University of Alberta

Mechanisms of MUC1/ICAM-1 signaling in Breast Cancer Metastasis

by

Ashlyn Jolene Bernier

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Medical Sciences

Laboratory Medicine and Pathology

Fall 2011
Edmonton, Alberta

Dedication

I dedicate this Thesis to my parents, Randy and Darcey Bernier. Their unwavering support and unconditional love have allowed me to learn from my mistakes, embrace every opportunity, and believe in my limitless potential. Everything I have accomplished is because of you.

Abstract

Breast cancer is the second leading cause of cancer death in Canadian women. In these patients, mortality is due to metastasis of cancer cells from the breast to distant organs, resulting in impairment of function. To metastasize, cells must move through the stroma of the breast, enter the circulation, survive transit, exit the circulation, and form a secondary tumor. A critical component of this metastatic cascade is cancer cell motility. It is not fully understood how breast cancer cells gain the ability to move or what signaling pathways mediate these events, and identification of critical components of these pathways would represent potential targets for anti-metastatic therapies.

The MUC1 glycoprotein is expressed on the apical membrane of normal breast epithelia. In many human breast carcinomas, MUC1 is overexpressed and loses apical polarization, events that correlate with increased metastasis. The contribution of MUC1 overexpression to increased metastasis is not completely understood, with the majority of studies attributing an anti-adhesive role to MUC1. Several critical steps of the metastatic cascade require cell adhesion, and it has been reported that MUC1 is a ligand for ICAM-1, which is expressed throughout the migratory tract of a metastasizing breast cancer cell. It was subsequently reported that MUC1/ICAM-1 binding initiates calcium oscillations, cytoskeletal reorganization, and cell migration, suggesting that binding could be important in metastasis.

Here, we investigate the mechanism of MUC1/ICAM-1 binding induced signaling. We show that Src kinase is a critical component of the MUC1/ICAM-1 signalling axis, and that MUC1 forms constitutive dimers which are required for Src recruitment and ICAM-1 binding induced signaling. We show that MUC1 dimers are not covalently linked and do not require cytoplasmic domain cysteine residues, contrary to other reports. We show that MUC1 extracellular domain shedding is not required for

dimerization, Src recruitment, or ICAM-1 binding induced calcium oscillations, although it is required for migration. Lastly, we show that autoproteolytic cleavage of MUC1 is not required for normal function. These results reveal information on the mechanism of MUC1/ICAM-1 signalling, which can be used to identify novel targets and combinational strategies for anti-metastatic therapy in breast cancer.

Acknowledgements

This work would not have been possible without the support of the University of Alberta, Canadian Breast Cancer Society, and United States Department of Defense.

I would like to express heartfelt thanks to my supervisor, Dr. Judith Hugh, for her guidance, patience, and support during the duration of this project.

Thank you to the members of my supervisory committee, Dr. Andrew Shaw and Dr. X. Chris Le, for their insight, support, and advice on this project and my professional development.

Thank you to past and current students Dr. Jennifer Rahn, Dr. Qiang Shen, and Nirosha Gunasekara for advice and guidance on this project.

Thank you to Jing Zhang, Maxine Farr-Jones, Brian Maciejko, and Lindsay Finnie-Carvalho for excellent technical support.

Special reagents and cell lines, without which this work could not be completed, were made available by Dr. K.C. Kim, Dr. Erik Lillehoj, Dr. Don Fujita, Dr. Tony Pawson, Dr. Sandra Gendler, Dr. Ken Dimock, ICOS Corporation, and ARIAD Pharmaceuticals.

Thank you to Dr. Jason Acker, Dr. Jonathan Martin, and all academic and non-academic staff in the Department of Laboratory Medicine and Pathology who have supported me in countless ways during my program.

Thank you to my friends and family for moral support, encouragement, and listening when I needed to share both frustrations and successes.

And lastly, thank you to Hart Stadnick, for always telling me what I need to hear whether I like it or not. I love you with all my heart.

Table of contents

Table of contents continued

Page

Table of contents continued

Table of contents continued

Table of contents continued

Table of contents continued

Table of contents continued

List of Figures

List of Figures continued

List of Figures continued

List of Abbreviations

aa- Amino acid

Ab – Antibody

ADAM10 – A disintegrin and metalloprotease-10

ADAM17 – A disintegrin and metalloprotease-17

ADP – Adenosine diphosphate

AP – Ammonium persulfate

ATCC – American type culture collection

ATP – Adenosine triphosphate

Ca^{2+} - Calcium

CaM – Calmodulin

CaOs – Ca^{2+} oscillation

CaMKII – CaM-dependant protein kinase II

CAM – Cell adhesion molecule

CD – Cytoplasmic domain

Chk – Csk homology kinase

CMFDA – 5-chloromethylfluorescein diacetate

COPD – Chronic obstructive pulmonary disorder

Csk – C-terminal Src kinase

DIC – differential interference contrast

DMEM – Dulbecco's modified eagle medium

DRM – detergent resistant membrane

DSS – Disuccinimidyl suberate

ECD – Extracellular domain

ECL – Enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

EGFR – Epidermal growth factor receptor

EMA – Epilthelial membrane antigen

EMT – Epithelial – mesenchymal transition

ER – Estrogen receptor

ERK – extracellular signal regulated kinase

ETA – Epithelial tumor antigen

F-actin – Filamentous actin

FAK – Focal adhesion kinase

List of Abbreviations continued

FBS – Fetal bovine serum

FITC – fluorescein isothiocyanate

FKBP – FK506 binding protein

G418 – Geneticidin

G-actin – Globular actin

GalNAc – *N*-acetylgalactoseamine

GalNAcT – *N*-acetylgalactoseaminyltransferase

GPCR – G-protein coupled receptor

GDP – Guanosine diphosphate

GlcNAc – *N*-acetylglucosamine

GlcNAcT – *N*-acetylglucosyltransferase

GTP – Guanosine triphosphate

HA – Hemagglutinin

HEK – human embryonic kidney epithelial cells

HER2 – Human epidermal growth factor receptor-2

HRP – Horseradish peroxidase

ICAM – Intercellular adhesion molecule

IL – Interleukin

INF – Interferon

IP3 – Inositol (1,4,5) triphosphate

IP3R – IP3 receptor

KLF4 – Kruppel-like factor 4

LSB – 4X Laemmli sample buffer

mAb – Monoclonal antibody

MβCD – methyl-betacyclodextrin

MEA – Membrane epithelial antigen

MEF – Mouse embryonic fibroblasts

MCA – Membrane carcinoma antigen

miRNA – MicroRNA

MLCK – Myosin light chain kinase

MMTV-PyV MT – Mouse mammary tumor virus-driven polyoma middle T-antigen

MT1-MMP – Membrane type-1 matrix metalloprotease

PAGE – Polyacrylimide gel electrophoresis

List of Abbreviations continued

PDGF – Platelet derived growth factor

PEM – Polymorphic epithelial mucin

PG – Protein G

PH – Pleckstrin homology

PI3K – Phosphatidylinositol 3-kinase

PIP2 – Phosphatidylinositol (4,5) bisphosphate

PKC – Protein kinase C

PLC – Phospholipase C

PMA – Phorbol 12-myristate 13-acetate

PR – Progesterone receptor

PSG1 – P-selectin glycoprotein-1

PTP – Protein tyrosine phosphatase

PUM – Peanut-reactive urinary mucin

ROI – Region of interest

RT – Room temperature

RTK – Receptor tyrosine kinase

SDS – Sodium dodecyl sulphate

SEA – Sea urchin, enterokinase, and agrin

SFK – Src family non-receptor tyrosine kinase

SH – Src homology

SHP – Src homology region 2 domain containing kinase

sialylT – sialyltransferase

siRNA – Small interfering ribonucleic acid

$sLe^{a/x}$ – Sialyl Lewis a/x

SYF – Src/Yes/Fyn

TBS – Tris-buffered saline

TBS-T – TBS with 0.05% Tween-20

TEM – Transendothelial migration

TF – Thomsen-Friedenreich antigen

TLDU – Terminal lobular – ductal unit

TMD – Transmembrane domain

TNF – Tumor necrosis factor

Tris-HCl – Trizma hydrochloride

List of Abbreviations continued

Tween-20 – polyoxyethylene-sorbitan monolaurate

VCAM – Vascular cell adhesion molecule

VNTR – Variable number tandem repeat

WCL – Whole cell lysate

Chapter 1. Introduction

1.0. Thesis overview

Breast cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related mortality in Canadian women with an estimated 5,300 deaths in 2010 [1]. In these patients, mortality was due to metastasis of cells from the primary breast tumor to distant sites such as bone, liver, and brain, where metastatic tumors form, leading to impairment of organ function [2, 3]. In order to generate secondary tumors, cancer cells must complete several steps in the metastatic cascade, including movement through the stroma, entry into circulation, survival during transit, and movement through endothelial cells and the basement membrane at distant sites. A key requirement during this process is cell motility, a tightly orchestrated process involving numerous cell signalling pathways, actin cytoskeletal reorganization, and disruption and formation of focal adhesions between the migrating cell and underlying substratum [4]. It is not clear how breast cancer cells develop the ability to move, or what proteins and signalling pathways mediate each step. Clarification of the mechanism(s) underlying the inappropropriate or persistent activation of migratory signalling in breast cancer cells is a critical step in the development of therapies which can be used clinically to reduce breast cancer related mortality.

The MUC1 transmembrane glycoprotein is expressed on the apical membrane of normal breast epithelia and consists of a heavily glycosylated extracellular domain (ECD), single pass transmembrane domain (TMD), and a 72-amino acid (aa) cytoplasmic domain (CD). In many human breast carcinomas, MUC1 is overexpressed, underglycosylated and loses apical polarization, events that correlate with increased metastasis [5-7]. A large body of research has emerged investigating the role of MUC1 in carcinogenesis, cell survival, motility, and growth, with several promising clinical developments investigating the potential for MUC1 in cancer detection and therapy [8-

10]. However, the correlation between increased MUC1 expression and increased metastasis is not completely explained by the majority of existing studies that attribute an anti-adhesive role to MUC1 due to its large, negatively charged ECD. Although anti-adhesive properties would contribute to cell motility at certain points in cancer metastasis, other steps require cell-cell adhesion, a role that is at odds with MUC1's proposed anti-adhesive function. In this regard, we were the first to report that MUC1-ECD binds to ICAM-1, a protein present on activated stromal and endothelial cells, key points in the path of a metastasizing breast cancer cell [11, 12]. Subsequently, we reported that MUC1/ICAM-1 binding leads to generation of cellular calcium oscillations (CaOs), cytoskeletal rearrangements, and cell motility in MUC1-transfected cells and in MUC1 expressing breast cancer cells [13-15]. The mechanism of MUC1/ICAM-1 induced signalling has not been fully described, although our previous studies implicated the activity of Src kinase, a non-receptor tyrosine kinase, in transduction of the ICAM-1 signal. Although the mechanism of Src recruitment and activation is unclear, Src targeted anti-cancer therapies have been developed and tested clinically, and there is great potential for the rational combination of drugs targeting multiple components of a single pathway [16-18]. Therefore, investigation of the role of Src kinase in transmission of the MUC1/ICAM-1 signal may lead to development of therapeutic strategies which effectively target breast cancer metastasis.

In recent years, a body of research has emerged indicating that MUC1 forms dimers which are dependent on membrane proximal cysteine residues [19]. Inhibitors of MUC1 dimerization have been shown to result in cancer cell death *in vitro* and *in vivo* [20-22], although the mechanism of MUC1 dimerization and the role of dimerization in other cell processes, such as metastasis, is unclear. As our reported MUC1 ligand, ICAM-1, exists as a dimer, it is plausible that MUC1 dimerization plays a role in transmission of

the ICAM-1 binding induced signal. Investigation of the role of MUC1 dimerization in transmission of the ICAM-1 signal could reveal additional clinical applications for the inhibitors of MUC1 dimerization already in existence, as well as provide targets for the development of novel therapies.

MUC1 undergoes several cleavage events, the roles of which in MUC1 signalling have not been described. Following translation of the MUC1 protein, conformational stress results in autoproteolytic cleavage, resulting in a protein consisting of two associated portions at the cell surface [23]. In addition, MUC1 present at the cell surface is subject to cleavage by the proteases ADAM17 and MT1-MMP, releasing the ECD into the extracellular space [24, 25]. It has been reported that this cleavage is dependent on a post-translational cleavage, although the mechanism of this dependency is not understood [26]. As high levels of serum MUC1-ECD has been correlated with a poor prognosis, the role of these cleavage events in MUC1 signalling is of clinical importance [27, 28].

In this study, we investigate the importance of Src kinase, MUC1 dimerization, and MUC1 cleavage in transmission of the ICAM-1 binding induced signal. We report that Src kinase is a critical component of the MUC1/ICAM-1 signalling pathway, highlighting the importance of investigation of the mechanism of Src recruitment and activation. MUC1 dimers form constitutively, are not covalently linked, and are independent of membrane proximal cysteine residues, contrary to previous reports. Inhibition of MUC1 dimerization results in decreased Src binding and disruption of ICAM-1 binding induced CaOs and cell migration. Further, we report that inhibition of MUC1-ECD shedding does not interfere with dimerization, Src recruitment, or ICAM-1 binding induced CaOs, although it does disrupt ICAM-1 binding induced migration. We also investigated the activity of two MUC1 mutants, which do not undergo autoproteolytic cleavage, in ICAM-1 binding induced signalling, and we found that

although autoproteolytic cleavage is not required for transmission of the ICAM-1 signal, the nature of the mutation conferring cleavage resistance is a determinant of protein activity. We interpret these findings to indicate that MUC1 may dimerize by distinct mechanisms in different cellular contexts, although dimerization is clearly an important factor in MUC1 function. Importantly, we report that MUC1 dimerization is required for Src recruitment and ICAM-1 binding induced events, a novel and clinically significant finding, as inhibition of MUC1 dimerization therefore represents a potential target for anti-metastatic therapies. The discovery that MUC1-ECD shedding is required for ICAM-1 binding induced cell migration, but not CaOs, provides both an additional potential therapeutic target as well as information on the potential mechanism of ICAM-1 induced signalling. We conclude our study with a proposed mechanism of MUC1/ICAM-1 signaling, which will provide hypothesis for future studies and potential targets for clinical therapies.

1.1. Breast cancer

1.1.1. The normal breast

The structure and function of a normal adult breast is complex and dynamic, changing with age, menstrual, and reproductive status. In both males and females, breasts begin to develop early in gestation with the appearance of ectodermal thickenings known as the "milk lines", which extend from the axilla to the medial thigh [29]. These thickenings will regress with the exception of the region over the fourth intercostal space, which will eventually develop into the breast. By the end of gestation, the mammary epithelium will have invaginated into the underlying stroma, developing into rudimentary ductal trees. Interestingly, this stage of ductal development occurs independently of hormonal stimulus, and occurs normally in mice deficient in estrogen, progesterone,

prolactin, and growth hormone receptors, all which contribute to later stages of ductal development [30, 31].

The mammary gland established *in utero* is quiescent until puberty. In females, onset of menstruation initiates a series of structural and functional changes in the breast tissue. Hormonal influences result in a period of mammary growth, with deposition of fat and connective tissue occurring concurrently with elongation and further branching of the ductal systems established *in utero* [30]. At the terminus of each duct, an alveolar "bud" will develop, consisting of division-competent stem cells which can differentiate into epithelium and myoepithelium, and are the initiation point for branching [32]. Following this period of growth and development, the mammary gland will be organized into terminal lobular – ductal units (TLDU) within the stromal tissue (Fig 1.1), which are capable of milk secretion. With each menstrual cycle, new alveolar buds form, resulting in a highly developed TLDU by age 35, with up to 80 alveoli per TLDU.

Full functionality of the mammary gland is only achieved under the influence of pregnancy and lactation. During pregnancy, hormonal changes result in growth of the breast and an increase in the proportion of glandular tissue. Following parturition, withdrawl of progesterone and high levels of prolactin stimulate the production of milk. Lactation is maintained by the action of prolactin on the luminal epithelial cells, stimulating production of milk, and oxytocin on the myoepithelial cells, stimulating ejection of milk [33]. Upon termination of breastfeeding, glandular involution occurs, with cell death and remodelling of the mammary tissue to nearly its pre-pregnancy state. Following menopause, lack of stimulation by estrogen and progesterone results in involution of the mammary gland, shortening of the ducts, atrophy of the lobules, and a predominance of fatty tissue.

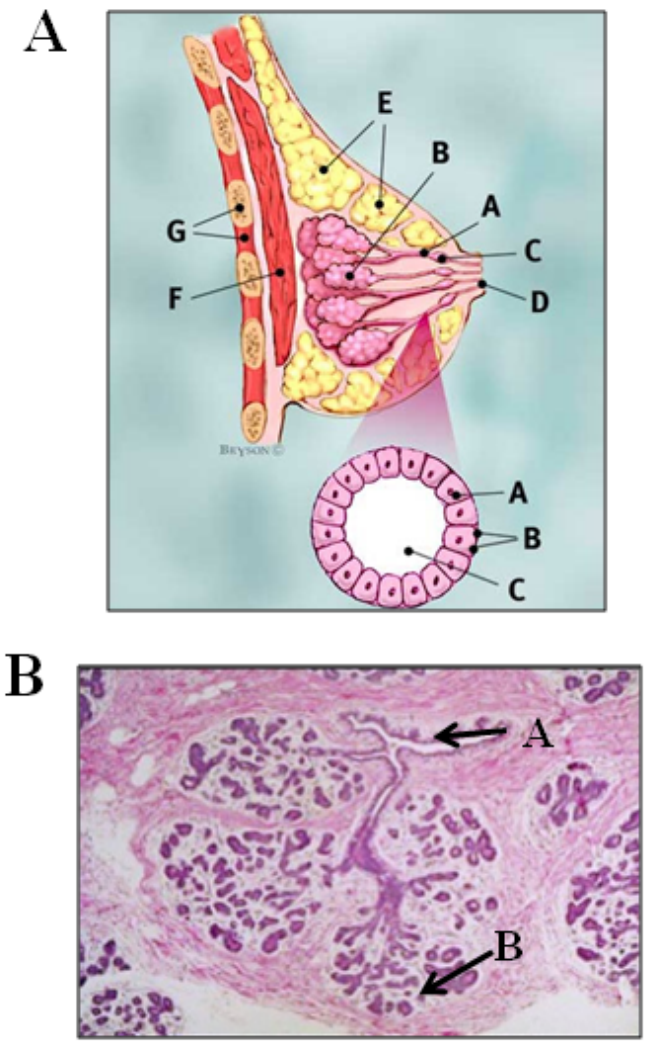


Figure 1.1. The normal human mammary gland. A. Schematic of the normal human breast. Breast profile: (A) Ducts (B) Lobules (C) Sinus (D) Nipple (E) Fat (F) Pectoralis major muscle (G) Chest wall/rib cage. Enlargement: (A) Normal duct cells (B) Basement membrane (C) Lumen. Adapted from http://www.breastcancer.org/symptoms/understand_bc/what_is_bc.jsp. B. Histology of the normal mammary gland. (A) Ducts (B) Terminal lobules. Adapted from http://www.breastpath.net/page1/page1.html.

Various cell types are represented in the mature breast. The ducts and lobules consist of the luminal epithelial cells and basal myoepithelial cells, which form a net-like structure around alveoli. A basement membrane surrounds the epithelial and myoepithelial cells of the TDLUs, consisting of extracellular matrix proteins [34]. The breast stroma consists primarily of fibroblasts, which have the potential to differentiate into myofibroblasts following injury [35] and adipocytes. The breast also contains vascular smooth muscle and endothelial cells of the blood and lymphatic systems, with each vessel surrounded by a basement membrane.

1.1.2. The cancerous breast

1.1.2.1. Classification of breast cancer

Breast cancer was the most diagnosed malignancy in Canadian women in 2010, with an estimated 23,200 diagnosed and 5,300 deaths [1]. Breast cancer originates from stem cells of the ductal epithelia, and can be histologically classified as ductal-like (ductal carcinoma) or lobular-like (lobular carcinoma), and were historically classified based on this differentiation [36]. Ductal carcinomas account for approximately 80% of diagnosed breast cancers, tend to form more glandular structures, and proliferate more rapidly [37]. Lobular carcinomas are hallmarked by loss of E-cadherin expression, and are often estrogen receptor (ER) and progesterone receptor (PR) positive [38].

To completely classify a breast cancer sample, the sample is fixed, sectioned, and stained, usually with a hematoxylin and eosin stain. The sections are then examined and assayed for several standardized parameters. Grading focuses on the appearance of the cancer cells compared to normal breast tissue, and is an indication of the level of differentiation of the tumor, with poor differentiation indicating a worse prognosis [39]. Further examination of the tumor sample, as well as of lymph nodes in the axilla, can

determine tumor stage, or level of progression, which is discussed in Section 1.1.2.2, and is also a predictor of prognosis [40]. Lastly, tumor samples are also analyzed histologically for expression status of certain receptors, namely ER, PR and HER2/neu/ErbB2. Determination of the presence of these receptors is indicative of prognosis and will direct management and treatment course [41-43].

In recent years, maturity of the field of tissue molecular analysis has made it apparent that breast cancer is actually a group of many diseases, and classification should reflect this. A paradigm shifting paper by Perou et al. [44] showed that microarray analysis of breast tumors resulted in the emergence of six main groups, based on differences in global gene expression. The initial division is based on ER receptor status, with ER-positive tumors being further subdivided into Luminal A and a worse prognosis Luminal B. ER-negative tumors are divided into ErbB2 positive, and a basal-like subtype characterized by being ``triple-negative`` for ER, PR and ErbB2. Recently, another subtype, claudin-low, has been identified [45] and characterized [46]. Further analysis revealed that these different subtypes have distinct rates of incidence [47, 48], survival [44], sites of metastasis [49], and response to certain chemotherapies [46, 50-53]. This evolution of breast cancer diagnostics has allowed for the rational and intelligent treatment of breast cancers based on their biology.

