI-ToF MS

1399	SERPINB5	gil62738526	Chain B, The 2.8 A Structure Of A Tumour Suppressing Serpin	0	24.7	5.7	43.0	7	MALDI-ToF MS
1405	CAPG	gil21730367	Macrophage Capping Protein Cap G	1.9E-10	19	5.3	38.7	5	LC-MS/MS
1411	SERPINB5	gil547892	Serpin B5 precursor	5.8E-5	23.2	5.6	42.1	9	LC-MS/MS
1450	TSSC1	gil84029603	Tumor-suppressing subchromosomal transferable fragment candidate gene 1 protein		11.11	4.7	43.6	4	LC-MS/MS
1468	ANXA1	gil442631	Annexin I	0.002	21	7.9	38.6	6	MALDI-ToF MS
	ANXA1	gil442631	Annexin I	0.002	21	7.9	38.6	6	MALDI-ToF MS
1469	ANXA1	gil113944	Annexin-1	60E-10	37.28	6.6	38.6	12	LC-MS/MS
1502	AKR1A1	gil62738430	Chain A, Apo R268a Human Aldose Reductase	0.004	20.3	6.3	36.0	5	MALDI-ToF MS
1488	TALDO1	gil6648092	Transaldolase	3.8E-7	42.43	6.3	37.5	14	LC-MS/MS
1513	ANXA3	gil47115233	Annexin A3		39.3	5.6	36.4	17	MALDI-ToF MS
1527	TALDO1	gil6648092	Transaldolase	8.1E-7	30.2	6.3	37.5	10	LC-MS/MS
	QPRT	gil1060907	Quinolinate phosphoribosyl transferase	0.004	10.4	5.8	31.23	5	MALDI-ToF MS
1552	QPRT	gil21903445	Quinolinate phosphoribosyltransferase	1.1E-7	22.9	5.7	30.7	6	LC-MS/MS
1707	PSME1	gil30581141	Proteasome activator subunit 1 isoform 2	0	35.2	6.3	28.7	9	MALDI-

									ToF MS
1722	DCI	gil1169204	2-trans-enoyl-CoA isomerase, mitochondrial precursor	6.5E-9	10.93	8.6	32.7	3	LC-MS/MS
1734	PSME1	gil30581141	Proteasome activator subunit 1 isoform 2	0	53.6	6.3	28.7	13	MALDI-ToF MS
1732	PSME1	gil30581141	Proteasome activator subunit 1 isoform 2	1.0E-8	47.79	5.7	28.7	12	LC-MS/MS
1743	CLIC1	gil4588526	Nuclear chloride channel	0	60.2	5.0	27.2	11	MALDI-ToF MS
1752	ERP29	gil6015110	Endoplasmic reticulum protein ERp29 precursor	2.2E-9	33.33	7.2	78.9	8	LC-MS/MS
1753	CTSD	gil5822091	Cathepsin D At Ph 7.5	0.002	19.9	5.3	26.4	4	MALDI-ToF MS
1781	TPI1	gil66360366	Chain B, Human Triosephosphate Isomerase Of New Crystal Form	0	68	6.5	26.9	13	MALDI-ToF MS
1798		gil662841	Heat shock protein 27	0.004	33.7	8.1	22.4	6	MALDI-ToF MS
1800	HSP27	gil46397655	Heat-shock protein 27kDa protein	6.5E-6	29.7	8.1	27	8	LC-MS/MS
1832	HMGB1	gil48145843	HMGB1 Protein	0.008	13.5	5.6	25.0	5	MALDI-ToF MS
1971	SOD1	gil5822074	Superoxide Dismutase Mutant With Lys 136 Replaced By Glu, Cys 6 Replaced By Ala And Cys 111 Replaced By Ser (K136e,	0.002	27.5	5.4	15.8	4	MALDI-ToF MS

			C6a, C111s)						
2139	NME1	gil35068	Nm23 protein	0.002	26.7	7.1	20.7	5	MALDI-ToF MS
2424	CLP	gil75765235	Coactosin-Like Protein	0	30.5	5.5	15.9	7	MALDI-ToF MS
2477		gil42542978	Galectin-1	0	73.9	5.3	14.9	8	MALDI-ToF MS
		gil126155	Galectin-1	3.2E-11	80.74	5.1	14.1	8	LC-MS/MS

Appendix 5

Table 8: Detailed information (gene symbol, accession number, E-value, % of coverage of peptide for identified proteins, pI, molecular weight and number of peptide matched for protein identification) for all identified proteins from the conditioned media 2D-DIGE study using MALDI TOF MS and/or LC-MS/MS. * p-value of identified protein if protein identified using LC-MS/MS and expectation value (confidence in the database match) if protein identified using MALDI TOF MS.

DIGE No.	Gene Symbol	Accession No	Protein name	p-value/E-value*	Coverage	PI	MW	No of peptide matched	ID by
231	LAMC2	gi|157419138	Laminin, gamma 2 isoform a precursor	0	18.6	5.8	134.8	22	MALDI-ToF MS
	LAMC2	gi|90185107	Laminin subunit gamma-2 precursor	1.0E-30	28.9	5.7	130.8	26	LC-MS/MS
232	GSN	gi|121116	Gelsolin precursor	1.4E-7	7.8	5.8	85.6	3	LC-MS/MS
	C1R	gi|115204	Complement C1r subcomponent precursor	1.6E-6	6.1	5.8	80.0	3	LC-MS/MS
267	GSN	gi|4504165	Gelsolin isoform a precursor	0.004	15.2	5.9	56.0	9	MALDI-ToF MS
326	TPTE2	gi|74749506	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase	0.007	13.6	9.2	61.4	6	MALDI-ToF MS
346	CBF	gi|134105218	Complement Factor B	0	14.2	6.8	85.6	7	MALDI-ToF MS

350	CBF	gi|584908	Complement factor B precursor	1.9E-8	14.6	6.6	85.4	9	LC-MS/MS
	RDX	gi|464541	Radixin	7.2E-9	11.4	5.9	68.5	5	LC-MS/MS
380	HSPA5	gi|16507237	Heat shock 70kDa protein 5	0	32.9	5.1	72.4	17	MALDI-ToF MS
	HSPA8	gi|123648	Heat shock cognate 71 kDa protein	2E-9	7.89	5.2	70.8	4	LC-MS/MS
384	HSP90	gi|17865718	Heat shock protein HSP 90-beta	3.3E-12	34.5	4.8	83.2	24	LC-MS/MS
392	EZR	gi|21614499	Ezrin	0.002	25.8	5.9	69.5	14	MALDI-ToF MS
411	HSPA8	gi|5729877	Heat shock 70kDa protein 8 isoform 1	0	35.1	5.1	71.1	16	MALDI-ToF MS
429	HSPA1B	gi|167466173	Heat shock 70kDa protein 1B	0.005	16.1	5.5	70.3	7	MALDI-ToF MS
431	PGM2	gi|63055049	Phosphoglucomutase 2	0.002	15.4	6.3	68.7	7	MALDI-ToF MS
	PLS3	gi|2506254	Heat shock 70 kDa protein 6	1.0E-9	22.8	5.4	70.3	9	LC-MS/MS
437	RNPEP	gi|20137480	Aminopeptidase B	6.9E-11	21.0	5.2	72.5	11	LC-MS/MS

440	LCP1	gi|1346733	Plastin-2	0	19.9	5.2	70.8	10	MALDI-ToF MS
	LCP1	gi|1346733	Plastin-2	3.6E-10	36.3	5.0	70.2	21	LC-MS/MS
443	LCP1	gi|1346733	Plastin-2	0	20.7	5.2	70.8	11	MALDI-ToF MS
	LCP1	gi|1346733	Plastin-2	6.2E-13	40.9	5.0	70.2	16	LC-MS/MS
451	PDIA4	gi|119530	Protein disulfide-isomerase A4 precursor	9.4E-08	6.2	4.8	72.8	3	LC-MS/MS
478	P4HB	gi|20070125	Prolyl 4-hydroxylase, beta subunit precursor	0.001	12.6	4.8	57.5	5	MALDI-ToF MS
480	SERPINB1	gi|50363217	Serine (or cysteine) proteinase inhibitor, clade A member 1	0.006	20.8	5.4	46.8	5	MALDI-ToF MS
483	NUCB1	gi|20070228	Nucleobindin 1	0.005	24.7	5.1	53.8	8	MALDI-ToF MS
	NUCB1	gi|90110780	Nucleobindin-1 precursor	5.7E-6	10.8	5.0	53.8	5	LC-MS/MS
484	NUCB1	gi|20070228	Nucleobindin 1	0	42.3	5.1	53.8	16	MALDI-ToF MS
486	NUCB1	gi|20070228	Nucleobindin 1	0	31.9	5.1	53.8	12	MALDI-ToF MS