1.1.2.2. Progression of breast cancer

All cancers are initially ***in situ***, meaning the tumor mass has not yet penetrated through the basement membrane and into the stromal tissue [54]. At this stage, the tumor is too small to be detected by breast examination, although mammography may detect deposits of calcium within the tumor mass, which occurs when calcium salts from the bloodstream diffuse into necrotic areas within the tumor [55]. Eventually, the tumor mass

will invade through the myoepithelial cells and basement membrane into the stroma, at which point the cancer is classified as **invasive** (Fig 1.2). Invasive carcinoma is of particular clinical significance due to the potential for the tumor cells to enter the blood or lymphatic systems and metastasize to other organs in the body, a process which is discussed in detail in Section 1.1.3. Establishment of metastatic breast cancer cells at secondary sites, such as lung, liver or brain, results in loss of functionality and subsequent patient mortality.

1.1.3. Metastasis of breast cancer

1.1.3.1. The metastatic cascade

A well defined sequence of events must occur in order for cells from a primary tumor mass to metastasize to a distant site in the body (Fig 1.3). This complex sequence of steps begins with major changes in the phenotype of cancer cells within the primary tumor. In order to metastasize, breast carcinoma cells of epithelial origin must aquire the ability to *invade* the surrounding stroma, a process requiring motility. In order for breast cancer cells to invade the stroma, they must lose the epithelial phenotype and shift to a more fibroblastic or mesenchymal phenotype. This process, termed the epithelial-mesenchymal transition (EMT), is also involved in non-pathologic events such as embryonic development and wound healing [56]. Several classes of proteins have been implicated in EMT, namely extracelluar matrix components, adhesion proteins, DNA binding proteins, and growth factors [57]. Specifically, loss of E-cadherin expression and cell polarity [58], and upregulation of N-cadherin, P-cadherin [59] and matrix metalloproteases [60-62] are hallmarks of EMT.

As breast cancer cells move through the stromal tissue, they may encounter a blood or lymphatic vessel. Movement of the cell through the basement membrane and

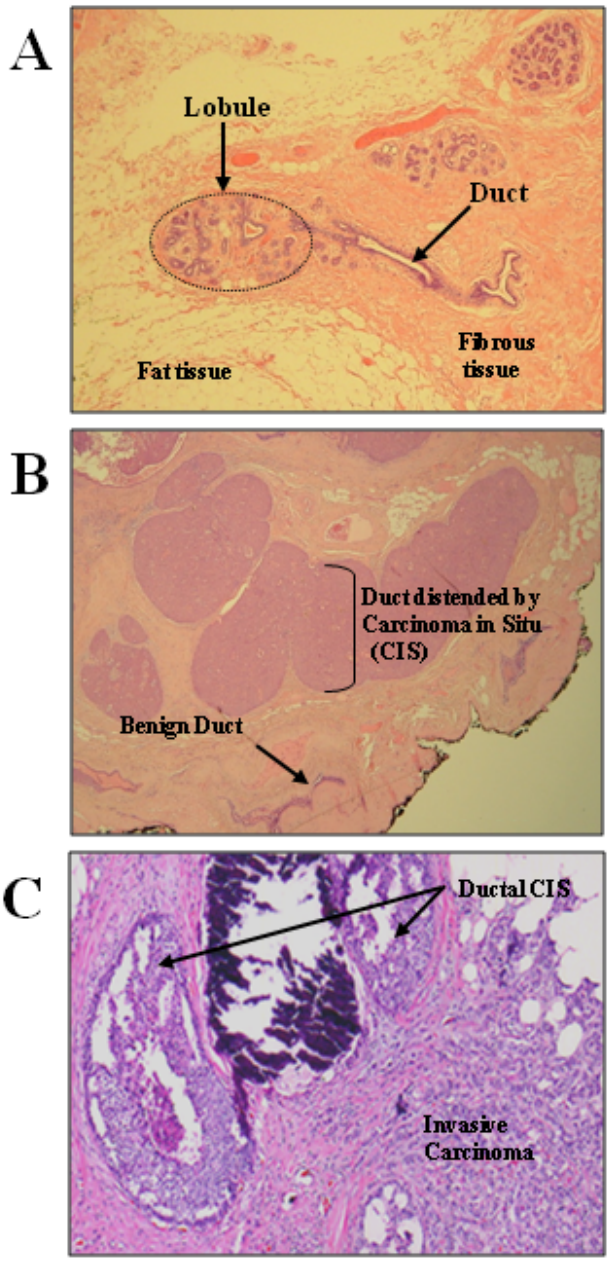


Figure 1.2. Histology of breast cancer subtypes. Photomicrographs of (A) Normal breast. (B) Ductal carcinoma *in situ*. (C) Invasive ductal carcinoma. Samples were stained with Hematoxylin and Eosin. Pictures courtesy of J. Hugh.

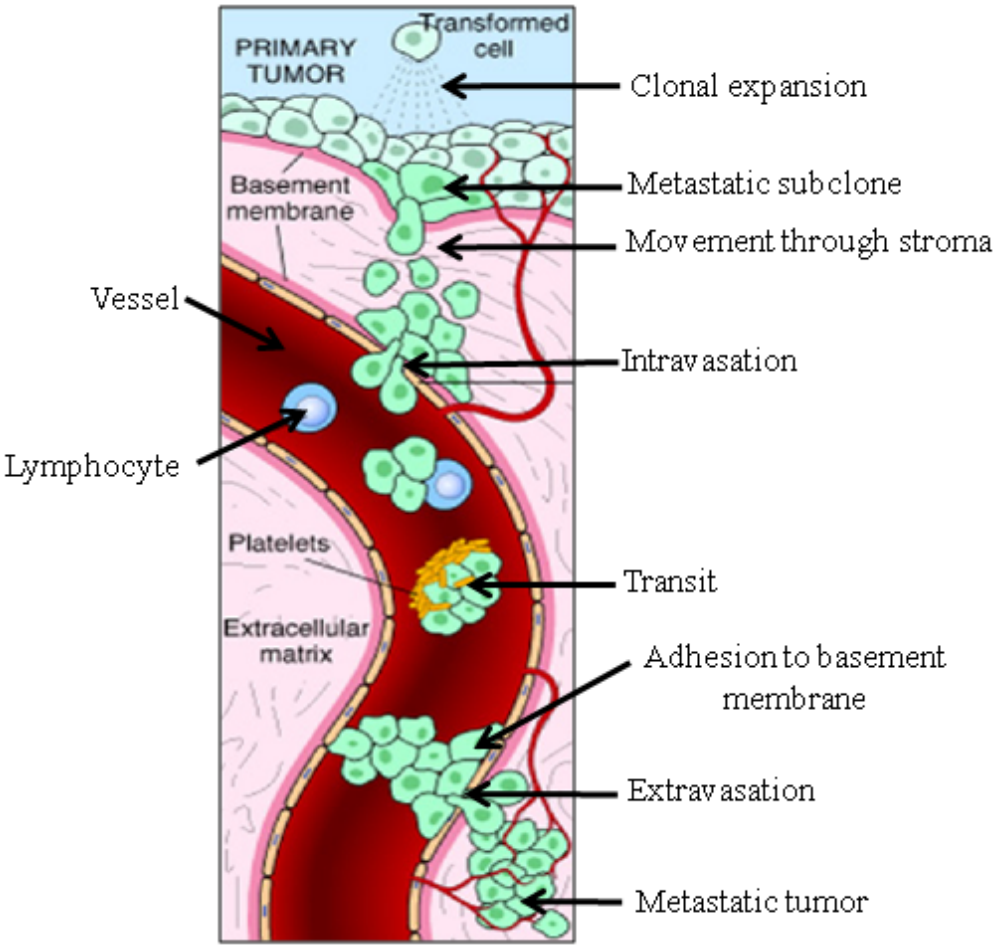


Figure 1.3. The metastatic cascade. Adapted from http://www.aquaticpath.umd.edu/appliedtox/carcinogenesis2.pdf

endothelial cells of the vessel is a process termed *intravasation*, and is the next step in metastasis. In the lumen of the vessel, cancer cells are subjected to hostilities that may result in cell death. For example, loss of stromal support may result in anokis, a form of apoptosis triggered by loss of cell attachment. Also, stromal cells in the primary tumor environment support survival and metastasis of tumor cells by upregulating expression of a variety of genes involved in angiogenesis, invasion, and growth [63-67]. Additionally, the dangers of both hemodynamic shear stress and immune surveillance are unavoidable for the circulating cancer cell.

If a metastasizing cancer cell survives transit through the circulation, it can potentially adhere to the endothelial cells of a vessel wall and *extravasate* into a secondary site. Tumor cell preference for the site of extravasation and the process of metastatic tumor establishment is not entirely understood. Breast cancer most commonly metastasizes to bone, lung, liver and brain, with different prognosis [68] and genetic profiles [49, 69, 70] between sites. Although the mechanism behind this bias is not known, several theories have been proposed. In 1889, Stephen Paget's "seed and soil" theory postulated that it is not only the tumor cell ("seed") which determines the site of metastasis, but also the microenvironment of the metastatic site ("soil"). Basically, only tissues which provided the appropriate setting for a specific tumor cell would be susceptible to metastasis by that tumor [71]. Another theory proposes that specific ligand-receptor interactions direct circulating cells to certain "addresses" within the body. Human breast cancer cells have increased expression of chemokine receptors CXCR4 and CCR7, and sites of preferential breast cancer metastasis express high levels of the respective ligands CXCL12 and CCL21 [72]. Therefore it is plausible that circulating breast cancer cells will arrest upon transit through these organs and increase the likelihood for extravasation and development of a metastatic tumor.

An additional theory proposes that cancer cells utilize a mechanism of extravasation similar to that of leukocytes, where a series of critical adhesions occur between the leukocyte and the activated endothelium (Fig 1.4) [73], resulting in leukocyte movement into tissue. Tissue inflammation results in the production of local factors which have several effects, including localized "activation" of the vessel endothelial cells by interleukins (IL) -1, -6, -8 and -12, and tumor necrosis factor (TNF)-α [74-76]. Activation of the endothelium results in upregulation of vascular adhesion proteins such as selectins, which can bind to sialyl Lewis a/x ($sLe^{a/x}$) containing glycoproteins on leukocytes such as P-selectin glycoprotein-1 (PSG1) [77]. This adhesion is weak, and results in leukocyte "rolling" across the endothelium. A more stable adhesion is mediated by endothelial cell adhesion molecules (CAMs) such as intercellular adhesion molecules (ICAM) and vascular adhesion molecule (VCAM), with expression stimulated by interleukin (IL)-1β, IL-4, TNF-α and interferon (INF)-γ [78]. β1- and β2-integrins on leukocytes bind to VCAM and ICAM, respectively, resulting in arrest of the rolling leukocyte [79, 80]. Following firm adhesion, leukocytes will undergo cell spreading, invadapodia formation, and transendothelial migration (TEM) [81].

Many parallels can be found between the adhesion molecules and receptors used by leukocytes and metastasizing cancer cells. The correlation between inflammation and cancer metastasis has been the subject of recent interest [82, 83], as many of the endothelial cell surface receptors recognized by cancer cells are a product of the inflammatory response. For example, the selectins used by leukocytes in initial attachment are also receptors for $sLe^{a/x}$ containing glycoproteins on cancer cells. Increased expression of $sLe^{a/x}$ structures has been correlated with cancer progression, and many cancer proteins, primarily of the mucin family, are known to have altered expression and structure of this and many other potential selectin ligands [84-86]. In

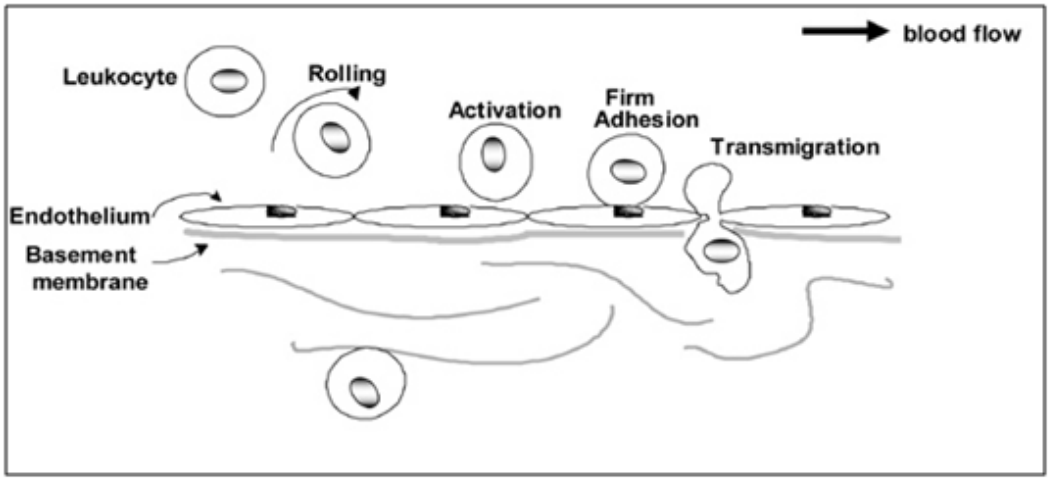


Figure 1.4. The multi-step model of leukocyte extravasation. Adapted from http://nieronline.org/index.php?title=Renal_Heparan_Sulfate_Proteoglycans.

addition to potential binding to selectins, many cancer cells also possess ligands for the CAMs involved in firm adhesion of leukocytes. ICAM-1 is a ligand for the mucin MUC1 [12, 87], a protein commonly overexpressed in many cancers of epithelial origin and associated with poor prognosis [88-91]. In addition, several endothelial cell integrins are receptors for cancer cell molecules [73]. Thus, the evidence for a leukocyte-like migration mechanism in cancer cell metastasis is accumulating and will guide both future research and therapeutic development [92].

1.1.3.2. Cell migration

Several steps of the metastatic cascade require cell motility, a complex and tightly regulated process. In the normal breast, epithelial cells are non-motile, and therefore breast carcinoma cells must develop the ability to move in order to metastasize. Cell movement can be precipitated by a variety of non-mutually exclusive factors, including environmental growth factors and cytokines, EMT, and escape from oxidative stress [93-95]. The currently accepted model of cell motility can be summarized as (1) protrusion of lamellipodium at the leading edge; (2) membrane anchoring to substratum; (3) disassembly of membrane anchors at the rear; and (4) contraction of cell from the rear [96, 97] (Fig 1.5).

In a resting cell, the actin cytoskeleton is in a steady state characterized by equilibrium between polymerisation of globular-actin (G-actin) at the "barbed" (+) end and depolymerisation at the "pointed" (-) end of filamentous-actin (F-actin) [93]. Initiation of cell motility via activation of growth factor receptors is largely controlled by members of a group of proteins belonging to the Rho family of guanosine triphosphate (GTP)-ases. Upon stimulation, guanosine diphosphate (GDP) is exchanged for GTP, resulting in an "active" state, facilitating downstream activation of effector molecules and

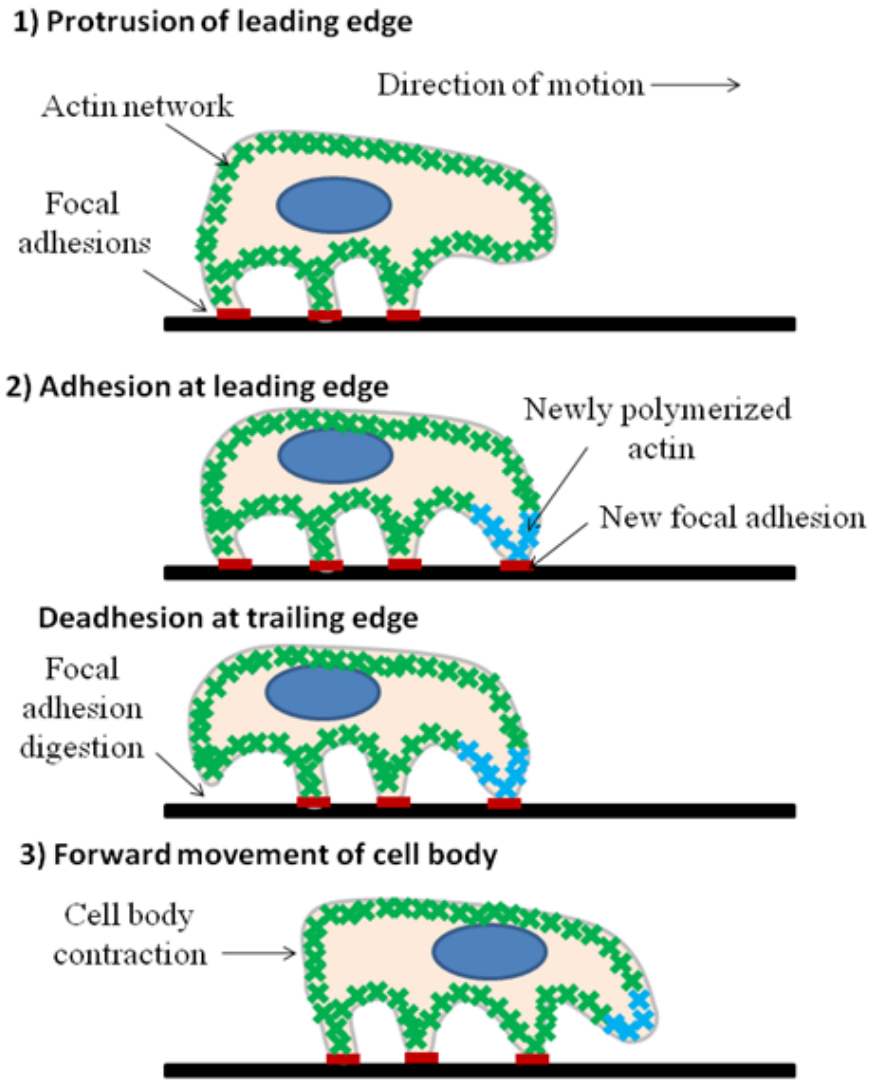


Figure 1.5. Schematic of the process of cell motility. Cell motility involves several, highly complex steps, beginning with protrusion of the cell body at the leading edge. This is followed for the simultaneous formation of new focal adhesions at the leading edge and digestion of focal contacts at the trailing edge. Cell body contraction results in forward movement of the cell body. Repeated cycles of these steps result in directional cell motility. Based on [96].

leading to reorganization of the actin cytoskeleton [98]. The main substrates for active Rho GTPases are the WAVE/WASP family of Arp2/3 complex activators [99], which facilitate actin polymerization at the leading edge by forming "nucleation cores", the first step in creation of a new filament. Rho family GTPases have been reported to be overactive in cancer cells [100], and increased expression of RhoA has been associated with breast cancer progression [101].

Actin filaments can adopt different conformations to generate either lamellipodia or filopodia. Lamellipodia are thin, broad protrusions that are the basis of directional migration, and are created by Arp2/3 mediated actin polymerisation and branching [102]. Filopodia, by contrast, are long, narrow protrusions that consist of actin filaments which are not branched. Activation of different members of the RhoGTPase family determines if lamellipodial or filipodial protrusions will develop [102]. Family member Rac initiates lamellipodia protrusive motility, while Cdc42 activation results in filipodial protrusions. Following protrusion formation at the leading edge, focal contacts must be established to anchor the cell membrane to the underlying substratum. Integrins, transmembrane receptors composed of non-covalently associated α and β subunits, are key components of this process as they can associate with numerous components of the extracellular matrix (ECM) [103]. An important characteristic of focal contacts is the ability for turnover, as contacts need to be established and dissolved rapidly for optimal cell migration [104]. The protein tyrosine kinase focal adhesion kinase (FAK) plays a major role in the generation of focal contacts, and can serve as a scaffold protein to bring other components within proximity, facilitating interactions [96]. FAK is activated by autophosphorylation on Y^{397} in response to integrin binding to ECM components, activation of growth factors receptors or G-protein coupled receptors (GPCRs), or phosphorylation at Y^{397} by activated Src kinase [105, 106]. This phosphorylation event

leads to recruitment of Src homology (SH) 2 domain containing proteins such as Src kinase, triggering a cascade of phosphorylation and recruitment events. Linkage to the actin cytoskeleton occurs through proteins such as talin and paxillin [107]. FAK and Src have been implicated in the progression and metastasis of colorectal [108], breast [109], and prostate [110] cancers, and several chemotherapeutic compounds have been developed to specifically inhibit this interaction [111-113].

In addition, other types of linkages exist between the actin cytoskeleton and components of the ECM, with differing components, function, and *in vitro* vs. *in vivo* existence [107]. Cell-cell adhesion (adherens junctions) is also controlled by Src kinase activity, as activated Src can induce degradation, cleavage, or dissociation of adherens junctions [114]. Increased Src activity has been correlated with decreased expression of E-cadherin, a critical component of adherens junctions, and decreased cell-cell adhesion [115].