	NUCB1	gi|90110780	Nucleobindin-1 precursor	1.5E-7	9.3	5.0	53.8	4	LC-MS/MS
497	HSPA6	gi|34978357	Heat shock 70 kDa protein 6	1.5E-9	7.6	5.7	70.9	4	LC-MS/MS
509	CCT8	gi|9988062	T-complex protein 1 subunit theta	2.6E-8	12.5	5.3	59.5	11	LC-MS/MS
513	HSP60	gi|129379	60 kDa heat shock protein	1.6E-7	13.4	5.5	61.0	8	LC-MS/MS
557	PDIA3	gi|2507461	Protein disulfide-isomerase A3 precursor	2.8E-09	42.3	5.9	56.7	20	LC-MS/MS
	BAT1	gi|2500529	Spliceosome RNA helicase BAT1	1.6E-7	21.0	5.3	48.9	10	LC-MS/MS
	FKBP4	gi|399866	FK506-binding protein 4	4.8E-7	23.0	5.2	50.7	9	LC-MS/MS
558	PDIA3	gi|21361657	Protein disulfide isomerase-associated 3 precursor	0.009	16.4	6.0	57.1	6	MALDI-ToF MS
561	PDIA3	gi|21361657	Protein disulfide isomerase-associated 3 precursor	0	39.6	6.0	57.1	16	MALDI-ToF MS
563	PDIA3	gi|21361657	Protein disulfide isomerase-associated 3 precursor	0	27.1	6.0	57.1	10	MALDI-ToF MS
590	PDIA3	gi|2507461	Protein disulfide-isomerase A3 precursor	7.9E-9	25.7	5.9	56.7	7	LC-MS/MS

628	TUBB	gi|29788785	Tubulin beta	0	29.3	4.8	50.1	10	MALDI-ToF MS
629	NUCB2	gi|462693	Nucleobindin-2 precursor	1.8e-7	19.7	4.8	50.1	4	LC-MS/MS
661	CTSA	gi|20178316	Lysosomal protective protein precursor (Cathepsin A)	0.001	14.8	6.2	54.9	5	MALDI-ToF MS
677	GSS	gi|4504169	Glutathione synthetase	0.001	34.4	5.7	52.5	11	MALDI-ToF MS
	GSS	gi|1346191	Glutathione synthetase	1.6E-8	20.2	5.5	52.3	8	LC-MS/MS
687	ACTR3	gi|5031573	ARP3 actin-related protein 3 homolog	0	21.5	5.6	47.8	7	MALDI-ToF MS
691	SERPINF1	gi|20178323	Pigment epithelium-derived factor precursor	0	18.7	6.0	46.5	7	MALDI-ToF MS
	SERPINF1	gi|20178323	Pigment epithelium-derived factor precursor	5.6E-10	24.8	5.9	46.3	12	LC-MS/MS
697	CTSD	gi|4503143	Cathepsin D preproprotein	0	27.7	6.1	45.0	8	MALDI-ToF MS
705	CTSD	gi|4503143	Cathepsin D preproprotein	0	27.7	6.1	45.0	8	MALDI-ToF MS
711	GDI2	gi|13638228	Rab GDP dissociation inhibitor beta	4.8E-11	31.9	6.0	50.6	8	LC-MS/MS

	SSB	gi|125985	Lupus La protein	6.4E-10	25.2	6.7	46.8	5	LC-MS/MS
	AHCY	gi|20141702	Adenosylhomocysteinase	4.4E-9	15.7	5.9	47.6	5	LC-MS/MS
726	PA2G4	gi|13632817	Proliferation-associated protein 2G4	6.8E-6	13.9	6.1	43.7	4	LC-MS/MS
731	SERPINB7	gi|20139982	Serpin B7	4.8E-9	11.5	6.3	42.8	4	LC-MS/MS
736	HSPA5	gi|16507237	Heat shock 70kDa protein 5	0	32.9	5.1	72.4	17	MALDI-ToF MS
	EIF4A	gi|46397463	Eukaryotic initiation factor 4A-I	3.1E-8	22.6	5.1	46.1	10	LC-MS/MS
748	CPA4	gi|85544285	Chain A, Procarboxypeptidase A4	0	38.9	6.2	45.7	13	MALDI-ToF MS
763	CTSB	gi|157833437	Chain A, Recombinant Procathepsin B	0	26.2	5.9	35.8	9	MALDI-ToF MS
790	ACTB	gi|4501885	Beta actin	0.002	32.8	5.3	42.0	8	MALDI-ToF MS
791	ACTB	gi|4501885	Beta actin	0.005	26.7	5.3	42.0	8	MALDI-ToF MS
795	INHBA	gi|4504699	Inhibin beta A precursor	0.003	31.5	8.9	48.2	8	MALDI-ToF MS

796	VIM	gi|62414289	Vimentin	0	44.0	5.1	53.6	18	MALDI-ToF MS
799	NUDC	gi|62287138	Nuclear migration protein nudC	5E-10	19.6	5.1	38.2	5	LC-MS/MS
	KRT18	gi|125083	Keratin, type I cytoskeletal 18	1.7E-8	18.8	5.2	48.0	6	LC-MS/MS
807	GLUL	gi|145580062	Chain A, Glutamine Synthetase	0.006	17.2	6.5	43.9	6	MALDI-ToF MS
809	ACTG1	gi|4501887	Actin, gamma 1 propeptide	0	20.8	5.3	42..1	6	MALDI-ToF MS
	KRT10	gi|21961605	Keratin, type I cytoskeletal 10	2.6E-7	6.24	5.0	59.4	3	LC-MS/MS
818	VIM	gi|62414289	Vimentin	0	37.3	5.1	53.6	18	MALDI-ToF MS
854	SERPINB1	gi|13489087	Serine (or cysteine) proteinase inhibitor, clade B, member 1	0	20.3	5.9	42.8	6	MALDI-ToF MS
	SERPINB1	gi|266344	Leukocyte elastase inhibitor	6.0E-10	25.0	5.8	42.7	11	LC-MS/MS
855	SERPINB5	gi|547892	Serpin B5 precursor (Maspin)	2.5E-5	5.60	14.6	42.0	6	LC-MS/MS
867	CANT1	gi|66774052	Soluble calcium-activated nucleotidase 1	3.8E-8	19.2	5.6	44.8	9	LC-MS/MS

869	CTSB	gi|157833437	Procathepsin B	0	23.7	5.9	35.8	9	MALDI-ToF MS
	CTSB	gi|68067549	Cathepsin B precursor	1.2E-12	41.5	5.8	37.7	11	LC-MS/MS
916	TALDO1	gi|5803187	Transaldolase 1	0	33.8	6.4	37.6	11	MALDI-ToF MS
930	DDAH1	gi|34783629	DDAH1 protein	0.006	31.3	5.2	27.4	7	MALDI-ToF MS
969	CAPZA	gi|5453597	F-actin capping protein alpha-1 subunit	0.001	39.3	5.4	33.0	7	MALDI-ToF MS
972	APOE	gi|114039	Apolipoprotein E precursor	8.7E-12	11.6	5.5	36.1	3	LC-MS/MS
1014	THBS1	gi|40317626	Thrombospondin 1 precursor	0	10.9	4.7	133.3	10	MALDI-ToF MS
	THBS1	gi|117949802	Thrombospondin-1 precursor	2.0E-10	6.92	4.5	129.3	6	LC-MS/MS
1060	ANXA3	gi|4826643	Annexin A3	0.003	36.1	5.6	36.5	8	MALDI-ToF MS
	ANXA3	gi|113954	Annexin A3	7.9E-10	42.0	5.5	36.3	10	LC-MS/MS
1070	TSP	gi|117949802	Thrombospondin-1 precursor	2E-9	10.0	4.5	129.3	7	LC-MS/MS

1109	CAPZB	gi|13124696	F-actin-capping protein subunit beta	4.9E-09	31.4	5.2	31.3	10	LC-MS/MS
1118	NP	gi|157168362	Nucleoside phosphorylase	0	48.1	6.5	32.3	10	MALDI-ToF MS
1131	TPI1	gi|39932641	Triosephosphate isomerase	1.4E-10	30.9	6.5	26.6	6	LC-MS/MS
1140	CLIC1	gi|14251209	Chloride intracellular channel 1	0.008	18.3	5.1	27.2	4	MALDI-ToF MS
	CLIC1	gi|12643390	Chloride intracellular channel 1	1.1E-8	27.8	4.9	26.9	7	LC-MS/MS
1191	HC8	gi|4506183	Proteasome alpha 3 subunit isoform 1	0.001	30.6	5.2	28.6	8	MALDI-ToF MS
1252	PRDX6	gi|4758638	Peroxiredoxin 6	0	42.9	6.0	25.1	9	MALDI-ToF MS
1309	GSTP1	gi|2554831	Chain A, Glutathione S-Transferase P1- 1[v104]	0.009	45.2	5.4	23.5	7	MALDI-ToF MS
	GSTP1	gi|121746	Glutathione S-transferase P	5.2E-06	28.1	5.3	23.3	5	LC-MS/MS
1318	SERPINA1	gi|1703025	Alpha-1-antitrypsin precursor	3.9E-9	39.7	5.2	46.7	9	LC-MS/MS
1328	HSPG2	gi|24212664	Basement membrane-specific heparan sulfate proteoglycan core	3.9E-12	2.37	6.0	46.8	8	LC-MS/MS