Emerging evidence has demonstrated that calcium (Ca^{2+}) plays a critical role in actin cytoskeletal "treadmilling", which provides the protrusive force at the leading edge as well as retraction at the rear (Fig 1.6). Non-pathologic cell migration in mammalian leukocytes [116], spermatozoa [117], and neurons [118, 119] is controlled by Ca^{2+}, and actin assembly in myofibrillogenesis of *Xenopus* embryos is dependent on the generation of a Ca^{2+} oscillatory signal [120]. As a signalling mechanism, Ca^{2+} oscillation allows for specific activation of downstream effectors depending on frequency, amplitude and spatiality [121]. Calcium release is stimulated by activation of transmembrane receptors such as receptor tyrosine kinases (RTKs), GPCRs and integrins, resulting in local activation of phosphatidylinositol 3-kinase (PI3K) at the leading edge. PI3K can be activated by association with the active G-protein Ras or through association of the SH2 domain with a phosphorylated tyrosine [122]. The phosphoryated tyrosine residue can

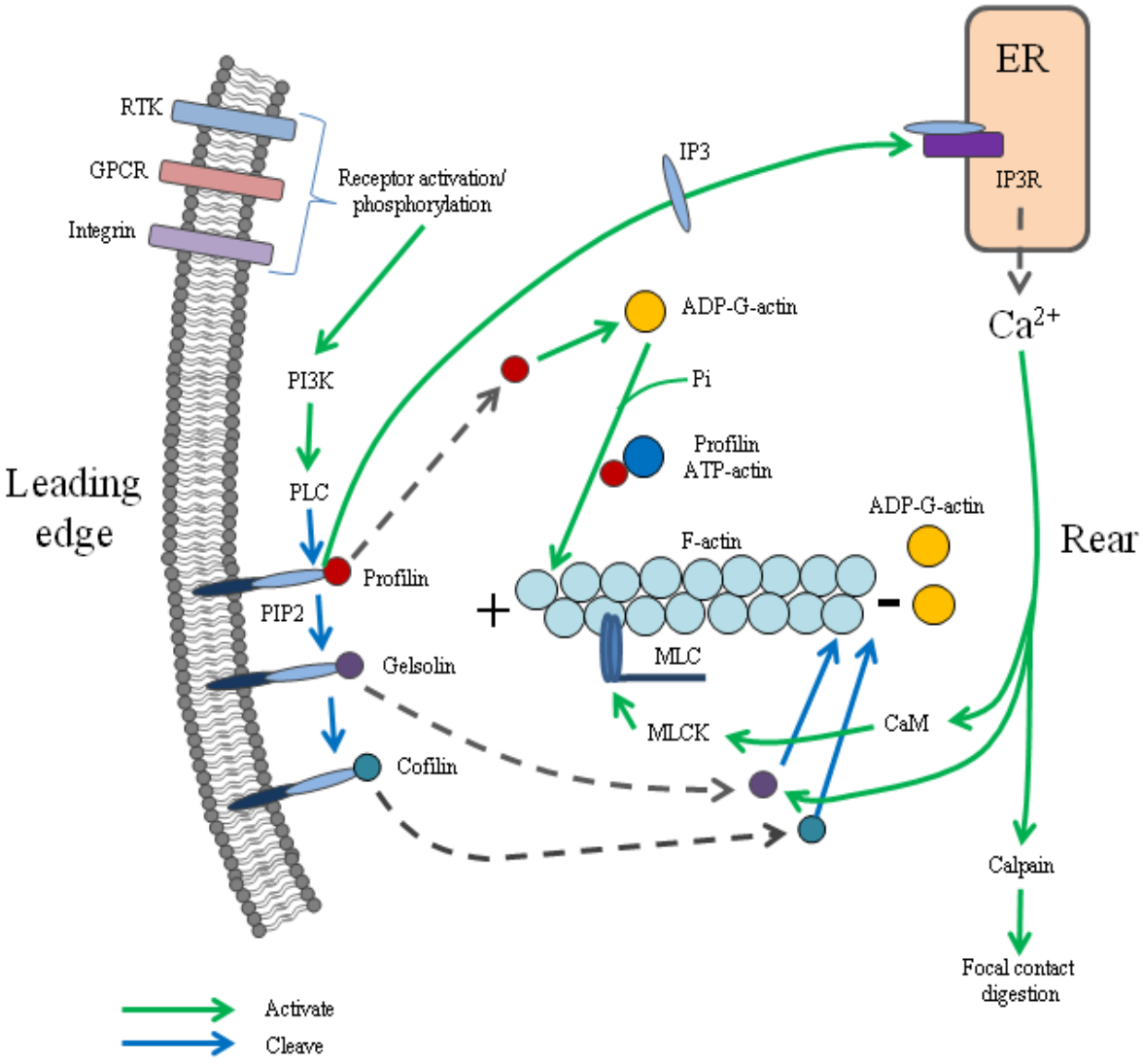


Figure 1.6. Actin cytoskeletal reorganization by PLC and calcium. Based on [96,102,126].

either be internal to the PI3K protein, with tyrosine-688 being a substrate for activated Src kinase [123], or present on an activated membrane anchored receptor such as platelet derived growth factor (PDGF) [122]. These interactions result in both PI3K activation and localization to the cell membrane, where its substrates are located. Phosphorylation of phosphoinosidtides by PI3K generates docking sites for proteins containing pleckstrin homology (PH) domains, such as phospholipase C (PLC) [96]. Active PLC can then hydrolyse phosphatidylinositol (4,5) bisphosphate (PIP2), generating several products, such as profilin, which facilitates G-actin adenosine diphosphate (ADP)/adenosine triphosphate (ATP) exchange, resulting in addition of G-actin to F-actin at the barbed end. Gelsolin and cofilin, two other proteins released by PLC activity, act to sever F-actin from the pointed end. Cleavage of PIP2 also generates inositol (1,4,5) triphosphate (IP3), which binds to the IP3 receptor (IP3R) on the endoplasmic reticulum, stimulating release of internal Ca^{2+} stores [124]. Further activation of PLC by Ca^{2+} results in the characteristic Ca^{2+} oscillation, which diminishes in amplitude as intracellular stores are depleted [125].

The impact of the Ca^{2+} oscillatory signal on cell activity is not completely understood and is the subject of much interest, as Ca^{2+} as second messenger is widespread in cells in spite of its potential toxicity [121, 126]. In the context of cell migration, Ca^{2+} is known to bind to calmodulin (CaM), facilitating activation of CaM-dependant protein kinase II (CaMKII) and myosin light chain kinase (MLCK), promoting cell contraction [96]. Ca^{2+} can also activate calpain, which targets components of focal contacts, releasing these contacts at the pointed end and causing forward movement. Other factors contributing to focal contact turnover include phosphorylation of FAK at S^{910} by extracellular signal regulated kinase (ERK), which inhibits FAK-paxillin binding and dissociates the ECM-actin cytoskeleton linkage. In addition, ERK upregulates proteases

involved in cleavage of extracellular and intracellular contacts [96], digesting mature contacts and allowing for forward movement of the cell body.

Investigation of the process of cancer cell motility, a critical step in the metastatic cascade, has revealed several potential targets for cancer therapeutics. In particular, the correlation between increased Src kinase activity and breast cancer metastasis has attracted much interest into the function of Src kinase in normal and pathologic states and the potential of Src inhibitors in a clinical setting [127-129].

1.2. Structure and function of the MUC1, ICAM-1 and Src molecules

1.2.1. The mucin family of proteins

Mucins are high molecular weight, heavily glycosylated proteins expressed on the epithelial luminal surfaces of the body. Their primary function in normal tissue is to form a protective gel-like coating, shielding epithelial surfaces from desiccation, pathogens, and other insults [130, 131]. Several of the known mucin family members lack a transmembrane domain (TMD) and are secreted from cells (MUC2, 5, 6, and 7), while others possess a single-pass TMD and cytoplasmic domain (CD), in addition to the large, negatively charged extracellular domain (ECD) (MUC1, 3, 4, 12, 16, and 17) (Figure 1.7). The ECD of transmembrane mucins is composed of a variable number of tandem repeats (VNTR); and MUC1 3, 12, 13 and 17 also contain a sperm protein, enterokinase, and agrin (SEA) domain, which contains an autoproteolytic cleavage site [132-134]. In these variants, immediately following translation, conformational stress results in fracture, followed by non-covalent reassociation and export to the cell surface. The physiological significance of this cleavage event has yet to be fully elucidated, but it has been hypothesized that this cleavage event permits rapid shedding of the ECD,

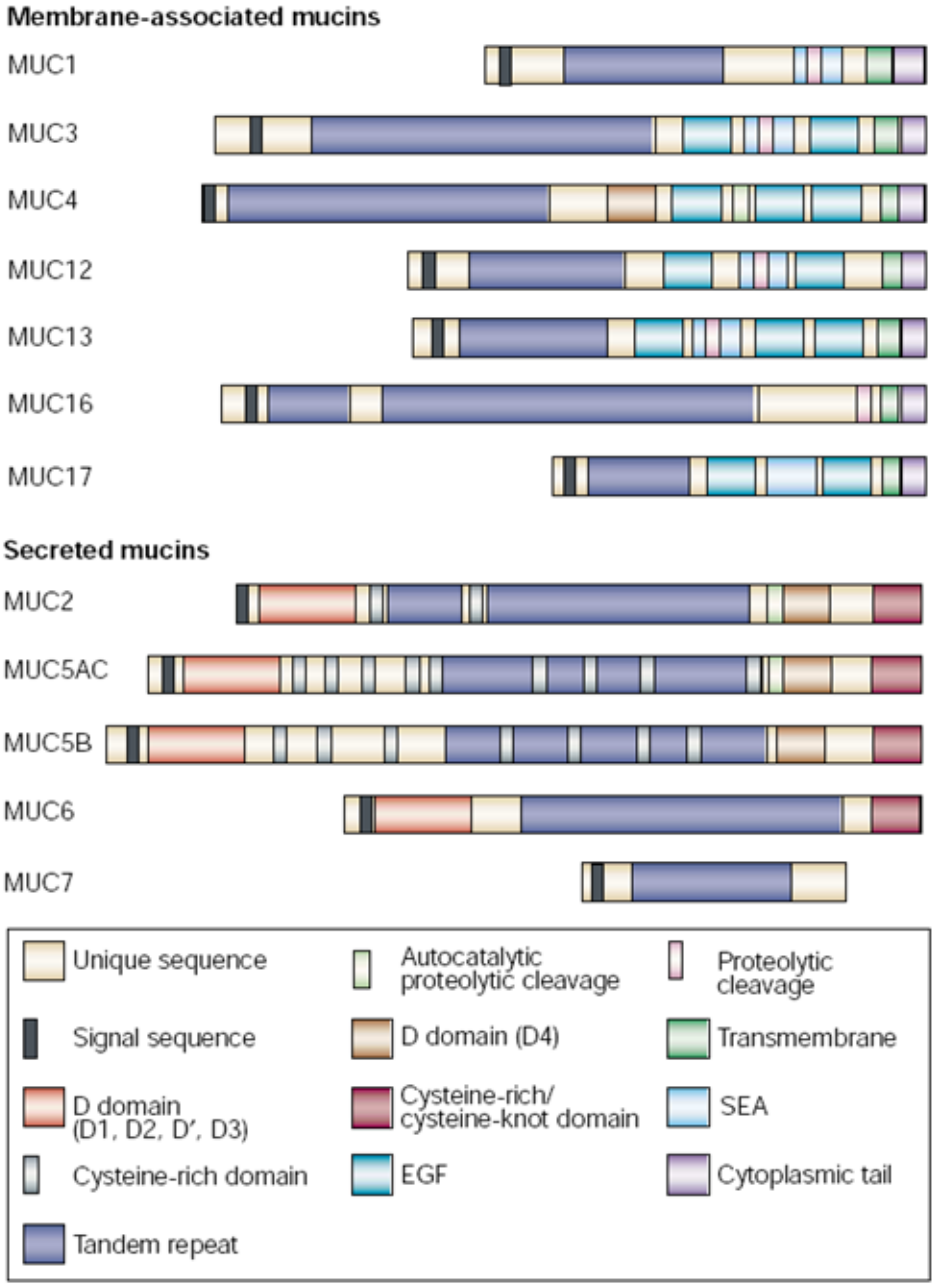


Figure 1.7. Structure of the mucin family glycoproteins.
Adapted from [131] with permission from the publisher.

translating extracellular conditions to the cytoplasm [8]. The potential mechanism and consequences of this cleavage event will be further discussed in Section 1.2.2.4.

Overexpression of membrane-tethered mucins has been implicated in several respiratory pathologies, such as cystic fibrosis, asthma, and chronic obstructive pulmonary disorder (COPD) [135]. Additionally, tethered mucins have been the subject of intense study over the last decade with reference to their role in cancer, and a clear relationship between overexpression and glycosylation changes and prognosis has emerged [136-142]. The most thoroughly understood mucin, MUC1, is overexpressed in breast cancer [7, 143, 144], has been implicated in tumor growth, metastasis and immune suppression [145-148], and will be discussed at length in Sections 1.2.2. and 1.2.3.

1.2.2. MUC1 structure

1.2.2.1. MUC1 Extracellular domain

MUC1 (also known as CA15-3, CD227, episialin, EMA, ETA, DF3, MEA, MCA, PEM and PUM) was initially purified and cloned from human breast milk [149, 150]. It consists of a large ECD, containing the SEA domain cleavage site, a single-pass TMD and a 72 amino acid (aa) CD. MUC1-ECD is highly O-glycosylated, with 50 to 90% of its mass due to carbohydrate moieties, and can weigh 250-500kDa depending on the number of VNTRs and level of glycosylation. The MUC1-ECD contains an N-terminal 23-aa signal sequence, VNTRs of a 20-aa sequence (Fig 1.8), with repeat number varying from 40-90 depending on allelic polymorphism, and flanked by degenerate repeats [151-153]. Each tandem repeat contains five serine and threonine residues, which are potential sites for the attachment of O-linked glycoproteins. This glycosylation imparts a negative charge to the MUC1-ECD, and results in a rigid structure that may extend 200-500nm from the cell surface [154, 155]. C-terminal to the

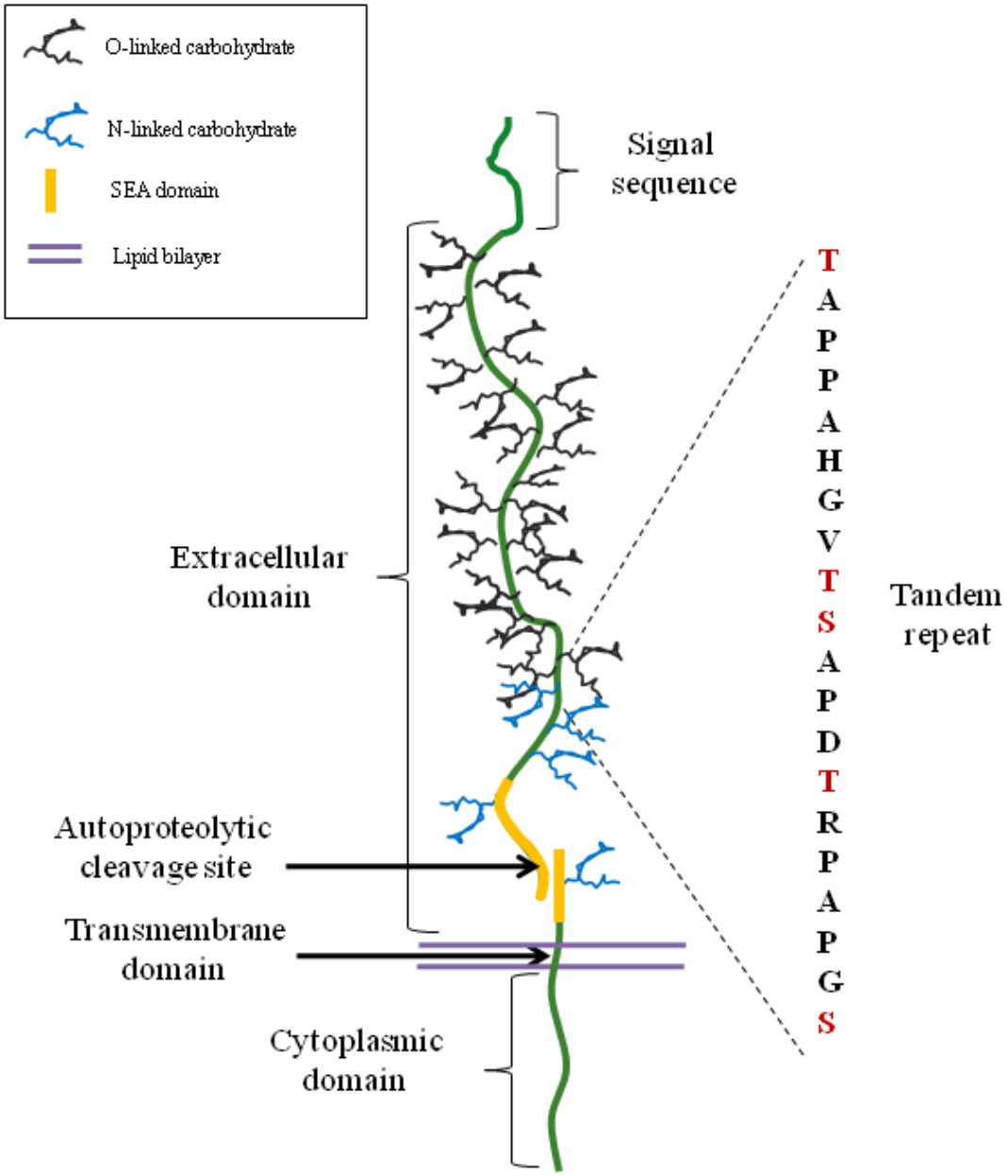


Figure 1.8. Schematic of MUC1 structure. From the N-terminus, MUC1 consists of a large extracellular domain containing a signal sequence, a VNTR region, and an autoproteolytic cleavage site. The 28-aa transmembrane domain is followed by a 72-aa cytoplasmic domain.

VNTRs is the 120-aa SEA domain, containing the autoproteolytic cleavage site. Following cleavage, the two subunits reassociate as a heterodimer [132] of the large, VNTR containing portion and the membrane-spanning portion, which contains 58 extracellular domain residues (Fig 1.8). The SEA domain and this cleavage event will be discussed further in Section 1.2.2.4. Glycosylation of MUC1 occurs in the Golgi complex, with N-glycosylation potentially occurring on five sites C-terminal to the tandem repeat region [153, 156] (Fig 1.8). O-glycosylation of the VNTR region is carried out by a number of enzymes of the *N*-acetylgalactoseaminyltransferase (GalNAcT) family. The specific type and extent of glycosylation will depend on the expression profile of the various enzymes in the cell, as well as the physiological context [135]. In normal breast epithelial cells, addition of *N*-acetylgalactoseamine (GalNAc) to the MUC1 peptide backbone by β1,3-GalNAcT forms the "Tn antigen" (Fig 1.9). This is followed by addition of galactose, which forms the "core 1" structure, also known as T antigen or Thomsen-Friedenreich (TF) antigen (Fig 1.9). These sugar side chains are a substrate for "core 2" enzymes, which add *N*-acetylglucosamine (GlcNAc), followed by chain extension, resulting in polylactoseamine chains which can be linear or branched. Chains are terminated with addition of sugars such as sialic acid, fucose and GalNAc [6, 157].

In human breast cancers, both the composition and distribution of O-glycans on the MUC1 protein is altered. Glycans are often shorter and core 1 based, with siaylation occurring soon after initiation, and resulting in the generation of new antigens such as sialyl-T [6, 157, 158] (Fig 1.9). In the breast cancer cell line T47D, the majority of the glycans are T antigen, with sialyl-T and Tn antigen making up the remainder [159]. Additionally, T47D cell MUC1 is glycosylated on nearly all potential VNTR sites, compared to about 50% in normal tissues, possibly explained by increased accessibility of sites due to the truncated O-glycans [157, 160, 161]. These differences in

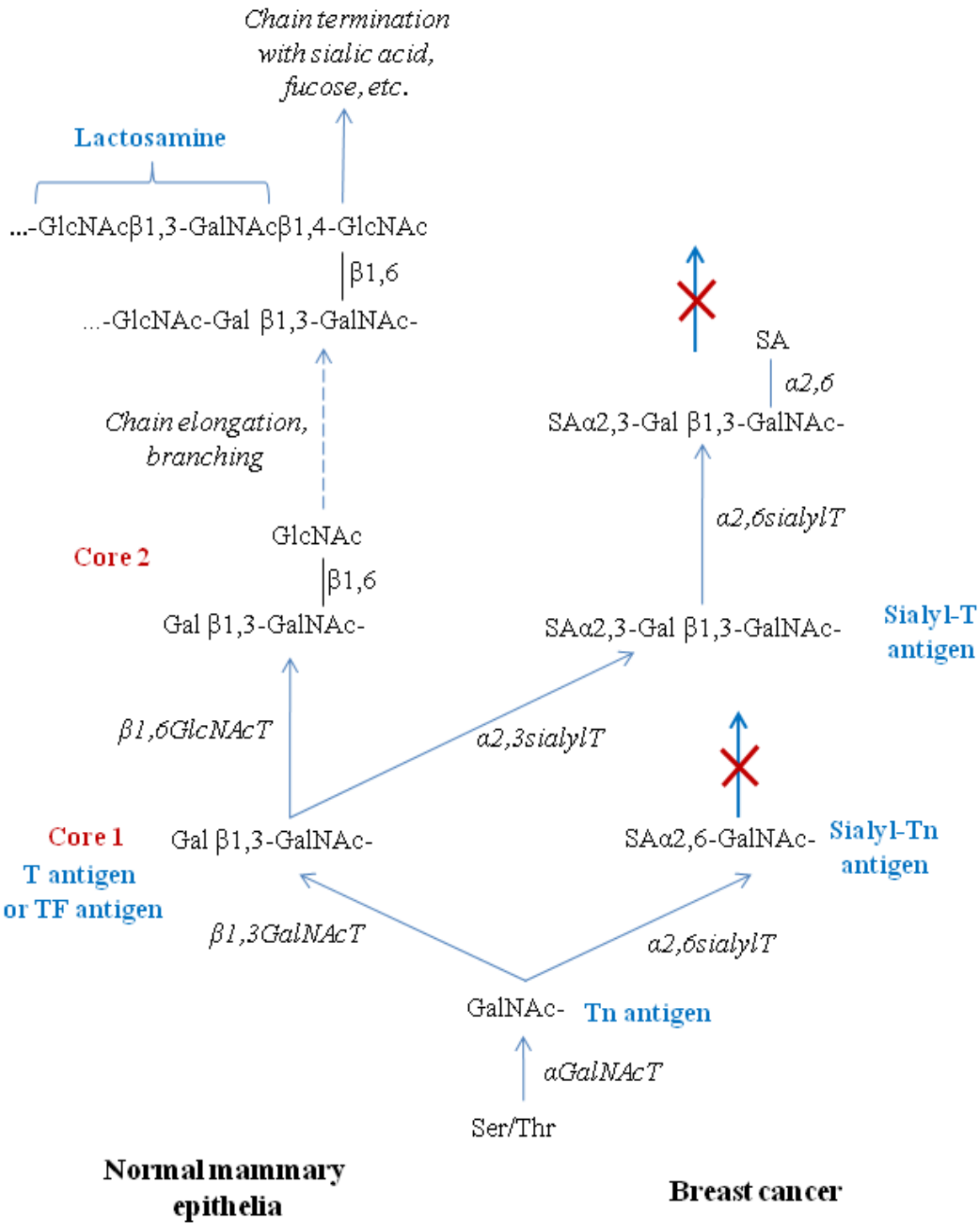


Figure 1.9. MUC1 O-glycosylation structure and enzymes in normal and cancerous breast epithelia. Based on [157-159].

glycosylation patterns between normal and cancer cell MUC1 can be attributed to the differing expression profiles of the enzymes responsible for initiation and extension of O-glycans. The activity of β1,6-*N*-acetylglucosyltransferase (GlcNAcT) has been found to be decreased in breast cancer tissues, while the activity of α2,3-sialyltransferase (sialylT) is increased [6], resulting in premature sialylation of O-glycans, terminating chain extension. Recently it was shown that overexpression of α2,3-sialylT could promote mammary tumorigenesis in mice [162], indicating that the presence of the sialyl-T antigens could confer a growth advantage. Rughetti et al. found that recombinant MUC1 protein containing high levels of sialyl-T O-glycans impaired the function of dendritic immune cells, suggesting immunosupression as a potential mechanism for the observed effects [163]. Additionally, TF antigens present on cancer associated MUC1 [164, 165] have been shown to be ligands for circulating galectin-3, and binding results in polarization of MUC1, increasing endothelial adhesion by revealing previously occult surface ligands [166-168].

The dysregulation of MUC1 O-glycosylation results in unique epitopes present on breast cancer cells which are candidates for use in the development of cancer vaccines and treatments. Antibodies raised to the protein backbone of MUC1 were found to react with over 90% of breast carcinomas assayed, with little or no reactivity to normal breast epithelia [169], indicating that in cancer the peptide backbone is exposed and potentially immunogenic. Recent work by Ryan et al. has suggested that the combination of O-glycan and peptide backbone neoepitopes may elicit a greater immune response than the peptide backbone alone, an important consideration in the design of vaccines [170]. Numerous peptide, cell, DNA, and glycopeptide based MUC1 vaccines have been developed, with promising results from initial testing and trials [9, 171-175].

1.2.2.2. MUC1 transmembrane domain

MUC1 contains a single-pass, 28-aa TMD [143]. The length of the MUC1-TMD is longer than the average TMD of 20-aa [176], which may have several implications on protein trafficking, membrane localization, and processing. For example, cleavage of amyloid precursor protein by γ-secretase is affected by the length of the TMD [177], and MUC1 has been recently identified as a γ-secretase substrate [178].

1.2.2.3. MUC1 cytoplasmic domain

In contrast to its lengthy TMD, MUC1-CD is relatively short, at only 72-aa (Fig 1.10), and has an atomic mass between 25-15kDa, depending on *N*-glycosylation and cleavage [178]. The first three aa of the MUC1-CD, termed the CQC motif, has been a subject of interest in recent years. An earlier report by Pemberton et al. demonstrated the necessity of the cysteine residues in membrane localization [179], while a subsequent study demonstrated that the cysteine residues are sites of palmitoylation and are involved in MUC1 recycling from endosomal compartments [180]. In conflict with Pemberton's study, they also reported that cysteine mutation does not prevent MUC1 trafficking to the cell membrane or association with detergent resistant membranes (DRM). DRM is a broad definition for cholesterol enriched domains of the cell membrane which have unique lipid and protein composition. Although the classification, structure and function of DRMs is still a topic of debate, the importance of DRMs in cellular activity is well established [181, 182].

In recent years, focus has shifted to the role of the CQC motif in MUC1 nuclear localization and dimerization. Extensive research on this topic, performed by the Kufe group, demonstrates that the CQC motif is necessary for oligomerization of truncated MUC1 (containing only the cytoplasmic domain) *in vivo* [19]. Further work led to the

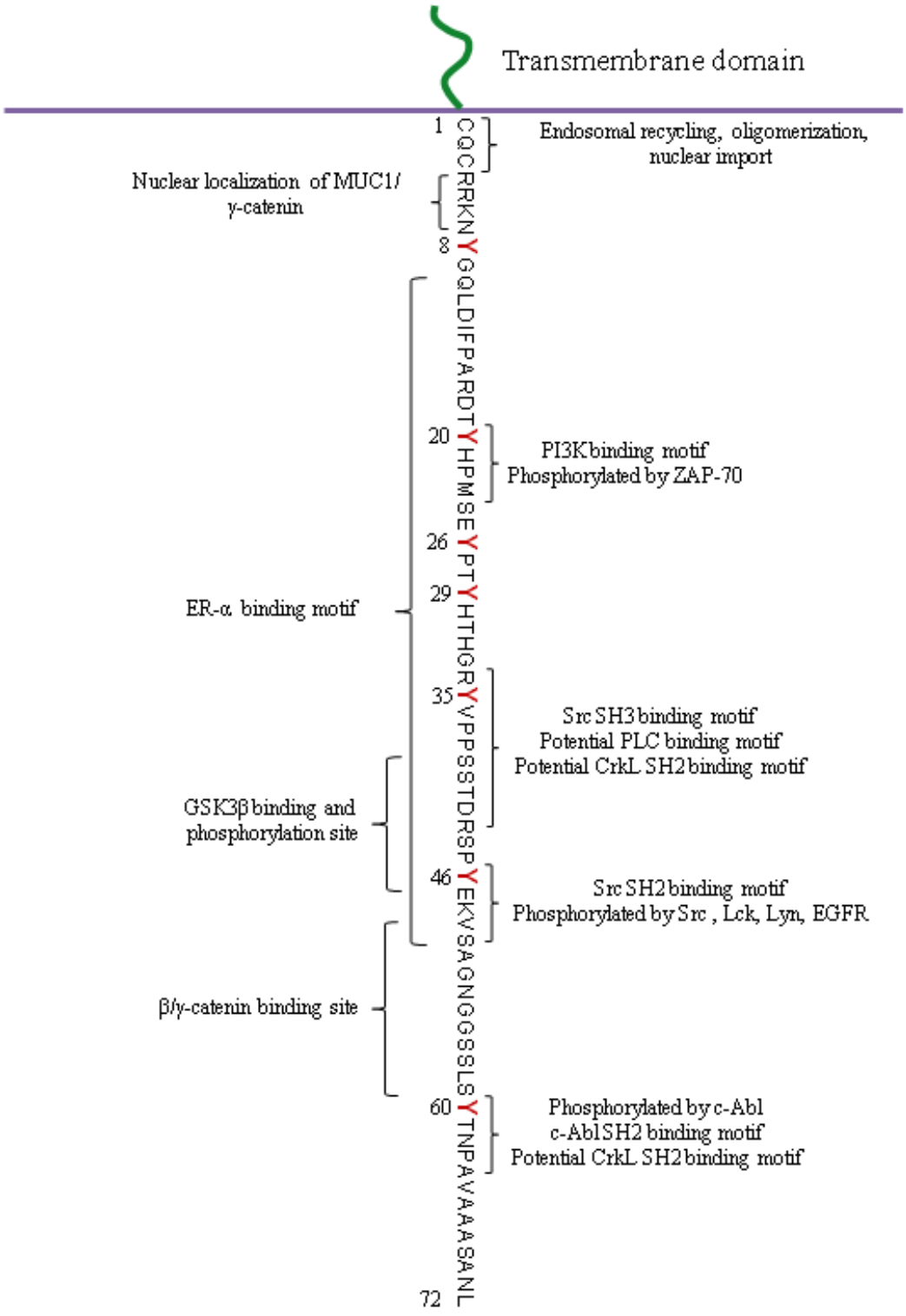


Figure 1.10. Schematic of MUC1 cytoplasmic domain. Selected characterized motifs and binding partners are indicated. Based on [19, 179, 180, 186-196].

development of an inhibitor which blocks oligomerization and nuclear localization of MUC1, disrupts redox balance, and induces necrotic cell death in several solid and hematologic malignancies [20-22, 183, 184].

Interest in the MUC1-CD as a signalling molecule was sparked when it was discovered that tyrosine phosphorylation occurred at several sites [185]. As MUC1-CD does not harbour tyrosine kinase activity itself, research began to focus on interactions between MUC1-CD and potential kinase effector proteins. Subsequently, MUC1-CD has been shown be a substrate for several tyrosine kinases, including c-Src, Lck, Lyn, c-Abl, and epidermal growth factor receptor (EGFR) [186-189] (Fig 1.10). Tyrosine phosphorylation of MUC1-CD generates potential binding motifs for several proteins such as Src, c-Abl, PI3K, PLC, Grb2, and CrkL [185, 186, 190-193] (Fig 1.10). Additional signalling and structural molecules that MUC1-CD has been demonstrated to interact with include ER-α, β-catenin, γ-catenin, and ezrin, which suggests linkage between MUC1-CD and the actin cytoskeleton [186, 194-198]. Interestingly, many of these interactions have been found to be altered in cancerous cells, and will be discussed further in Section 1.2.3. Taken together, these studies indicate that MUC1 likely plays a role in many cell signalling pathways, but the exact function of MUC1 in these pathways is not fully understood.

1.2.2.4. MUC1 cleavage

In both normal and cancerous epithelial cells, MUC1 undergoes an autoproteolytic cleavage event (S1) post-translationally, resulting in two subunits that reassociate non-covalently [132, 199] (Fig 1.9). The site of cleavage has been identified as G^{1097}/S^{1098}VVV within the SEA domain [23], and involves attack of the glycine carbonyl group by the serine hydroxyl group, followed by hydrolysis which forms novel

N- and C-termini [200] (Fig 1.11). This cleavage occurs due to the strained conformation of the newly synthesized protein, disrupting proper hydrogen bonding for β-sheet conformation. S1 cleavage results in a conformation favourable for hydrogen bonding and β-sheet conformation, also potentially explaining the mechanism for maintained association between the two subunits [200].

S1 cleavage can be disrupted by several mutations at the G/SVVV site. Insertion of 2-4 glycines just N-terminal to G/SVVV resulted in lack of S1 cleavage and maintenance of native folding [201], due to relief of conformational stress at the G/S site. Another experiment involved mutation of the catalytic serine to alanine, and resulted in an uncleaved protein which was unable to adopt a native structure [200], as determined by molecular modelling. In a separate study [26], *in vivo* analysis of a serine to alanine mutant also concluded that the mutant exists as a full-length, uncleaved protein, but did not investigate the effect of mutation on MUC1 folding, although they did confirm that the mutant was expressed at the cell surface. They also constructed several mutants with both glycine and serine residues mutated, with similar results.

The exact role (if any) that MUC1 S1 cleavage has in protein functionality has yet to be determined, but a useful model may be the better characterized NOTCH protein (Fig 1.12). NOTCH receptors are single-pass transmembrane proteins which function in transcriptional activation of genes involved in differentiation, proliferation, and survival [202]. NOTCH undergoes S1 cleavage by a furin-like protease during maturation, resulting in two associated subunits present at the cell surface [203], similar to MUC1. In contrast to MUC1, however, S1 cleavage has been found to be important for cell surface expression [204]. Studies investigating the role of this cleavage in NOTCH function have yielded contradictory results, indicating that S1 cleavage may only be important in response to certain NOTCH ligands [205, 206].

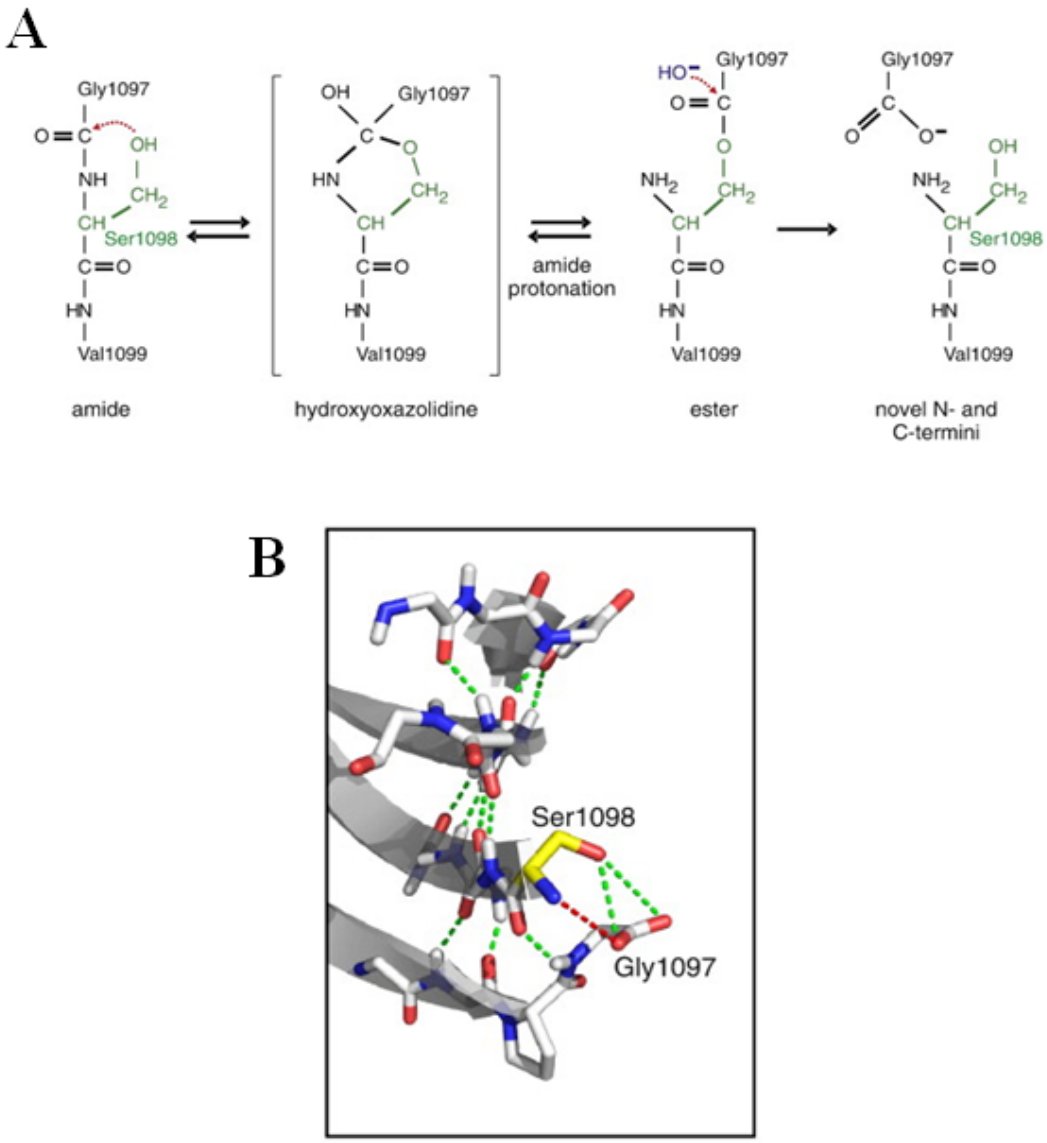


Figure 1.11. Autoproteolysis of MUC1 at S1 cleavage site. **A.** Cleavage at the site G'SVVV occurs by attack of the glycine carbonyl group by the serine hydroxyl group and results in novel N- and C-termini. **B.** The cleaved products remain reassociated non-covalently, potentially due to several hydrogen bonds formed between the two products. Adapted from [200] with permission from the publisher.

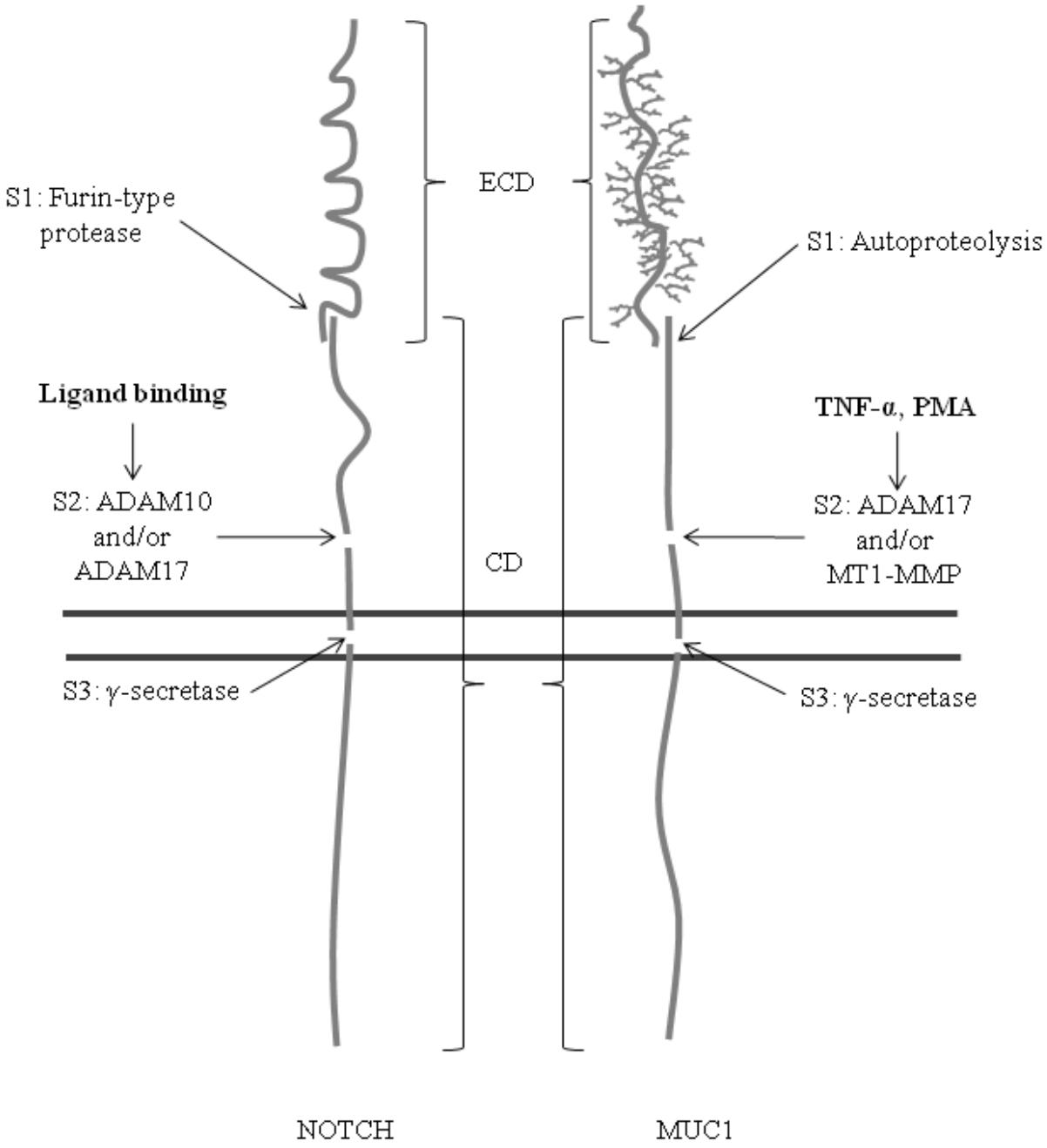


Figure 1.12. Comparison of cleavage sites in NOTCH and MUC1. NOTCH and MUC1 both undergo a series of cleavage events that are important in receptor structure and function. Based on [24, 25, 178, 203, 210, 213, 214, 218, 219].

The presence of high levels of MUC1-ECD in human serum has been correlated with cancer progression and has been used clinically as a diagnostic and prognostic tool [7, 27, 207-209]. For MUC1-ECD to enter the circulation, several steps must be taken. First, MUC1 must lose apical polarity, which occurs in most MUC1 overexpressing carcinomas and has been correlated with a poor prognosis [5, 89, 137]. Secondly, the MUC1-ECD must be shed from the cell, either by dissociation of the non-covalently linked subunits generated by S1 cleavage, or by a second cleavage event (S2). Thathiah et al. reported in 2003 that MUC1-ECD is released from the cell surface following S2 cleavage by a disintegrin and metalloprotease-17 (ADAM-17) [24]. They subsequently identified a second, pervanadate stimulated, MUC1 protease, membrane-type 1 matrix metalloprotease (MT1-MMP) [25], and also demonstrated that TNF-α treatment stimulated MUC1-ECD release [210]. Interestingly, a separate 2003 study found that mutation of the S1 cleavage site to generate an uncleaved form inhibited S2 cleavage and MUC1-ECD release, suggesting a functional consequence of S1 cleavage [26]. The site(s) of cleavage by these sheddases have not been identified, although substrate specificity for both ADAM17 and MT1-MMP have been described [211]. The function of S2 cleavage of MUC1 is not fully understood, but several interesting hypotheses can be formulated. Release of the large, negatively charged ECD could facilitate dimerization of MUC1-CD (discussed in Section 1.2.2.3), as steric hindrance imparted by the ECD could prevent dimers from forming. Further, MUC1-CD has been localized to the nucleus while the ECD has not, indicating that a cleavage event occurred prior to import [212]. Additional research is needed to ascertain the role of MUC1 cleavage in both normal and cancerous cells.