			protein precursor						
1395	FHC	gi|56682959	Ferritin, heavy polypeptide 1	0	51.9	5.3	21.3	9	MALDI-ToF MS
1580	SOD1	gi|134611	Superoxide dismutase	9.3E-10	27.92	5.6	15.9	4	LC-MS/MS

Appendix 6

Table 9: Detailed information (gene symbol, accession number, E-value, % of coverage of peptide for identified proteins, pI, molecular weight and number of peptide matched for protein identification) for all identified proteins from the membrane fraction 2D-DIGE study using MALDI TOF MS and/or LC-MS/MS. * p-value of identified protein if protein identified using LC-MS/MS and expectation value (confidence in the database match) if protein identified using MALDI TOF MS.

DIGE no	Gene Symbol	Accession No	Identification	p-value/E-value*	Coverage	MW	pI	No of peptides Matched	ID by
83	GANAB	gi|54037162	Neutral alpha-glucosidase AB precursor	0.008	8.3	107.3	5.7	5	MALDI-ToF MS
84	GANAB	gi|38202257	Alpha glucosidase II alpha subunit isoform 2	0	13	107.3	5.7	11	MALDI-ToF MS
149	GANAB	gi|577295	KIAA0088	0.001	15.5	107.2	5.7	8	MALDI-ToF MS
177	GANAB	gi|54037162	Neutral alpha-glucosidase AB precursor	1.3E-10	4.45	106	5.7	4	LC-MS/MS
	GANAB	gi|54037162	Alpha glucosidase II alpha subunit isoform 2	0.007	15	107.3	5.7	11	MALDI-ToF MS
178	GANAB	gi|577295	KIAA0088	0	20.3	107.3	5.7	13	MALDI-ToF MS
209	HSPD1	gi|129379	60 kDa heat shock protein, mitochondrial precursor (Hsp60)	3.1E-5	7.1	61.1	5.5	4	LC-MS/MS
218	ITGA3	gi|11467963	Integrin alpha-3 precursor	1.1E-5	2.72	118.6	6.6	3	LC-MS/MS

	ITGA3	gi|11467963	Integrin alpha-3 precursor	0.007	10.9	119.8	6.6	10	MALDI-ToF MS
219	ABCB3	gi|26788068	novel protein similar to human transporter 2, ATP-binding cassette, sub-family B	0.008	5.4	79.5	8.7	5	MALDI-ToF MS
229	RHOT2	gi|108860798	Mitochondrial Rho GTPase 2 (MIRO-2)	3.0E-8	6.47	68	5.4	3	LC-MS/MS
243	IMMT	gi|29427676	Mitochondrial inner membrane protein (Mitofilin)	4.3E-10	11.61	83.6	6.0	7	LC-MS/MS
272	JUP	gi|130257	Junction plakoglobin (Desmoplakin-3)	0.001	12.9	82.4	6.0	6	MALDI-ToF MS
334	NT5E	gi|112825	5'-nucleotidase precursor (Ecto-5'-nucleotidase)	1.8E-8	5.4	63.3	6.6	4	LC-MS/MS
	NT5E	gi|4505467	5'-nucleotidase precursor (Ecto-5'-nucleotidase)	0.007	15.9	63.9	6.6	7	MALDI-ToF MS
346	ATP7A	gi|1351993	Copper-transporting ATPase 1	0.007	8.3	164.8	5.9	8	MALDI-ToF MS
351	NDUFS1	gi|33519475	NADH dehydrogenase (ubiquinone) Fe-S protein 1	0	22.6	80.4	5.9	17	MALDI-ToF MS
367	ATP5B	gi|114549	ATP synthase subunit beta, mitochondrial precursor	6.5E-10	27.4	56.5	5.1	11	LC-MS/MS
370	HSPA5	gi|16507237	Heat shock 70kDa protein 5	0	16.2	72.4	5.1	7	MALDI-ToF MS