NOTCH has also been identified as a substrate for both ADAM17 and another protease, ADAM-10, both which act to release NOTCH-ECD and activate the NOTCH

signalling pathway [213-215]. Ligand binding to NOTCH-ECD results in *trans*-endocytosis into the ligand expressing cell, revealing an occult site on NOTCH and allowing cleavage by ADAM10/17 [216]. The role of each of the ADAMs in NOTCH signalling is not fully understood, but appears to be dependent on cell context and activating stimulus [217]. S2 cleavage (by either ADAM) is necessary for a third cleavage event (S3), which is performed by an enzyme complex called γ-secretase and releases a fragment of NOTCH which travels to the nucleus, activating transcription of target genes [218-221]. MUC1 has recently been identified as a substrate for γ-secretase, extending the analogy between itself and NOTCH [178], although the role of MUC1 γ-secretase cleavage has yet to be elucidated.

Cleavage of a variety of cell surface receptors by ADAMs and γ-secretase has been implicated in cancer progression and metastasis and identification and evaluation of inhibitors is currently the subject of intense research [222-229]. The role of these cleavage events in MUC1 function is not fully understood but would be of significant clinical interest due to the involvement of MUC1 in cancer progression.

1.2.2.5. MUC1 splice variants

Alternative splicing of MUC1 mRNA results in the generation of several truncated variants. The first described is termed MUC1-Y, and lacks the entire VNTR domain but contains a portion of the SEA domain, and identical TMD and CD to MUC1 [230]. Although it contains the S1 cleavage site, it does not undergo autoproteolysis and exists as a single protein entity. MUC1-Y is a binding partner for another splice variant, termed MUC1-SEC, which contains the tandem repeat domain but lacks a TMD and is secreted from the cell. Binding between MUC1-Y and MUC1-SEC results in tyrosine phosphorylation of MUC1-Y cytoplasmic domain [231]. High levels of MUC1-Y have

been reported in breast, ovarian and prostate cancer, suggesting function for MUC1-Y in cancer initiation and/or progression [232-234]. Another isoform, MUC1-Z (also called MUC1-X), is similar to MUC1-Y but contains an additional 18aa in the ECD [235]. Presently, the expression profiles and significance of these alternate isoforms in normal and malignant tissue is unclear.

1.2.2.6. MUC1 expression in normal and cancerous tissue

The MUC1 gene is encoded on chromosome 1q21 [236, 237], and the promoter contains potential binding sites for several transcriptional regulators including Sp1, NF-1, NF-κB, ER, and PR [8]. MUC1 expression has been demonstrated to be upregulated in response to treatment with ethanol, IL-6, INF-γ, IL-7, and phorbol 12-myristate 13-acetate (PMA) [238-241].

Normal expression of MUC1 occurs in several different localities in the body. It is expressed nearly ubiquitously in secretory epithelial tissues such as that of the lung, uterus, eye, breast, and stomach [242], and is believed function as part of a protective barrier against environmental factors. MUC1 is also expressed on a variety of cells of hematopoietic origin and the MUC1 gene promoter contains *cis*-elements that are specific to both epithelial and hematopoietic cell types [243]. Expression of MUC1 in malignancies of both epithelial and hematopoietic origin has been the subject of much research, as well as the interaction between soluble MUC1 and immune cells. MUC1 is reported to be expressed on activated T-cells, and a role in T-cell migration has been suggested by the polarization of MUC1 at the leading edge of migrating T-cells [244, 245]. Interestingly, MUC1 tandem repeats, which could be secreted or cleaved from carcinoma cells, has been found to induce T-cell anergy, a process that is reversible by addition of IL-2 [245, 246]. Cross-linking of T-cell associated MUC1 with divalent

antibodies also inhibited T-cell proliferation, suggesting a role for MUC1 clustering in this process [247]. The immunosuppressive role of circulating MUC1 and anti-MUC1 antibody is still a topic of debate, and will be an important consideration in the design of MUC1-specific vaccines [248-250].

MUC1 is reported to be overexpressed in both epithelial and hematopoietic malignancies [8], and several mechanisms of overexpression have been reported. At the level of transcription, the MUC1 promoter contains a STAT site which has been shown to be responsive to proinflammatory factors such as INF-γ and IL-6 [238], suggesting a role for inflammation in MUC1 overexpression. The MUC1 gene has also recently been reported to be amplified in breast carcinoma [251], and a role for methylation of the MUC1 gene has also been demonstrated to be important in transcriptional regulation [252, 253]. A gene translocation, t(1;14)(q21;q32), has also been reported in B-cell lymphomas, bringing the MUC1 gene under control of the Ig heavy chain promoter [254, 255]. Taken together, the accumulating body of evidence reporting high levels of MUC1 in malignancies strongly suggests a role for MUC1 in tumorigenesis and/or tumor progression.

1.2.3. MUC1 function

1.2.3.1. MUC1 function at the molecular level

1.2.3.1.1. MUC1 as a cellular receptor

Although early investigations of MUC1 labelled it as an anti-adhesive protein due to its large, negatively charged ECD [256, 257], more recent work has demonstrated a pro-adhesive role. MUC1 is reported to interact with several extracellular ligands, such as the flagella of *Pseudomonas aeruginosa* [258, 259]. Further studies on this interaction revealed that MUC1 is a negative regular of airway inflammation, and suppresses the

inflammatory response stimulated by *P. Aeruginosa*. Additional work indicated that the MUC1-CD is a critical component of this anti-inflammatory effect and is phosphorylated upon *P. Aeruginosa* binding, adding weight to the notion of MUC1 as a cell signalling receptor [260, 261].

Due to the loss of apical polarization in many cancer tissues, MUC1 may potentially interact with many ligands it would otherwise not encounter. For example, MUC1 has been identified as a ligand for ICAM-1 [12], which is expressed on both stromal and endothelial cells [11, 262]. This binding has been found to initiate cell signalling in MUC1 expressing cells leading to cell migration and simulated transendothelial migration, and will be discussed further in Section 1.2.6.

MUC1 has also been reported to bind to circulating galectin-3, which is increased in the serum of cancer patients [263, 264], via the Tn antigen present on cancer-associated MUC1-ECD (discussed in Section 1.2.2.1). This binding results in polarization of cell surface MUC1, revealing previously occult cell surface adhesive proteins such as CD44 and E-cadherin. Increased homotypic cell adhesion via these proteins allows for cancer cell embolus formation, increasing survival of tumor cells in circulation through avoidance of anoikis. The MUC1-ECD also contains $sLe^{a/x}$ sugar side chains which have been shown to interact with selectin proteins present on activated endothelium, and this has been found to inhibit leukocyte attachment to endothelial cells [265, 266]. This binding parallels interactions between activated endothelial cells and leukocytes, which results in extravasation; therefore the possibility that MUC1 $sLe^{a/x}$ binding is involved in tumor cell metastasis is appealing. Although these data reveal additional ligands for MUC1-ECD, they support the notion that MUC1 functions primarily as an anti-adhesive molecule, and additional research is needed to reconcile these diverse roles.

1.2.3.1.2. MUC1 as a scaffold protein

Not only does MUC1-CD bind to several cellular proteins (discussed in Section 1.2.2.3), but it has been postulated to act as a scaffold protein, acting to promote signalling by recruitment of signalling mediators, such as kinase-substrate pairs, facilitating signal initiation. For example, MUC1 is a known substrate and binding partner of Src, with potential binding sites for both Src SH2 and SH3 motifs [267]. MUC1/Src interaction has been shown to potentiate interaction between Src and downstream effectors such as P13K and CrkL [14, 268]. Interaction between MUC1 and Src has been implicated in MUC1 targeting to the mitochondria, murine mammary tumorigenesis, calcium signalling, and cell motility, suggesting roles in redox balance, cell proliferation, and metastasis [14, 268, 269]. In addition to being phosphorylated by Src at Y^{46}, this site is also a substrate for the SFKs Lyn, in multiple myeloma cells [189], and Lck, in activated T-cells [187, 270].

Phosphorylation of MUC1-CD at Y^{46}, by Src, Lck, or Lyn, results in increased interaction between MUC1-CD and β-catenin [186, 187, 189, 195]. In epithelial cells, this results in decreased cell-cell adhesion, as MUC1 competes with E-cadherin for binding to β-catenin [271]. Association between MUC1-CD and β-catenin has been reported to increase nuclear levels of β-catenin, inhibit β-catenin degradation, promote cellular invasion and has been correlated with a poor prognosis [196, 272-274]. Contrary to this work supporting a role for MUC1-CD/β-catenin interaction in tumor progression, it has also been reported that this interaction can inhibit cell proliferation, indicating that cellular context may be an important factor [275].

Another important interaction occurs between MUC1 and EGFR, which is frequently overexpressed in breast cancers and is associated with a poor prognosis [276, 277]. EGF-binding induced activation of the ERK/MAPK pathway has been shown to be

increased in the presence of MUC1 in the mouse mammary gland [188]. MUC1-CD is phosphorylated at Y^{46} by EGFR in response to EGF treatment, resulting in Src SH2 domain recruitment and increased association between MUC1 and β-catenin [278]. As Src recruitment to MUC1 has been demonstrated to result in cell motility [14], and MUC1 binding to β-catenin decreases cell adhesion, the potential role for MUC1/EGFR interaction in cancer metastasis is clear. The influence of MUC1 on EGFR activity has been further demonstrated by studies showing that inhibition of MUC1 activity with antibodies or intercellular peptides reduces EGFR expression and function [279-282].

Of particular interest is the interaction between MUC1 and Src kinase, specifically the causes and implications of this relationship. Early investigations focused on the relationship between Src, MUC1 and EGFR, as discussed above. The MUC1-CD phosphorylation and binding site for Src SH2 domain has been identified, and although Src SH3 domain has been shown to bind to MUC1-CD, the specific binding site has not been identified [267]. Association of MUC1-CD and Src has been implicated in MUC1 mitochondrial targeting [269], tumorigenesis [268], and cell migration [14], with MUC1-CD/Src association induced by ErbB activation, MUC1 expression, and ICAM-1 binding to MUC1-ECD, respectively. The mechanism of Src activation and MUC1-CD phosphorylation at Y^{46} following cell stimulation has not yet been described. The question remains as to what is necessary and sufficient for MUC1/Src binding and activation, as several downstream effects of MUC1/Src association have been demonstrated without endogenous stimulation. For example, MUC1 expression is sufficient to potentiate Src-dependent tumorigenesis in mouse mammary tumor virus-driven polyoma middle T-antigen (MMTV-PyV MT) transgenic mice [268]. Other reports have demonstrated a necessity for growth factor receptor stimulation by heregulin [269], fibroblast growth factor [283], and MUC1 stimulation by ICAM-1 [13, 14] in

MUC1/Src mediated signalling. Tyrosine phosphorylation of MUC1-CD at Y^{46}, potentially by Src, has also been demonstrated in response to antibody-mediated dimerization of MUC1-CD [284]. Taken together, these data indicate that the MUC1/Src relationship is potentially regulated by numerous cellular and environmental factors with distinct downstream effects.

1.2.3.1.3. MUC1 in transcriptional regulation

Nuclear entry of MUC1-CD has been the subject of intense study, as it appears to not only induce the expression of genes involved in cancer progression, but also facilitates the nuclear entry of other transcription factors [281, 285, 286]. Also, a 35-gene MUC1-induced gene signature has recently been identified and shown to predict decreased survival in breast cancer patients [287]. As overexpression of MUC1 is sufficient for induction of anchorage-independent growth and tumorigenicity [144], investigation of the genetic impact of MUC1 is of great interest.

Nuclear MUC1-CD has been found to interact with β-catenin, STATs, ER-α, NF-κB, and p53, affecting transcription of genes preferentially involved in invasion, angiogenesis, and survival [288, 289]. For example, MUC1 colocalization with β-catenin in the nucleus results in activation of Wnt-target genes such as cyclin-D1, an effect that can be abrogated by mutation of MUC1-CD Y^{46} , implicating phosphorylation by EGFR or Src in this process [285]. Overexpression of cyclin-D1 has been shown to be correlated with overexpression of EGFR, and MUC1 has been implicated in the expression of both [279, 285]. The loss of MUC1 has been shown to decrease interaction between EGFR and the cyclin-D1 promotor, suggesting that MUC1 may promote cyclin-D1 expression several ways. Interestingly, another paper has reported that expression of the MUC1-CD inhibits cellular proliferation and cyclin-D1 expression [290], indicating that cellular and

environmental context is critical in experimental design and interpretation. Illustrating this, a study using MUC1 siRNA reported cell-line specific differences in transcriptional regulation by MUC1 [291].

Nuclear entry of MUC1 has been attributed to the nucleoporin Nup62, and requires an intact $C^{1}QC$ motif in the MUC1-CD which is postulated to facilitate dimerization [19]. Several inhibitors have been developed targeting MUC1-CD dimerization, and therefore nuclear entry, have been identified, and an initial report has shown that inhibition of MUC1-CD dimerization results in decreased transcriptional activity, although additional research is needed to determine the mechanism of this effect [292].

1.2.3.2. MUC1 function at the cellular level

MUC1 has been implicated in a diverse array of cell signalling pathways involved in cell proliferation, survival, adhesion, and migration. The dysregulation of MUC1 expression and localization in cancer cells facilitates its involvement in these pathways and contributes to cancer progression. MUC1 has been identified as an oncogene, and overexpression has been shown to promote tumorigenesis *in vivo* [293], potentially though a combination of activation of Src-mediated pathways [268], alteration of β-catenin function [273], and transcriptional activation of growth promoting factors [291]. Additionally, MUC1 has been shown to protect cells against oxidative stress induced apoptosis by upregulation of anti-oxidant enzymes [294, 295]. As cancer cells are frequently subject to oxidative stress [94], the presence of high levels of anti-oxidant enzymes could promote cell survival.

As described in Section 1.1.3, the metastatic cascade requires cancer cells to both adhere to and invade through stromal and endothelial cells, and MUC1 has been

implicated in cell adhesion and motility both *in vitro* and *in vivo*. Both anti- and pro-adhesive functions have been attributed to MUC1 in these processes, and although the specific role of MUC1 is unclear, it has been identified as a definitive participant. Recent work using microRNA (miRNA) targeting MUC1 expression has demonstrated that suppression of MUC1 results in decreased cell *in vitro* invasion and *in vivo* metastasis [296]. Work using MUC1-CD targeted peptides has yielded contradictory results. A peptide containing both the Src/EGFR phosphorylation site and β-catenin binding site on MUC1-CD blocked MUC1/β-catenin and MUC1/EGFR interactions and was found to reduce *in vitro* invasion of breast cancer cells, as well as tumor recurrence in immunocompromised mice [280]. An earlier report found that similar peptides, with or without tyrosine phosphorylation, increased *in vitro* invasion of breast cancer cells, although the mechanism of this is unclear [273]. More recent work has shown that targeting MUC1-CD dimerization with peptides containing the $C^{1}QC$ motif inhibits cell cancer cell growth and survival *in vitro* and in tumor models [20, 184]. Additional work is needed to determine the mechanism of action of these agents and their potential clinical applicability.

1.2.4. ICAM-1 structure and function

1.2.4.1. ICAM-1 structure and expression

Human ICAM-1 (also known as CD54) is encoded on chromosome 19p13 and is a single-pass transmembrane glycoprotein of the immunoglobulin (Ig) superfamily [297]. The extracellular domain contains five "Ig-like domains", followed by a hydrophobic TMD and a short CD (Fig 1.13). Each Ig-like domain is folded into anti-parallel β-sheets and disulfide bridging within each domain creates characteristic "loops" [298], with several potential N-glycosylation sites present [297]. ICAM-1 reportedly exists as both a

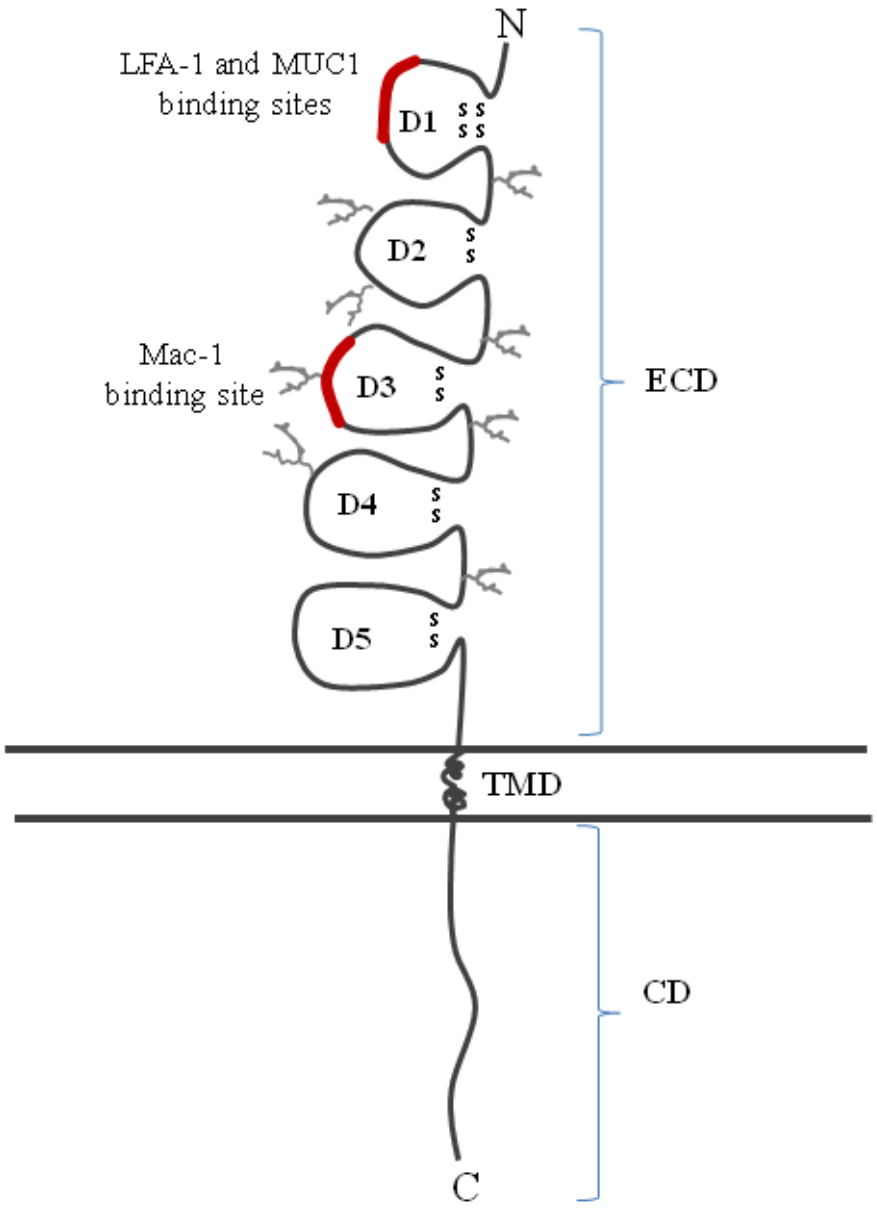


Figure 1.13. Schematic of ICAM-1 monomer. ICAM-1 consists of an ECD containing five cysteine-linked "Ig-like domains", followed by a TMD and CD. Potential N-glycosylation sites are shown, as well as reported binding domains for known ligands. Based on [297, 298, 311, 312].

monomer and a homodimer at the cell surface [299], but the mechanism of dimerization is unclear. Domain-1, 4, 5 and the TMD have all been implicated in dimerization [300-302] and may not be mutually exclusive [298]. Upon ligand binding, clustering of ICAM-1 has been reported, which can result in association of ICAM-1 with DRMs, potentially bringing it into contact with signalling partners [303-305].

The role of ICAM-1 in leukocyte transendothelial migration in response to inflammation is well characterized. It is expressed normally in cells involved in immune responses, and expression is upregulated, in these and other cell types, by pro-inflammatory cytokines such as TNF-α, IL-1 and INF-γ [297, 306]. ICAM-1 expression has been reported to be upregulated in the tumor microenvironment [11], and increased levels of serum ICAM-1 have been correlated with progression of colorectal, gastric, lung, and breast carcinomas [307-310].

1.2.4.2. ICAM-1 function

The role of ICAM-1 in leukocyte extravasation into tissue at sites of inflammation is well understood and is discussed in Section 1.1.3.1. [297]. ICAM-1 contains binding sites for the leukocyte integrins LFA-1 and Mac-1 on domains 1 and 3, respectively [311-313]. Following binding to leukocyte LFA-1, clustering of both LFA-1 and ICAM-1 has been reported, and dimeric ICAM-1 has been demonstrated to have greater affinity for LFA-1

than the monomeric form [301, 314]. Following binding of ICAM-1 to its ligand, an intracellular calcium flux has been reported, as well as activation of cell signalling mediators such as Src, paxillin, protein kinase C (PKC), Rho, and MLCK, all of which are implicated in cell motility (See Section 1.1.3.2) [315, 316]. It is therefore not surprising that retraction of ICAM-1 expressing endothelial cells at sites of leukocyte

attachment have been reported, facilitating extravasation [317, 318]. Interestingly, interaction of breast cancer cells with endothelial cells initiates Ca^{2+} signalling and adhesion dynamics, suggesting that circulating tumor cells could extravasate in a manner similar to leukocytes [319, 320]. This is especially significant in light of the interaction between ICAM-1 and breast cancer associated MUC1, a relationship that will be discussed further in Section 1.2.6.

1.2.5. Src structure and function

1.2.5.1. Src structure and expression

c-Src (Src) is the most well characterized member of the SRC family of non-receptor tyrosine kinases (SFK), which contains eight other members. Src weighs approximately 60kDa and the mature form consists of a myristoylated SH4 domain, an SH3 domain, an SH2 domain, an "SH2-kinase linker", and a protein-tyrosine kinase domain (SH1 domain), and a C-terminal regulatory segment (Fig 1.14) [321]. The 14-carbon myristoyl group is attached to an N-terminal glycine residue and facilitates localization of Src to the cell membrane and is required for functionality [322]. The SH3 domain consists of about 60aa and binds to ligands which adopt a polyproline type II helical conformation, which generally contains the sequence "PXXP", with arginine residues lying either N- or C-terminal to the polyproline motif [323]. Analysis of various SH3 containing proteins has revealed considerable variation in the composition and structure of ligands, and in fact the intramolecular SH3 ligand which maintains Src in an autoinhibited state does not contain a PXXP motif (Fig 1.14) [323, 324]. Src SH2 domain binds to phosphorylated tyrosine residues, favouring the sequence "pYEEI" but other sequences have been reported to be ligands for Src SH2, and again the intramolecular SH2 ligand pY^{527} does not conform to this motif (common amino acid

numbering based on chicken Src) [323]. The "linker" region between Src SH2 and SH1 (kinase) domains contains the Type II helix that binds to Src SH3 in an autoinhibited state (Fig 1.14). The SH1 domain is bilobal, a characteristic of all tyrosine kinases, with nucleotide binding and phosphotransfer occurring in the cleft between the two lobes. The "activation loop" is located in the C-terminal lobe and contains Y^{416}, a residue critical in regulation of Src activity. The C-terminal of Src contains another important residue, Y^{527}, important in maintainence of the autoinhibitory state of Src. Viral Src, encoded by the avian oncogene of Rous sarcoma virus, lacks this residue and is therefore exists in a perpetually active state [321]. Significantly, 12% of human colon cancers contain a mutant Src protein which also lacks this regulatory residue, and this activating mutation has been correlated with tumorigenesis and metastasis [325].

Src is expressed nearly ubiquitously in human cells, and is involved in cellular signalling processes controlling proliferation, survival, and motility. Src is activated in response to stimulation of cytokine receptors, GPCRs, RTKs, and integrins [326]. Overexpression of Src has been reported in a variety of human carcinomas, although amplifications and translocations are rare, indicating that overexpression of Src likely occurs at the transcriptional level [327].

1.2.5.2. Src activation

Src is maintained in an inactive state by intramolecular interactions between various domains and binding motifs (Fig 1.14). Binding occurs between Src SH3 domain and the Type II helix of the linker region, and between the SH2 domain and the phosphorylated pY^{527}. Phosphorylation of Y^{527} is controlled by C-terminal Src kinase (Csk) or Csk homology kinase (Chk), which are controlled by the activity of several cellular phosphastases, several of which have been found to be dysregulated in cancer [328, 329]. Collectively, these interactions act to "clamp" Src shut, preventing substrate

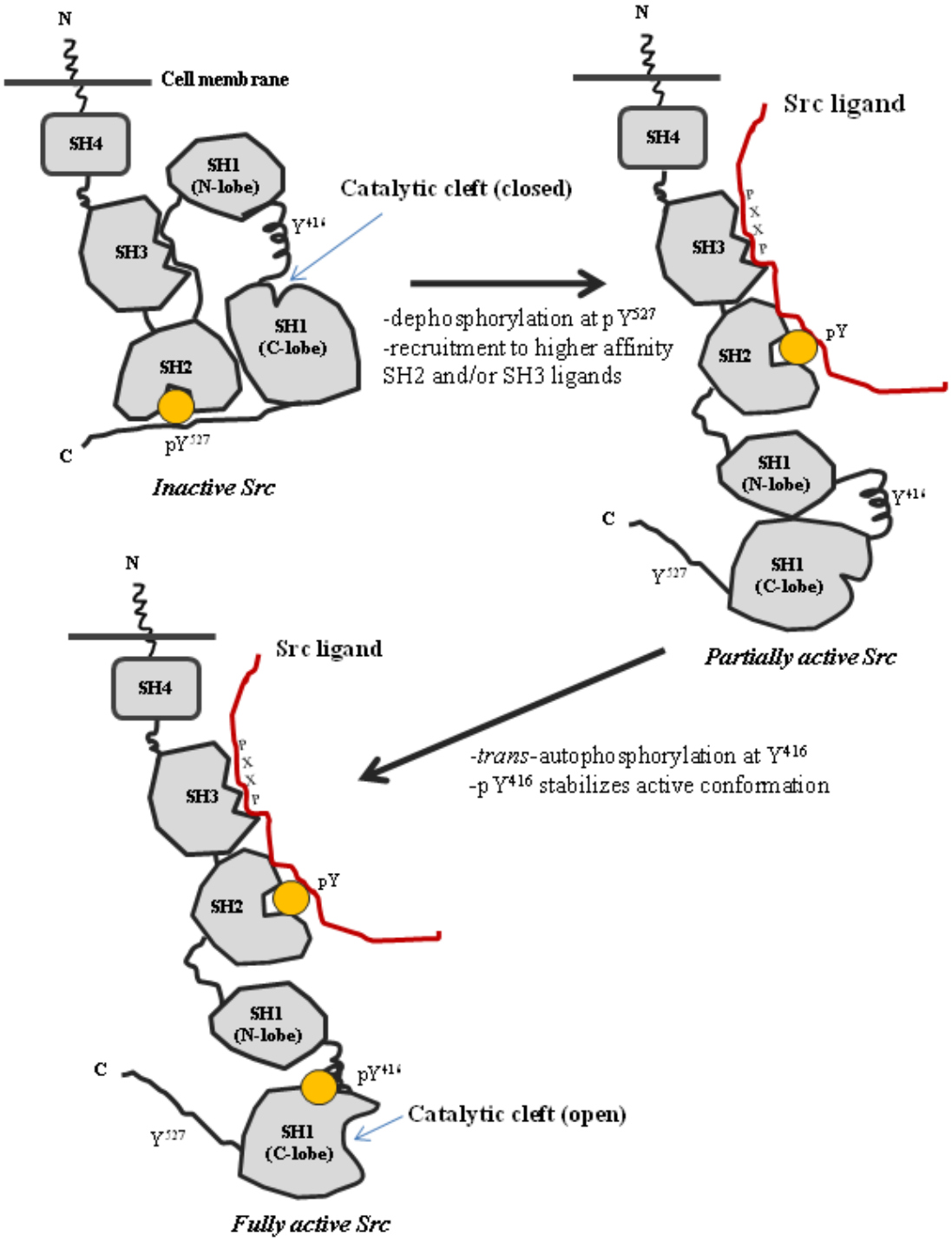


Figure 1.14. Schematic of Src structure and activation. Src is fully activated in a stepwise manner, with phosphorylation of pY^{416} required for maximal activity. Amino acid numbering based on chicken Src. Based on [321, 323].

binding by burying Y^{416} of the activation loop between the lobes of the kinase domain and preventing other key interactions [321].

Src can be activated by several, non-mutually exclusive events. Competition for Src SH2 and SH3 binding by higher affinity ligands can displace intramolecular interactions, effectively "unfolding" the enzyme. The intramolecular ligands for Src SH2 and SH3 domains must be of low affinity to allow for competition by exogenous ligands, demonstrated by the observation that neither ligand fits the consensus sequence [330]. Many substrates of Src contain SH2 and/or SH3 binding domains, which is especially effective as activation and recruitment of Src can occur concomitantly. Cooperative activation by SH2 and SH3 domain ligands has also been observed, meaning that the presence of one ligand "primed" the SFK for interaction with the second ligand, suggesting that ideal Src substrates may contain ligands for both domains [331-333]. Activation of Src through binding to either an SH2 or SH3 ligand has also been demonstrated [334, 335]. Taken together, these data indicate that although recruitment of either the SH2 or SH3 domain away from intramolecular interaction may be sufficient for partial activation, full functionality of Src may require ligation of both domains, in addition to other requirements, as described below.

For full activation, Src must be phosphorylated within the activation loop at Y^{416}, which is hypothesized to occurs by *trans*-autophosphorylation [321], (although *in silico* work has suggested otherwise [336]) indicating that clustering of Src may be an important component of full activation. For Y^{416} phosphorylation to occur, Src must be dephosporylated at Y^{527}. This is performed by enzymes such as protein tyrosine phosphatase (PTP)-α, PTP1, Src homology region 2 domain containing kinase (SHP)-1, and SHP-2, several of which have been implicated in cancer progression [337-339].

1.2.5.3. Src in cancer

Src is involved in many processes that are known to be altered in cancerous cells, such as growth, proliferation, and migration. Increased activity and/or expression of Src has been implicated in cancer initiation and progression, and numerous mechanisms of Src activation/inactivation which are altered in cancer cells have been described [128, 337, 340]. Increased Src activity has global consequences for cell function, and extensive work has been done on this topic. Src has been characterized as a positive regulator of EMT, likely through suppression of E-cadherin mediated cell adhesion [341-344]. The involvement of Src in pathways controlling cell motility and adhesion is well established [345]. For example, FAK is a known substrate and binding partner of Src, and binding of Src to FAK leads to Src activation through displacement of intramolecular interactions [333]. Coexpression of Src and FAK has been implicated in cancer progression [108, 109]. The intimate relationship between these two kinases has been demonstrated by the observation that inhibition of one kinase has implications on the activity of the other, and combined inhibition has showed significant promise as a therapeutic strategy in cancer [109-111, 346-348]. Similar observations have been made between Src and other substrates such as EGFR [349, 350] and c-Met [351-353]. Collectively, these observations suggest that synergistic inhibition of Src and Src effectors/substrates has significant clinical potential and several promising findings have been recently reported [16, 17, 354-357].

1.2.5.4. Src and cell motility in cancer

The known role of Src in normal cell migration is discussed in Section 1.1.3.2. Src is a critical component of pathways involved in assembly and disassembly of cell-cell and cell-matrix adhesions and increased activity of Src is implicated in metastasis of

numerous epithelial cancers [128, 345, 358]. Due to the diversity of proteins with which Src interacts, Src overactivation in breast cancer has far reaching implications, many of which can contribute to cell motility and metastasis. Abberant Src activation by growth factor receptors, many of which are overexpressed or dysfunctional in epithelial cancers, results in overactivation of pro-migratory pathways which are tightly regulated in normal cells. For example, Src interaction with β-catenin and E-cadherin result in adherens junctions disassembly, promoting cell invasion [114, 115, 359]. Association with FAK promotes downstream signalling involving p130Cas, paxillin, and the GTPase Rho, promoting actin cytoskeletal reorganization and cell migration [345]. Suppression of Src activity with PP2 or siRNA in breast cancer cells resulted in upregulation of E-cadherin, downregulation of Slug and Snail, consistent with reversal of EMT, a critical step in cancer metastasis [343]. Treatment of breast cancer cells with PP2 is also reported to restore the E-cadherin/ β-catenin interaction and reduce metastasis in murine models [360]. The literature clearly defines a role for Src in breast cancer cell motility and metastasis, and additional research will reveal specific interactions that are potential targets for anti-metastatic therapies.

1.2.6. MUC1 and Src

As MUC1-CD does not harbour tyrosine kinase activity, interaction of MUC1-CD with cytoplasmic tyrosine kinases has long been a subject of research attention. As MUC1-CD contains seven tyrosine residues as potential sites of tyrosine phosphorylation and signal initiation [185], it has been proposed that MUC1 functions akin to cytokine receptors, with phosphorylation events occurring following ligand binding by a non-receptor tyrosine kinase associated with the cytoplasmic domain, initiating cell signalling cascades. As the MUC1-CD was known to contain potential phosphorylation and binding

sites for Src kinase, an early study investigated this relationship and found that MUC1 is phosphorylated by c-Src *in vitro*, primarily at the site **Y**^{46}EKV, and that this phosphorylated motif binds to Src SH2 domain, resulting in increased association between MUC1 and β-catenin [267]. However, unphosphorylated MUC1-CD did not bind to Src SH2 *in vitro*, although it did bind to Src SH3 domain, demonstrating for the first time that MUC1 contains an SH3 binding site, although the MUC1-CD residues responsible were not identified. Also, MUC1 phosphorylation and β-catenin association with MUC1-CD was also observed in the absence of c-Src or in a Y^{46}F mutant *in vivo*, indicating that other kinases and phosphorylation sites are involved in β-catenin association. A later study demonstrated that EGFR is also capable of phosphorylating MUC1 at **Y^{46}**EKV, resulting in association between MUC1 and Src SH2 domain and β-catenin [361]. Although these works identified interactions which are important in the study of MUC1, the initiating stimuli or effects of these interactions on cell behaviour *in vivo* were not investigated. Interestingly, two subsequent publications investigating the use of MUC1-CD peptides containing the Y^{46}EKV motif reported conflicting effects of the peptides on cellular invasion [273, 280]. A major difference between these peptides was the inclusion of the potential CrkL binding site, Y^{60}TNP (Fig 1.10), in the peptide which increased cellular invasion levels. As Src association with MUC1-CD at Y^{46} could facilitate Src activation and phosphorylation of this peptide at Y^{60}, it is possible that this peptide initiated Src-CrkL mediated cell signalling cascades, resulting in cell migration. However, the effect of these peptides on MUC1-CD/Src association and activation of downstream signalling mediators such as CrkL was not investigated, although decreased association between MUC1-CD and β-catenin was reported following treatment with the peptide that inhibited cell invasion [280].

The first report of MUC1/Src interaction following stimulation of MUC1 with a physiological ligand found that ICAM-1 binding induced CaOs in MUC1 expressing cells were inhibited by treatment with PP2, a SFK inhibitor [13]. A subsequent paper investigating ICAM-1 binding induced signalling in MUC1 expressing cells also reported that a MUC1/Src/CrkL binding complex forms following ICAM-1 binding, and a Src-CrkL-Rac1/Cdc42 mediated signalling pathway results in cytoskeletal reorganization following ICAM-1 ligation [14]. However, the motifs involved in interaction between MUC1/Src and MUC1/CrkL were not investigated.

These works can be interpreted to demonstrate that MUC1-CD interacts with both Src SH2 and SH3 domains, possibly resulting in Src phosphorylation of MUC1 and recruitment of downstream signalling mediators such as β-catenin and CrkL. These interactions would promote cell migration by facilitating dissociation of cell adhesion and cytoskeletal rearrangements, respectively. However, to date, no inhibitors of the MUC1/Src interaction have been developed, which would be of potential clinical use in the treatment of breast cancer metastasis. To achieve this, the nature of the MUC1/Src interaction with regards to both SH2 and SH3 binding domains would need to be identified.

1.2.7. The MUC1/ICAM-1 signalling axis

1.2.7.1. MUC1/ICAM-1 binding

Binding between breast cancer associated MUC1 and endothelial cell ICAM-1 was first described in 1996 by Regimbald et al. [12]. This finding was significant in the context of cancer metastasis, as MUC1 was known to be upregulated in many epithelial cancers, and ICAM-1 was a characterized participant in leukocyte extravasation. In addition, circumferential expression of MUC1 in breast cancer cells, which would

facilitate interaction between MUC1 and ICAM-1 present on breast stromal cells, is correlated with increased lymph node metastasis [5]. Since this report, MUC1/ICAM-1 binding has also been shown to be involved in macaque trophoblast transendothelial migration, demonstrating a non-pathologic role for this interaction [362]. Binding between pancreatic tumor cell associated MUC1 and ICAM-1 expressed on B lymphocytes has also been demonstrated, prompting the authors to suggest a role for MUC1 in immunosurveillance [363].

The interaction between MUC1 and ICAM-1 has been further characterized, and it is now known that ICAM-1 binds to the VNTR peptide backbone of MUC1, which is exposed in breast cancer due to the presence of shorter O-glycans [6, 87]. It has been demonstrated that antibodies targeting MUC1 VNTRs or ICAM-1 domain 1, peptides consisting of at least two MUC1 tandem repeats, or recombinant ICAM-1 protein can inhibit MUC1/ICAM-1 binding [12, 87, 364]. These findings are especially significant in light of more recent data implicating this interaction in cancer progression, as inhibition of MUC1/ICAM-1 binding represents a potential therapeutic strategy.

1.2.7.2. MUC1/ICAM-1 signalling

Due to the potential implications of MUC1/ICAM-1 binding in cancer metastasis, research downstream of this interaction has focused on cell motility. Several years after the initial report, Rahn et al. found that ICAM-1 ligation triggered a cytosolic CaOs in MUC1 expressing breast cancer cells [13], which was significant due to the established role of Ca^{2+} in cell motility (see Section 1.1.3.2.). Interestingly, studies on the role of MUC1 in Jurkat cells found that activation of cells with anti-CD3 also resulted in a calcium influx, which was reduced and also became less oscillatory following MUC1 siRNA knockdown [270]. Subsequent reports revealed that following ICAM-1 induced

CaOs, MUC1 expressing breast cancer cells exhibited cytoskeletal rearrangements and migrated through a simulated endothelial cell monolayer [14, 15]. Significantly, the observed transendothelial migration was not facilitated by addition of chemoattractants, indicating the MUC1/ICAM-1 binding leads to directional cell migration, which is what would occur in extravasation of cancer cells.

Investigation of the signalling axis involved in these events has revealed that transmission of the MUC1/ICAM-1 signal involves well characterized pathways. Through the use of inhibitors, the CaOs signal was found the involve Src kinase, PI3K, PLC and IP3 receptor (IP3R), and DRMs. MUC1 contains potential binding and phosphorylation sites for Src [186], which can result in PI3K recruitment and activation (Fig 1.10), suggesting potential mechanisms for the observed downstream CaOs (Section 1.1.3.2). Active Src can also phosphorylate the regulatory p85 subunit of PI3K, activating PI3K activity, suggested an alternative or parallel mechanism of PI3K activation [123]. The actin cytoskeletal rearrangements observed following MUC1/ICAM-1 binding have been found to involve a Src-CrkL-Rac/Cdc42 pathway [14], again implicating Src kinase in signal transmission. Following ICAM-1 ligation, increased association between MUC1 and Src has been reported, as well as redistribution of Src into lipid rafts [14, 365]. How ICAM-1 ligation to MUC1-ECD facilitates initiation of signalling is not fully understood, but the role of Src kinase as a key factor is an appealing possibility in light of the implication of Src in breast cancer progression and the interest in Src inhibitors as components of combinational therapy regimes [16, 128, 354, 366].

1.3. Dimerization of cell membrane receptors

1.3.1. Constitutive dimerization – a paradigm shift

Classically, it was believed that non-kinase membrane receptors existed as monomers until ligand binding induced dimerization of the receptors, allowing trans-activation of receptor associated kinases and the triggering of signal initiating phosphorylation cascades [367, 368]. In recent years, a new paradigm has emerged in which receptors exist as pre-formed ligand-independent dimers, and has been described for a number of receptors including growth hormone receptor (GHR), erythropoietin receptor (EpoR), and EGFR family members [369-371]. The mechanisms of constitutive dimerization is not completely understood, but has been reported to involve the transmembrane domains of all receptors investigated [369, 371, 372]. Upon ligand binding, structural changes such as extracellular cysteine linkage, association with DRM fractions or resistance to cleavage result in signal initiation [373-375]. Compounds preventing the homodimerization of EGFR, ErbB2, and prolactin receptor have been developed and have demonstrated promise as chemotherapeutic agents in the treatment of breast cancer [376-379].

1.3.2. Dimerization of MUC1

The first indication that MUC1 dimerization may play a role in the function of MUC1 as a cell membrane receptor stemmed from a study utilizing a chimera of CD8 ECD and TM domains fused to MUC1-CD beginning at R^4RK [380]. They reported that treatment of this chimera with anti-CD8 antibody resulted in tyrosine phosphorylation of MUC1-CD at Y^{20}, Y^{29}, Y^{46}, and Y^{60}, and activation of ERK1/2 [284, 381]. Antibody treatment can result in dimerization of target receptors, a strategy that has been used in investigations of other cell membrane receptors [382-384]. Therefore it is possible that anti-CD8 treatment mediated dimerization, or clustered existing dimers, of the CD8/MUC1 chimeras, facilitating phosphorylation events and downstream signalling.

The role of MUC1 dimerization in signalling was specifically investigated by another group which reported that a truncated version of MUC1, consisting of a 72aa construct of MUC1-CD only, formed covalently linked dimers *in vitro* and dimers *in vivo* which were dependent on the membrane proximal cysteine residues [19]. Subsequently, they developed an inhibitor to MUC1-CD dimerization which displayed activity against several types of human tumors *in vivo* [20-22]. However, the mechanism of action of this inhibitor has not been described, nor has the mechanism of MUC1-CD dimerization. Also, the role of MUC1-CD dimerization in recruitment of important downstream signalling mediators such as Src, or in other pathways involving MUC1-CD implicated in cancer progression, have not been investigated. This information is required for full appreciation of the applicability of this inhibitor or the development of novel MUC1-CD dimerization inhibitors.

1.4. Hypothesis and objectives

The aim of this thesis is to investigate the mechanism of MUC1/ICAM-1 binding induced signalling. MUC1 is known to be overexpressed in breast cancers, and has been correlated with increased metastasis [27, 143, 385], and ICAM-1 is known to be involved in leukocyte extravasation, a process that metastasizing breast cancer cells must also complete [73, 262]. Previous work has demonstrated that this interaction results in the generation of pro-migratory CaOs [13] and simulated transendothelial migration [15] in MUC1 expressing cells, suggesting a role for MUC1/ICAM-1 binding in cancer cell extravasation. Elucidation of critical events occurring before and after ICAM-1 ligation to MUC1 will represent potential therapeutic targets in MUC1 overexpressing carcinomas. Due to the body of evidence supporting a critical role for Src in MUC1/ICAM-1 signalling, the relationship between Src and MUC1 will be an area of

focus in this thesis. Additionally, as other reports [19, 184] have demonstrated that MUC1 exists as a cysteine linked dimer which is a druggable target in cancer, the relationship between MUC1 dimerization, ICAM-1 signalling and Src recruitment to MUC1 will also be investigated. Lastly, as MUC1 undergoes several cleavage events, which may be co-dependent [26], the roles of S1 and S2 cleavage in ICAM-1 binding induced signalling will be examined. Conclusions generated by these studies will provide information on the mechanism of MUC1/ICAM-1 induced signalling, providing targets for the future development of anti-metastatic therapies.