	HSPA5	gi|1143492	BiP	0.001	26.6	72.2	5.0	12	MALDI-ToF MS
386	HSPA9	gi|21264428	Stress-70 protein, mitochondrial precursor	2.6E-11	21.94	73.6	5.8	14	LC-MS/MS
	HSPA9	gi|21264428	Stress-70 protein, mitochondrial	0	22.7	73.9	5.9	13	MALDI-ToF MS
393	HSPA9	gi|21264428	Stress-70 protein, mitochondrial precursor	5.0E-12	24.15	73.6	5.8	17	LC-MS/MS
	HSPA9	gi|24234688	Heat shock 70kDa protein 9 precursor	0	22.7	73.9	5.9	13	MALDI-ToF MS
416	LMNB2	gi|27436951	Lamin B2	0.004	18.5	67.7	5.3	8	MALDI-ToF MS
431	LMNB1	gi|125953	Lamin-B1	2.0E-5	18.9	66.3	4.9	10	LC-MS/MS
	HSPA8	gi|5729877	Heat shock 70kDa protein 8 isoform 1	0	32.8	71.1	5.4	15	MALDI-ToF MS
434	RUVBL2	gi|49065440	RuvB-like 2	1.3E-7	27.2	51	5.3	9	LC-MS/MS
436	LMNB1	gi|125953	Lamin-B1	8.0E-6	7.68	66.3	4.9	4	LC-MS/MS
441	WDR1	gi|12643636	WD repeat-containing protein 1	6.8E-6	5.94	66.1	6.1	4	LC-MS/MS
481	CCT6A	gi|730922	T-complex protein 1 subunit zeta	1.2E-4	5.27	57.9	6.2	3	LC-MS/MS
499	TCP1	gi|135538	T-complex protein 1 subunit alpha	2.9E-12	8.63	60.3	5.7	4	LC-MS/MS
	TCP1	gi|36796	T-complex polypeptide 1	0.009	13.7	60.8	6.0	6	MALDI-ToF MS

527	CES1	gi|119576	Liver Carboxylesterase 1 precursor	1.1E-9	14.6	62.4	6.1	5	LC-MS/MS
546	HSPD1	gi|31542947	Chaperonin	0.002	19	61.2	5.7	8	MALDI-ToF MS
550	HSPD1	gi|31542947	Chaperonin	0.007	24.6	61.2	5.7	8	MALDI-ToF MS
558	PDIA3	gi|2507461	Protein disulfide-isomerase A3 precursor	5.6e-9	24.55	56.7	5.9	11	LC-MS/MS
600	PDIA3	gi|2507461	Protein disulfide-isomerase A3 precursor	2.9E-12	38.81	56.7	5.9	16	LC-MS/MS
	PDIA3	gi|2245365	Protein disulfide-isomerase A3 precursor	0	30.9	57.1	5.9	12	MALDI-ToF MS
606	3-PGDH	gi|21264510	D-3-phosphoglycerate dehydrogenase	1.5E-8	16.8	56.6	6.2	8	LC-MS/MS
	3-PGDH	gi|66361514	A Deletion Variant Of Human Glucose 6- Phosphate Dehydrogenase Complexed With Structural And Coenzyme Nadp	0.007	17	56.7	6.7	7	MALDI-ToF MS
619	FKBP5	gi|2851536	FK506-binding protein 5	2.4E-7	14.66	50.1	5.6	6	LC-MS/MS
628	FKBP5	gi|2851536	FK506-binding protein 5	5.7E-8	17.51	50.1	5.6	7	LC-MS/MS
646	KRT8	gi|90110027	Keratin, type II cytoskeletal 8	2.7E-5	11.3	53.6	5.3	5	LC-MS/MS
649	KRT8	gi|4504919	keratin 8	0.007	19.3	53.6	5.5	8	MALDI-ToF MS
669	TUBA1C	gi|14389309	Tubulin alpha 6	0.001	29.3	50.8	4.9	9	MALDI
671	RUVBL1	gi|28201891	RuvB-like 1	1.9E-6	9.2	50.1	6.0	4	LC-MS/MS
688	RUVBL2	gi|5730023	RuvB-like 2	0	26.1	51.3	5.5	9	MALDI-ToF MS