As our working hypothesis, we propose that the MUC1 cytoplasmic domain exists as a covalently linked dimer which is required for Src recruitment and is cleaved following ICAM-1 binding, releasing the extracelluar domain and inducing pro-metastatic signalling.

We will test this hypothesis by the following specific objectives:

1. Establish the role of Src kinase in the MUC1/ICAM-1 signalling axis.
2. Investigate the role of cysteine-linked MUC1-CD dimerization in Src recruitment and ICAM-1 binding induced signalling.
3. Investigate the role of MUC1 S1 and S2 cleavage in ICAM-1 binding induced signalling.

Chapter 2: Materials and Methods

2.1. Antibodies and Reagents

Armenian Hamster CT2 monoclonal antibody (mAb), directed against the last 17 C-terminal amino acids of MUC1-CD (hereafter referred to as anti-MUC1-CD), was generously provided by Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ) [386]. Mouse B27.29 mAb, directed against the PDTRPAP epitope of the MUC1-ECD (hereafter referred to as anti-MUC1-ECD), was generously provided by Fujirebio Diagnostics, Inc. (Malvern, PA). Rabbit anti-Src mAb, rabbit anti-Src^{pY416} polyclonal Ab (pAb), and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Cell Signalling (Danvers, MA). Goat anti-mouse and anti-Armenian hamster horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). Rabbit anti-CD8α pAb was from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Small interfering RNA (siRNA) was from Dharmacon (Lafayette, CO). Enhanced chemiluminescence (ECL) Plus western blotting reagents were from GE Healthcare (Baie d'Urfe, QC). Fluo-3 AM was purchased from Molecular Probes (Bulington, ON). 35mm glass-bottomed microwell dishes were from MatTek Corporation (Asland, MA). Mouse anti-tubulin mAb and rabbit anti-GAPDH pAb, Pluronic F-127, protease inhibitor cocktail, phosphatase inhibitor cocktail, Trizma hydrochloride (Tris-HCl), Tris-base, polyoxyethylene-sorbitan monolaurate (Tween-20), gelatin Type A, HEPES, D-glucose, $MgCl_2$, $CaCl_2$, sodium dodecyl sulphate (SDS), ethylenediaminetetraacetic acid (EDTA), and RIPA were from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified eagle medium (DMEM), Opti-MEM media, Geneticidin (G418), Blasticidin S, trypsin, fetal bovine serum (FBS), Cell Tracker Green 5-chloromethylfluorescein diacetate (CMFDA), 0.4% trypan blue solution, and Lipofectamine 2000 were from Invitrogen, Inc. (Carlsbad, CA). Sodium hydroxide (NaOH), sodium chloride (NaCl), methanol (MeOH), Triton X-100, ammonium

persulfate (AP) and disuccinimidyl suberate (DSS) were from Fisher Scientific (Nepean, ON). Glycine, 40% (w/v) acrylamide solution, bromophenol blue, and Bradford protein assay reagent were from Bio-Rad Laboratories Ltd. (Hercules, CA). Potassium phosphate (KH_2PO_4), sodium phosphate (Na_2HPO_4), and sodium chloride (KCl) were from Caledon Labs Ltd. (Georgetown, ON). TEMED was from EMD Chemicals Inc. (Gibbstown, NJ). Glycerol was from MP Biomedicals (Solon, OH). Protein G-Agarose was from Roche Diagnostics (Laval, QC). $AP20187^{D}$ and $AP21998^{M}$ were generous gifts from ARIAD Pharmaceuticals, Inc (Cambridge, MA).

2.2. Plasmids and constructs

The pC1-Neo-hMUC1-TR+ FLAG plasmid carrying the MUC1 gene was kindly provided by Dr. Sandra Gendler and was used to generate MUC1-CFP and its derivatives MUC1-CFP-FvHA, MUC1-CFP-FvHA (AQA), MUC1-CFP-FvHA ΔSH2 (Y46F), MUC1-CFP-FvHA ΔSH3(P37A, P38A), MUC1-CFP-FvHA ΔSH2/3 (Y46F/P37A, P38A), MUC1 (VP), and MUC1-CFP (G4) (work by J. Rahn, Q.Shen, J. Zhang, and A. Bernier) (Fig 2.1). pUC-CVM-MUCY plasmid was from Gene-Therapeutics Luckenwalde (Luckenwalde, Germany) and was used to construct MUCY-YFP-FvHA. MUC1-CFP and MUCY-YFP constructs were constructed by inserting the MUC1/MUCY genes into pECFP/pEYFP plasmids (ClonTech) respectively, at XhoI and SacII sites. The plasmid pC4-Fv1E encoding the FKBP F36V variant followed by a c-terminal hemagglutinin (HA) epitope was generously provided by ARIAD Pharmaceuticals Inc and is described in [387]. To generate the MUC1-CFP-FvHA and MUCY-YFP-FvHA fusion proteins, the FvHA domain of pC4-Fv1E was amplified by polymerase chain reaction (PCR) with a 5' primer (ATTTGTACAT

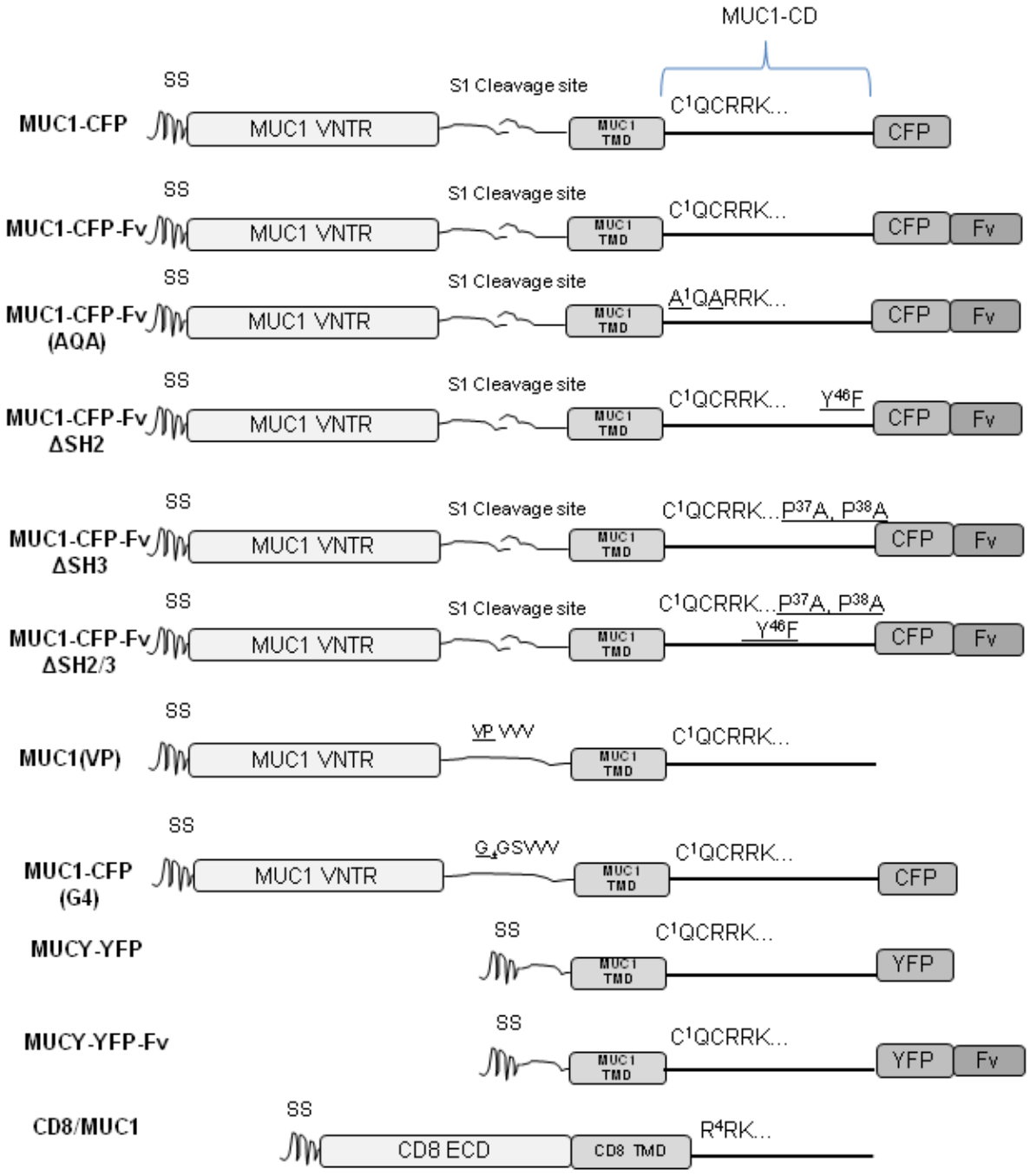


Figure 2.1. Schematic of MUC1 constructs used in this study. Mutations are underlined. "SS" denotes signal sequence.

GGCTTCTAGAGGAGTGC**)** and a 3' primer (CTCTTGTACACTGAAGTTCTCAGG ATCC**)** which introduced 3' and 5' BsrG1 restriction sites (underlined). The PCR product and MUC1-CFP/MUCY-YFP plasmids were digested with BsrG1, ligated, and sequenced to confirm insertion and orientation. MUC1-CFP-FvHA (CQC to AQA) was constructed by PCR of MUC1-CFP-FvHA the primer TTGGCTGTCGCTC AGGCCCGCCGAAAG containing the mutation (underlined). MUC1-CFP-FvHA ΔSH2, MUC1-CFP-FvHA ΔSH3 and MUC1-CFP-FvHA ΔSH2/3 were constructed by PCR of MUC1-CFP-FvHA using the primers TACCGATCGTAGCCCCTTTGAGAAGGTTTC, CGCTATGTGGCCGCTAGCAGTACCGATC or both sequentially, respectively. MUC1-CFP (G4), described in [200], was constructed by PCR of MUC1-CFP using the primer TTCAGGCCAGGAGGTGGTGGA GGATCTGTGGTG containing an additional four glycine residues (underlined) immediately N-terminal to the S1 cleavage site, G_4GSVVV. The pcDNA3.1MUC1 (VP) plasmid, described in [26], was kindly provided by Dr. K.C. Kim (Lovelace Respiratory Research Institute, AZ) and contains a mutation at the MUC1 S1 cleavage site encoding G/SVVV to VPVVV. MUC1 (VP) and MUC1-CFP plasmids. The pcDNA3.1-CD8/MUC1 plasmid, described in [380] and also kindly provided by Dr. K.C. Kim, encodes a construct containing the extracellular and transmembrane portions of CD8 and MUC1-CD, beginning at R^4RK. The c-Src and Y530F c-Src plasmids were kindly provided by Dr. T. Pawson (University of Toronto, Ontario).

2.3. Cell culture and transfection

Human breast cancer cell lines T47D and MCF-7 were from the American Type Culture Collection (ATCC) and were maintained in DMEM with 10% FBS and 6ug/ml insulin. 293T human embryonic kidney epithelial cells (HEK 293T) were from ATCC

and maintained in DMEM with 10% FBS. MEF SYF-/- cells were kindly provided by Dr. Don Fujita (University of Calgary, Alberta) and maintained in DMEM + 10%FBS. Mock (empty plasmid) and ICAM-1 transfected NIH 3T3 mouse fibroblast cells were a generous gift of Dr. Ken Dimock (University of Ottawa, Ontario) and were maintained in DMEM with 10% FBS and 5ug/ml Blasticidin S. Generation of MUC1 HEK 293T transfectants was performed using Lipofectamine 2000 and Opti-MEM according to manufacturer's instructions, and maintained in DMEM with 10% FBS and 200µg/ml G418. For certain experiments, transfectant populations were sorted by flow cytometry for stable clone selection, and others were used for experiments within three days of transfection. All cell lines were cultured at 37°C in a humidified incubator containing 5% CO_2 (Water-Jacketed Incubator, Forma Scientific, Marietta, OH).

2.4. Small interfering ribonucleic acid (siRNA) knockdown

Approximately 1 x 10^5 HEK 293T cells were plated in a 24-well plate (Corning Life Sciences, Lowell, MA) and allowed to adhere overnight to approximately 50% confluency. siRNA (Dharmacon) consisted of four pooled siRNA species targeting the following *SRC* sequences: GCAGUUGUAUGCUGUGGUU, GCAGAGAACCCGAG AGGGA, CCAAGGGCCUCAACGUGAA, and GGGAGAACCUCUAGGCACA. Transfection was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Lipofectamine 2000 reagent only or Scramble siRNA (provided by the manufacturer) were used as negative controls.

2.5. SDS-PAGE and Western blot analysis

Cells were lysed in ice-cold Triton X-100 buffer (20mM Tris-HCl pH7.4, 150mM NaCl, 2mM EDTA, 1% glycerol, 1% Triton X-100, with fresh 0.5% (w/v)

protease inhibitor cocktail and phosphatase inhibitor cocktail), homogenized with a 26 gauge needle and insoluble components pelleted by centrifugation at 14000 x g for 3 minutes. Supernatants were removed and assayed for protein concentration using Bradford assay (Bio-Rad), followed by boiling for 10 minutes in 4x Laemmli sample buffer (LSB) (0.5M Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, with 0.05% bromophenol blue for "reducing" conditions (standard) and omitted in "non-reducing conditions" (where indicated)). Samples were either stored at -20°C or subjected to 4-20% Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed using the Bio-Rad Mini-PROTEAN II system at 60mA per gel, 80 V (constant voltage) for 15 minutes followed by 120 V for the remainder in running buffer (25mM Tris-Base pH 8.3, 192mM glycine, 0.1% SDS). The proteins were then transferred to nitrocellulose membrane (Bio-Rad) using the Bio-Rad Mini Trans-blot system on ice for 60 minutes at 100 V and 350 mA in transfer buffer (25mM Tris-Base pH 8.3, 192mM glycine, 20% MeOH). Nitrocellulose membranes were then blocked in 5% (w/v) skim milk in Tris-buffered saline (TBS) (50mM Tris-Base pH 7.5, 150mM NaCl) with 0.05% (v/v) Tween-20 (TBS-T) for one hour at room temperature (RT) with gentle agitation. The membranes were then incubated with primary antibody overnight at 4°C at the concentration recommended by the manufacturer, followed by two 10 minute washes in TBS and one 10 minute wash in TBS-T. The membranes were then incubated with HRP-conjugated seconday antibody for one hour at RT at the concentration recommended by the manufacturer, following by another washing step identical to the first. Membranes were incubated with ECL plus western blotting reagent according to manufacturer's instructions, developed, scanned using a Canon Canoscan 8600F, imported into Image J (National Institutes of Health), analyzed for densitometry and/or

adjusted and cropped for presentation. Minimal adjustment for brightness and contrast was performed, but the edited image was representative of the original in all cases.

If membranes were to be probed by additional antibodies to proteins such as tubulin, used as a protein loading control, they were first stripped of all original antibody by incubation in stripping buffer (62.5mM Tris-HCl pH 6.7, 2% SDS, 100mM β-mercaptoethanol) for 30 minutes at 50°C with gentle agitation. Following a wash step and re-blocking, membranes were probed as described above.

2.6. Dimer detection

Human breast cancer cells or transfected HEK 293T cells were plated on 0.1% gelatin coated, UV-treated dishes and allowed to adhere overnight to approximately 70% confluency. Cells were then serum starved for 45 minutes in Imaging Buffer (152mM NaCl, 5.4mM KCl, 0.8mM $MgCl_2{\cdot}6H_2O$, 1.8mM $CaCl_2{\cdot}2H_2O$, 10mM HEPES, 5.6mM D-glucose). Where indicated, cells were then treated with $AP21998^M$, $AP20187^D$ or NIH-ICAM-1 cells in 37°C Imaging buffer, followed by aspiration of buffer and addition of 1mM DSS in ice-cold PBS for 10 minutes at 4°C. DSS is a membrane-permeable, irreversible crosslinker containing amine-reactive N-hydroxysuccinimide ester at each end of an 8-carbon spacer arm (11.4 Å) (Fig 2.2). It reacts with primary amines (ie. lysine) to form stable amide bonds between proteins. DSS does not induce dimerization of lysine-containing proteins, but rather aids in identification of protein complexes which are already formed upon treatment. DSS was aspirated, and cells resuspended in quenching solution (1M Tris, pH 7.5) and centrifuged at 2500rpm for 3 minutes. The cell pellet was resuspended in ice-cold lysis buffer, and prepared for Western blot analysis as described.

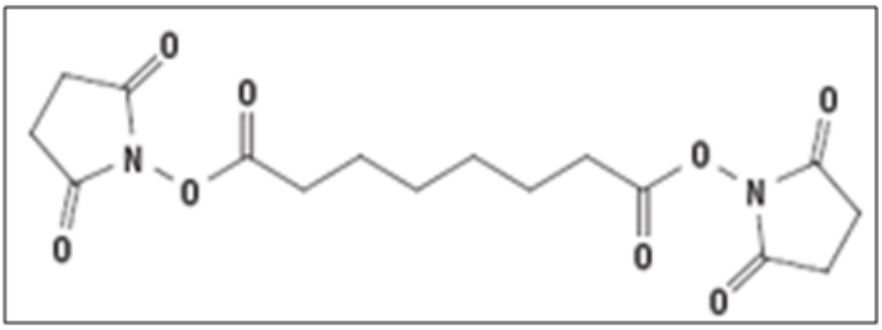


Figure 2.2. Structure of DSS. This membrane-permeable, irreversible crosslinker acts by reacting with primary amines to form new amide bonds between proteins. The 8-carbon space is 11.4 Å, indicating that only protein-protein pairs with primary amines within this distance will be crosslinked.

2.7. Dimer manipulation

Using the "ARGENT^{TM} Regulated homodimerization kit" [388] and the "RPD^{TM} Regulated secretion/aggregation kit" [389] (Ariad Pharmacauticals, Inc.), we developed a system for manipulation of MUC1-CD dimerization. The kits were designed to manipulate protein dimerization status by interacting an engineered "Fv dimerization domain" with monovalent and bivalent ligands. The "Fv domain" was created by a F36V mutation of naturally occurring FK506 binding protein (FKBP) to prevent binding of Fv ligands to endogenous FKBP [387] and a MUC1-CFP-Fv construct was creating using this plasmid, as described in Section 2.2. Importantly, the Fv domain itself does not induce dimerization, and addition of Fv domain ligands is required to manipulate dimerization status of Fv domain containing proteins. The bivalent Fv ligand AP20187^{D} was designed to induce dimerization of Fv domain containing proteins, while the monovalent Fv ligand AP21998^{M} was designed to disaggregate existing dimers (Fig 2.3). Through a Materials Transfer Agreement, we obtained a plasmid encoding the Fv domain, followed by a hemagglutinin (HA) tag (FvHA), (hereafter referred to as "Fv domain") and both AP20187^{D} and AP21998^{M}.

2.8. Cell viability assay

Cells were trypsinized, centrifuged, and the pellet resuspended in PBS. 20µl of cell solution was removed and combined with 20 µl of 0.4% trypan blue reagent. Two replicates of 10µl of mixture were applied to a hemacytometer and live cells for five fields (2 x 10^{-4} ml each) were counted and averaged. Cells were counted as "live" if they exhibited no cytoplasmic staining of trypan blue.

2.9. Immunoprecipitation

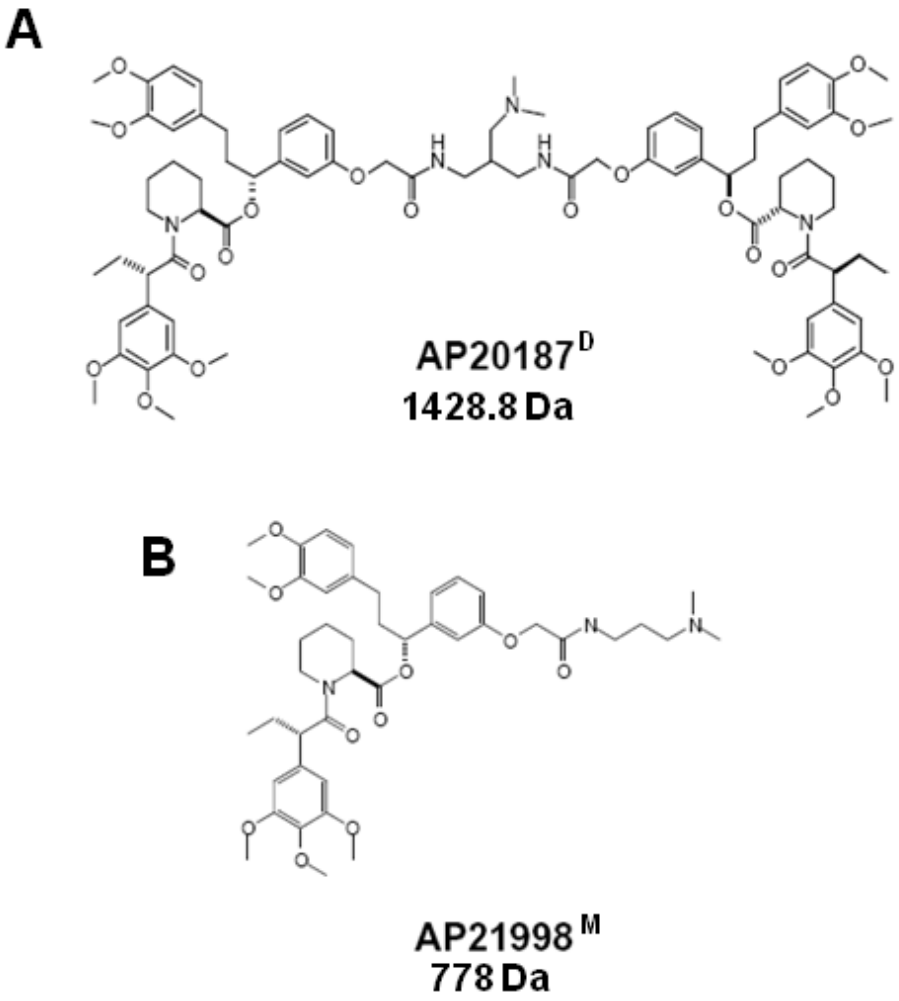


Figure 2.3. Structure of ARIAD Fv ligands. $AP20187^{D}$(A), the "dimerization compound" and $AP21998^{M}$(B), the "monomerization compound", both act by ligating an engineered "Fv domain".