695	ATP5B	gi|32189394	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor	0	57.5	56.5	5.3	18	MALDI-ToF MS
726	C20ORF3	gi|24308201	Chromosome 20 open reading frame 3	0	40.4	46.6	5.8	14	MALDI-ToF MS
746	GCN1L1	gi|4099605	Translational activator GCN1	0	10.4	109.1	5.9	8	MALDI-ToF MS
812	UQCRC1	gi|46593007	Ubiquinol-cytochrome c reductase core protein I	0.006	19.6	53.3	5.9	5	MALDI-ToF MS
827	AHCY	gi|20141702	Adenosylhomocysteinase	9.3E-10	13.6	47.6	5.9	6	LC-MS/MS
848	A2M	gi|112911	Alpha-2-macroglobulin precursor	0.008	6	164.6	6.0	7	
866	ACTA1	gi|55976646	Actin, alpha skeletal muscle	1.4E-5	8.49	42.0	5.1	3	LC-MS/MS
867	ACTA1	gi|55976646	Actin, alpha skeletal muscle	1.1E-6	8.49	42.0	5.1	3	LC-MS/MS
876	ACTB	gi|4501887	Actin, gamma 1 propeptide	0.005	32.8	42.1	5.3	8	MALDI-ToF MS
	ACTB	gi|4501887	Actin, gamma 1 propeptide	0	41.1	42.1	5.3	10	MALDI-ToF MS
877	ACTB	gi|4501885	Beta actin	0.002	24.5	42.0	5.3	5	MALDI-ToF MS
884	ACTB	gi|4501887	Actin, gamma 1 propeptide	0.003	23.5	42.1	5.3	6	MALDI-ToF MS
928	STOML2	gi|7305503	Stomatin (EPB72)-like 2	0.002	32.3	38.6	6.9	7	MALDI-ToF

									MS
929	CAPG	gi|729022	Macrophage-capping protein	5.6E-8	13.22	38.4	5.8	4	LC-MS/MS
934	CAPG	gi|729022	Macrophage-capping protein	9.6E-9	9.77	38.4	5.8	3	LC-MS/MS
950	SERPINB5	gi|547892	Serpin B5 precursor	1.2E-5	10.67	42.1	5.5	4	LC-MS/MS
	SERPINB5	gi|547892	Serpin B5 precursor	0	30.9	42.5	5.7	9	MALDI-ToF MS
955	SERPINB5	Sgi|547892	Serpin B5 precursor	1.4E-6	21.87	42.1	5.5	8	LC-MS/MS
	SERPINB5	gi|62738526	The 2.8 A Structure Of A Tumour Suppressing Serpin	0	36.3	43.0	5.7	11	MALDI-ToF MS
1029	NPM1	gi|114762	Nucleophosmin	3.9E-7	10.20	32.5	4.4	3	MALDI-ToF MS
1050	TALDO1	gi|6648092	Transaldolase	9.1E-6	18.40	32.0	6.3	7	LC-MS/MS
1053	ANXA3	gi|113954	Annexin A3	0	37.5	36.6	5.8		MALDI-ToF MS
1085	LDHB	gi|126041	L-lactate dehydrogenase B chain	4.4E-6	19.46	36.6	5.5	6	LC-MS/MS
	LDHB	gi|49259209	Chain A, Human B Lactate Dehydrogenase Complexed With Nad+ And 4- Hydroxy-1,2,5-Oxadiazole-3-Carboxylic Acid	0.007	22.8	36.8	5.9	7	MALDI-ToF MS
1153	ANXA3	gi|47115233	ANXA3	4.3E-8	22.60	36.3	5.5	8	LC-MS/MS
1181	NAPA	gi|116242794	Alpha-soluble NSF attachment protein	6.7E-9	20.68	33.2	5.1	6	MALDI-ToF MS

	NAPA	gi|3929617	Alpha SNAP	0.007	35.6	33.6	5.2	7	MALDI-ToF MS
1195	ANXA5	gi|113960	Annexin A5	7.4E-6	9.06	35.9	4.78	3	LC-MS/MS
	ANXA5	gi|49168528	Annexin A5	.003	24.7	36	4.9	8	MALDI-ToF MS
1225	PSME2	gi|18203506	Proteasome activator complex subunit 2	4.4E-6	14.64	27.3	5.3	4	LC-MS/MS
	PSME2	gi|6048403	Proteasome activator PA28 beta	0.001	24.3	27.5	5.4	6	MALDI
1270	CLIC4	gi|20141285	Chloride intracellular channel protein 4	4.2E-8	10.28	28.7	5.3	2	LC-MS/MS
		gi|75766221	Chain A, Crystal Structure Of A Soluble Form Of Clic4	0.007	31.1	30.5	5.7	6	MALDI-ToF MS
1277	PHB	gi|4505773	Prohibitin	0	36	29.8	5.6	8	MALDI-ToF MS
1303	PSME1	gi|1170519	Proteasome activator complex subunit 1	5.1E-9	27.71	28.7	5.7	6	LC-MS/MS
	PSME1	gi|30581141	Proteasome activator subunit 1 isoform 2	0	41.2	28.7	6.3	14	MALDI-ToF MS
1336	YWHAB	gi|78101741	Chain A, 14-3-3 Protein Beta	0	35.5	28.3	4.8	9	MALDI-ToF MS
1359	HSPB1	gi|19855073	Heat shock protein beta-1	1.3E-14	49.27	22.7	5.9	9	LC-MS/MS
	HSPB1	gi|4504517	Heat shock 27kDa protein 1	0.002	32.7	22.8	6.0	6	MALDI-ToF MS
1363	PNPO	gi|8922498	Pyridoxine 5'-phosphate oxidase	0	23.8	30.3	6.6	5	MALDI-ToF MS

1366	HSPB1	gi|19855073	Heat shock 27 kDa protein1	4.6E-9	45.37	22.7	5.9	8	MALDI-ToF MS
	HSPB1	gi|4504517	Heat shock 27kDa protein 1	0.002	32.7	22.8	6.0	6	MALDI-ToF MS
1386	HSPB1	gi|19855073	Heat shock 27 kDa protein1	7.8E-15	60.49	22.7	5.9	9	LC-MS/MS
	HSPB1	gi|4504517	Heat shock 27kDa protein 1	0.004	29.3	22.8	6.0	5	MALDI-ToF MS
1406		gi|116875831	Regulator of microtubule dynamics 1	0	27.4	36.0	8.9	7	MALDI-ToF MS
1408	HSPB1	gi|19855073	Heat shock 27 kDa protein1	2.9E-10	57.56	22.7	5.9	8	LC-MS/MS
	HSPB1	gi|4504517	Heat shock 27kDa protein 1	0.003	30.2	22.8	6.0	5	MALDI-ToF MS
1417	HSPB1	gi|19855073	Heat shock 27 kDa protein1	4.1E-13	40.49	22.7	5.9	7	LC-MS/MS
	HSPB1	gi|4504517	Heat shock 27kDa protein 1	0.009	44.4	22.8	6.0	7	MALDI-ToF MS
1469	GSTP1	gi|121746	Glutathione S-Transferase P1- 1[v104] Complexed With (9r,10r)-9-(S-Glutathionyl)-10- Hydroxy-9,10-Dihydrophenanthrene	4.2E-5	10.9	23.8	5.3	3	LC-MS/MS
	GSTP1	gi|2554831	Glutathione S-Transferase P1- 1[v104] Complexed With (9r,10r)-9-(S-Glutathionyl)-10- Hydroxy-9,10-	0.004	40.5	23.5	5.4	5	MALDI-ToF MS

			Dihydrophenanthrene						
1472	SAR1B	gi|7705827	SAR1a gene homolog 2	0	42.4	22.5	5.7	9	MALDI-ToF MS
1714	DIABLO	gi|18203316	Diablo homolog, mitochondrial precursor	2.4E-7	22.59	21.1	5.6	5	LC-MS/MS
1786	NME1	gi|127981	Nucleoside diphosphate kinase A	1.2E-10	46.71	17.1	5.7	7	LC-MS/MS
	NME1	gi|4557797	Non-metastatic cells 1, protein	0.008	31.6	17.3	5.8	4	MALDI-ToF MS
2353	S100A9	gi|115444	S100-A9	8.2E-8	42.6	18.4	5.6	4	LC-MS/MS
2482	S100A16	gi|20178113	S100-A16	1.7E-9	44.6	11.7	6.3	4	LC-MS/MS
	S100A16	gi|17933772	S100 calcium binding protein A16	0.007	35	11.8	6.3	4	MALDI-ToF MS
2509	S100A14	gi|20178118	S100-A14	3.1E-9	35.58	42.3	5.3	4	LC-MS/MS
2659	S100A6	gi|116509	S100-A6	2.9E-4	42.2	17.7	5.2	4	LC-MS/MS
2668	S100A2	gi|114152869	S100-A2	2.5E-4	28.5	11.1	4.5	2	LC-MS/MS