Following treatment(s), cells were lysed in Co-immunoprecipitation lysis buffer (50mM Tris pH 7.6, 100mM NaCl, 0.5mM EDTA, 0.5% Nonidet P-40, 0.5% protease and phosphatase inhibitors). Following homogenization and centrifugation, supernatant was placed in a 1.5ml Eppendorf tube. At this point, 30µl of cell extract could be removed, combined with 10µl of LSB, and boiled for 10 minutes, to be used as whole cell lysate (WCL). The remaining lysate was incubated with 10µg/ml of anti-MUC1-CD mAb for 2-4 hours at 4°C with gentle agitation. The lysate was then combined with 50µl of protein G-agarose, which was previously washed with lysis buffer to remove any soluble components, overnight at 4°C with gentle agitation. The immunocomplexes were then pelleted by centrifugation at 14,000 x g for 2 minutes, rinsed 3 times with Co-immunoprecipitation rinse buffer (50mM Tris pH 7.6, 100mM NaCl, 0.5mM EDTA, with fresh 0.5% (w/v) protease inhibitor cocktail and phosphatase inhibitor cocktail). The pelleted immunocomplex (~60µl) was then combined with 20µl of LSB and boiled for 10 minutes, followed by centrifugation at 14,000 x g for 10 minutes. The supernatant was collected and either run on SDS-PAGE immediately or stored at -20°C. As negative controls, samples containing only precipitating antibody (anti-MUC1-CD) +/- protein G-agarose was included in order to identify non-specific bands in Western blots.

2.10. Densitometry

In order to quantitate changes in protein levels revealed by western blot, densitometric analysis of protein bands was performed. Using Image J software (NIH), each band in a series was assayed for pixel density. For assays in which changes in total protein levels are of interest, each band of interest was normalized to a standard control such as tubulin, or in the case of anti-MUC1-CD immunoprecipitation, MUC1-CD. For assays in which changes in band ratio is of interest (ie. MUC1-CD dimer: total assays),

the ratio for each experimental condition was calculated and comparisons between conditions were made.

2.11. Calcium oscillation assay

35mm glass-bottomed MatTek microwell dishes were coated with 100µl of FBS (for breast cancer cell lines) or 0.1% (w/v) gelatin (for HEK 293T transfectants) and allowed to dry under UV-light for 2 hours. 100µl of human breast cancer cells or transfectants at 5-10 x 10^4 cells/ml were plated onto the wells and allowed to adhere overnight, resulting in about 70% confluency. For cleavage inhibition experiments, TAPI-0 containing media was added at this point and cells left for 24 hours (MUC1-CFP transfectants) or 40 hours (T47D cells). The culture media was then removed and replaced with DMEM and 10% FBS freshly supplemented with a mixture of 5mM Fluo-3 and 20% (w/v) Pluronic F-127 at a 1:1 ratio, diluted in the media 1/500, +/- 100µM TAPI-0. This results in a final concentration of 5µM Fluo-3 and 0.02% Pluronic F-127. The cells were incubated in this solution for 1 hour at 37°C, 5% CO_2, followed by rinsing with imaging buffer and left in imaging buffer (+/- 100µM TAPI-0) at 37°C, 5% CO_2 until experimentation. NIH ICAM-1 and NIH Mock treatment cells were prepared by suspension in imaging buffer at 1 x 10^7 cells/ml and incubation at 37°C, 5% CO_2 experimentation. Immediatly before experimentation, the MatTek dish containing the plated cells was placed on a 37°C stage warmer under the 20X Neo-Fluar objective of a Zeiss Axioscope Digital Imaging Microscope. A representative area of cells was focused, and a differential interference contrast image (DIC) (20ms exposure) was taken as the "before" image. Imaging buffer/TAPI-0 was then removed and cells were treated with 1uM $AP20187^D$ or $AP21998^M$ for one minute, if applicable to the experiment. Buffer was removed, and 60 images were taken under a fluorescein isothiocyanate (FITC) filter

(200ms exposure) at 3 second intervals using IonWave software (Empix Imaging Inc.). Following the first image, 100µl of NIH ICAM-1 or mock cells were added directly to the plated cells. A final DIC image was taken at the end of the experiment to ensure that plated cells had been covered by treatment cells. (Fig 2.4).

Quantitative analysis of each CaOs experiment (Fig 2.5) was performed by circling 40 random cells from the "before" DIC image (circled areas = regions of interest (ROI)). For each series of images (60 images per experiment), the change in average fluorescent intensity was plotted for each ROI and exported to MS Excel. For each ROI, an "oscillation factor" was determined by multiplying the number of oscillation cycles by the "amplitude factor" for each graph. The number of cycles was counted manually and the "amplitude factor" was calculated by plotting an Excel LOGEST trend line ($y = \text{intercept} \cdot \text{slope}^x$) from the start of the oscillations to the last recorded data point. The sum of the absolute values between the plotted trend line and the actual recorded data is the amplitude factor. Each experimental condition was repeated a minimum of 3 times, to make n=120 for statistical purposes.

2.12. Transwell migration assay

The upper membranes of 6.5mm diameter, 8µm pore size Transwell inserts (Corning Inc., Corning, NY) were coated with 0.1% gelatin and allowed to dry under UV light for 2 hours. Inserts were placed in a 24-well plate and 200µl of NIH ICAM-1/Mock cell suspension at 1.5 x 10^5 cells/ml was added to the upper chamber. Cells were allowed to adhere overnight at 37ºC, 5% CO_2, resulting in a single-cell monolayer of about 95% confluency the next day. For cleavage inhibition experiments, MUC1-CFP transfectants and T47D cells ("drop" cells) were pre-treated with TAPI-0 for 24 or 40 hours, respectively, prior to migration assay. Drop cells were suspended in serum-free DMEM

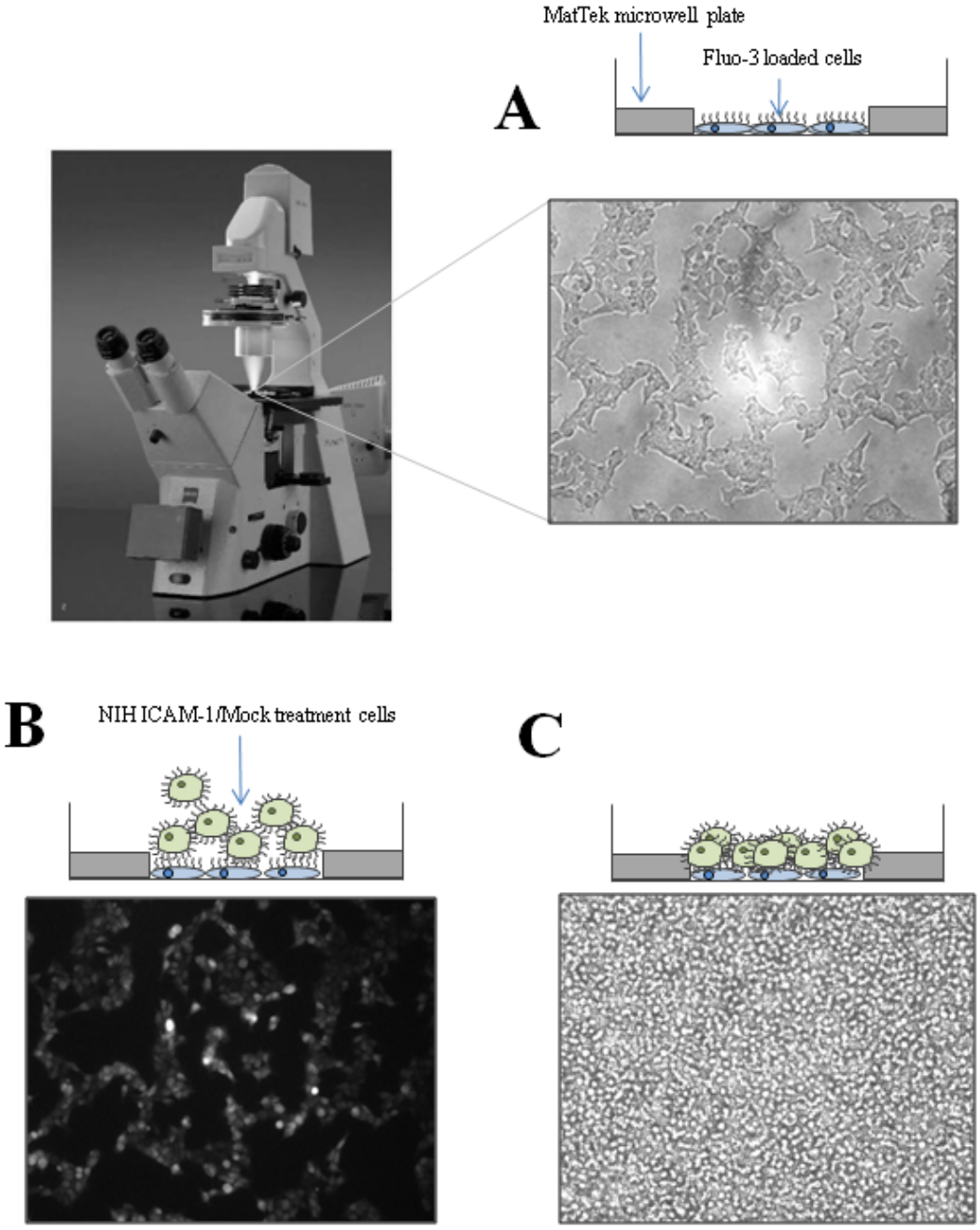
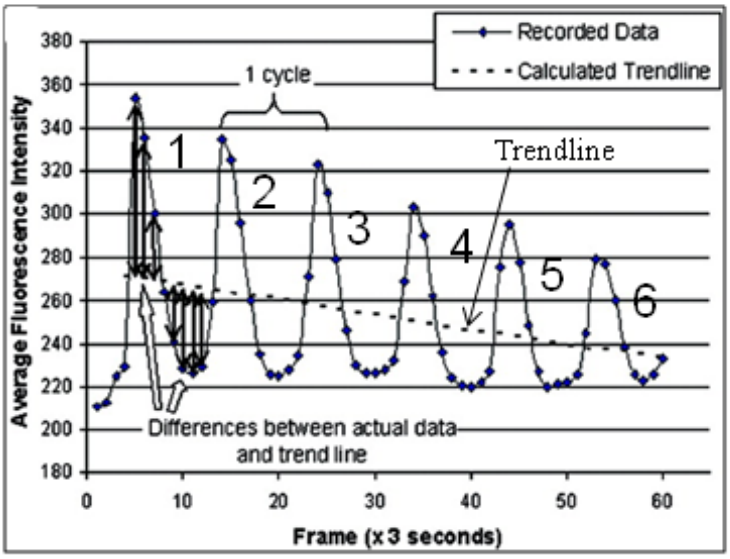


Figure 2.4. The calcium oscillation assay. Using a Zeiss Axioscope Digital Imaging Microscope, a DIC image of Fluo-3 loaded cells is taken **(A)** to use as a "before" image for quantification purposes. Following drop of treatment cells, imaging of cells under a FITC filter reveals changes in intracellular calcium levels (**B**). At the end of the experiment, an additional DIC image is taken to enusre dropped treatment cells have completely covered plated cells. Representative images shown.



Trendline = LOGEST function in Excel; $y = \text{intercept} \cdot \text{slope}^x$

Amplitude factor = Absolute value of differences between trendline and data points

Number of cycles = Manually counted number of up/down oscillations.

Oscillation factor = Amplitude factor x number of cycles

Figure 2.5. Quantitative analysis of calcium oscillation assay. A representative experimental result is shown for demonstration purposes. Amplitude factor was calculated by summing the absolute values of the differences between the "trendline" and the actual data points. Number of cycles was counted manually, and one cycle was considered an obvious "peak" in the graph. Multiplying the amplitude factor by the number of cycles generated the Oscillation Factor. Adapted from [13] with permission from the publisher.

containing 5 µM Cell tracker green CMFDA, +/- 100µM TAPI-0, for 30 minutes at 37°C, followed by incubation in serum free DMEM at 37°C for 30 minutes. Cell tracker green CMFDA is a cell-permeable thiol reactive probe which reacts with intracellular components to produce cells which are fluorescent under a FITC filter. Following incubation, cells were centrifuged at 2500rpm for 2 minutes and suspended in serum-free media at a concentration of 8 x 10^5 cells/ml. Chemical compounds such as $AP20187^D$, $AP21998^M$ or TAPI-0 were added at this point if appropriate. Media was removed from upper and lower chambers of Transwells, 200µl of cell suspension was added to the upper membrane of Transwell inserts and 500µl of fresh, serum-free DMEM was added to lower chamber, also containing $AP20187^D$, $AP21998^M$ or TAPI-0 if appropriate. Following incubation at 37°C overnight, media was removed and 2% paraformaldehyde in PBS was added to each chamber for 15 minutes. Cells were washed twice with PBS, and cells on the upper membrane of the insert were removed with a sterile cotton swab (Fig 2.6). The insert was then placed under a Zeiss Axioscope Digital Imaging Microscope using a FITC filter and 20x objective and images were obtained for five distinct fields of view. Cells were counted using the Northern Eclipse software (Empix) by applying psuedocolored maps to each image to aid in counting (Fig 2.7). To avoid bias, the first field of view was always at the "top" of the well, with each subsequent field of view directly below the last. When counting cells, a manual counting tool was used which numbered each cell as it was counted. As some of the Transwell membrane pores were visible in the fluorescent images, inclusion criteria for migrated cells was created. To be counted, a cell must either have: (a) have a significantly greater size than membrane pores; or (b) have significantly greater fluorescent intensity than membrane pores (Fig 2.7).

2.13. Flow cytometry

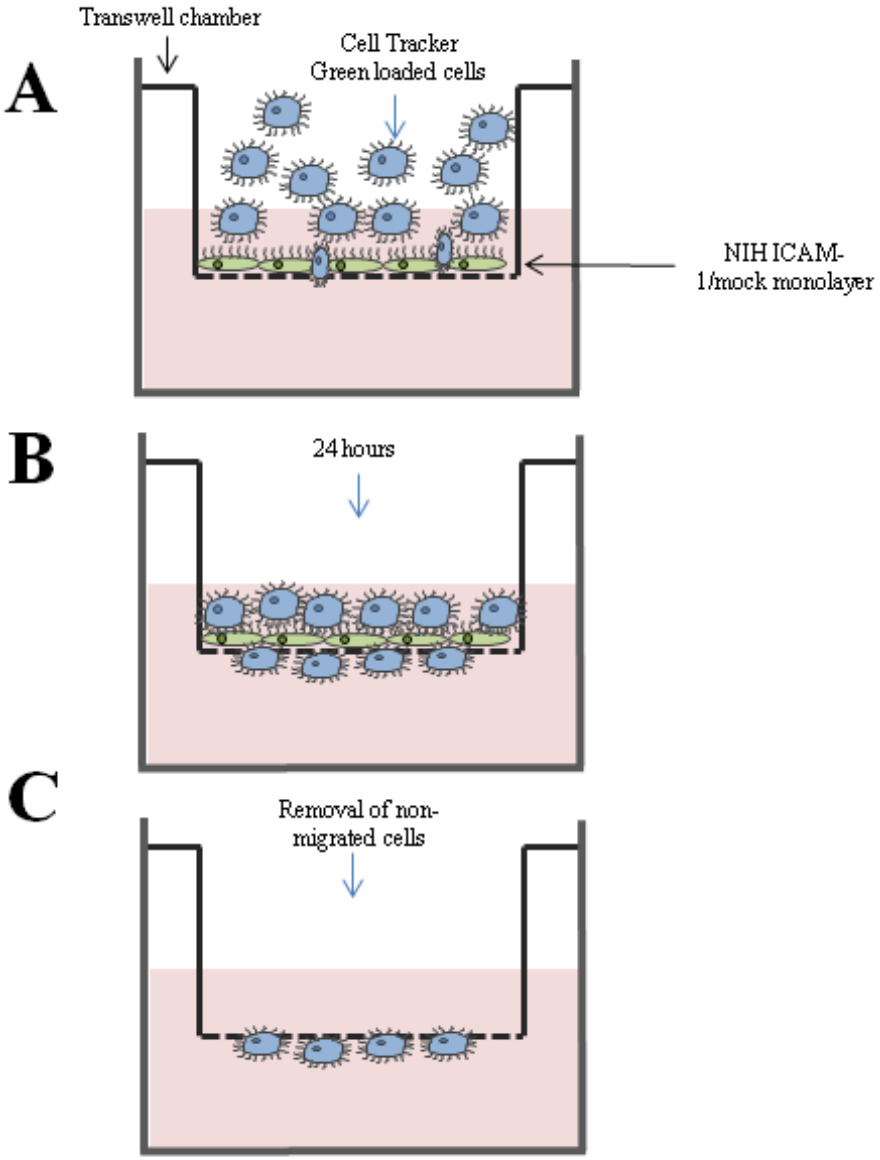


Figure 2.6. Schematic of Transwell invasion assay. Following loading with the fluorescent indicator Cell Tracker Green, human breast cancer or transfected HEK293T were dropped onto a monolayer of NIH ICAM-1/mock cells (**A**). After 24 hours (**B**), non-migrated cells were removed and migrated cells counted (C).

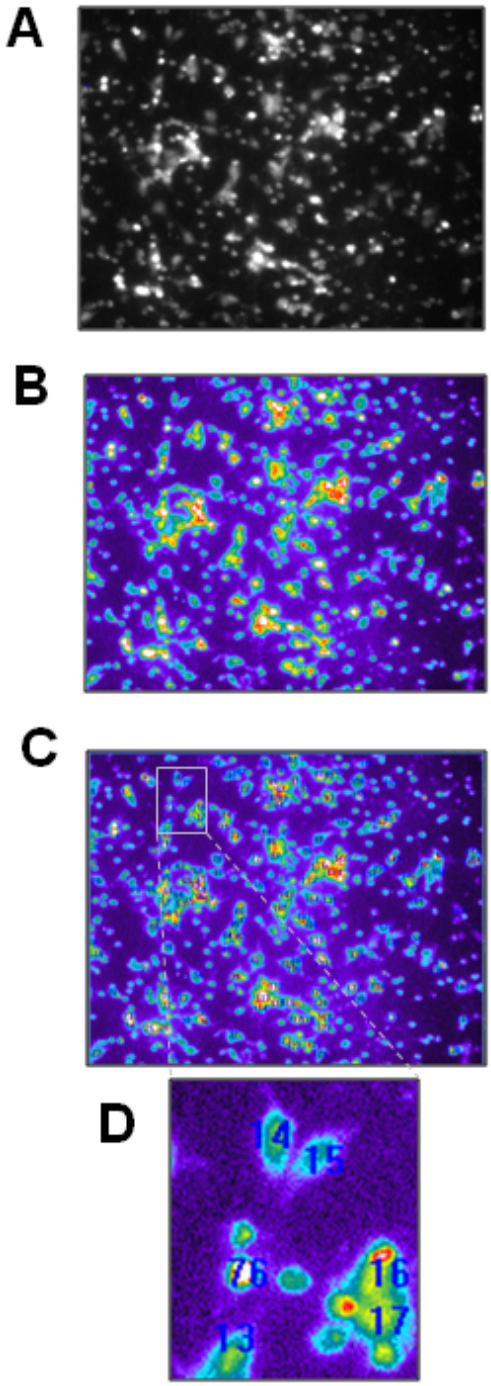


Figure 2.7. Counting migrated cells in Transwell assay. Images obtained using a FITC filter (A), are applied with a psuedocolored map (red = higher fluorescent intensity) (B). Migrated cells are counted using the manual count tool (C). An area is shown in larger magnification (D) to illustrate criteria for inclusion. Representative images shown .

For analysis of protein expression levels at the cell surface, flow cytometry was performed using FITC-conjugated anti-MUC1-ECD (FluoroTag^{TM} FITC conjugation kit, Sigma). Cells were suspended in ice-cold flow cytometry buffer (PBS, 0.5mM EDTA, 1% FBS) and 10µg/ml of FITC-conjugated anti-MUC1-ECD was added. Tubes were kept in the dark at 4°C with gentle agitation for 1 hour. Cells were then washed twice in ice-cold flow cytometry buffer to remove unbound antibody. As a control, MUC1-negative HEK 293T (parental) cells, both untreated and stained with FITC-conjugated anti-MUC1-ECD were submitted to account for autofluorescence and non-specific staining, respectively. For individual cell sorting, a FACSAria cell sorter was used and operated by Dorothy Kratochwil-Otto at the University of Alberta Flow Cytometry Facility. For analysis of protein expression, a FACSCalibur non-sorting dual laser instrument was used and operated by Ashlyn Bernier. The mean fluorescence for three independant experimental trials was obtained and used in analysis.

2.14. Shedding assay

1 x 10^6 human breast cancer or MUC1-transfected HEK 293T cells were plated in a 6-well plate, and allowed to adhere overnight to approximately 70% confluency. Media was removed and replaced with DMEM with 10% FBS containing treatment compound if applicable. If treatment duration exceeded 24 hours, media (containing treatment if applicable) was replaced 24 hours prior to end of treatment duration to control for time-dependant changes in MUC1 shedding. Following collection of conditioned media, cells were lysed as described in Section 2.5. Media was syringe-filtered through a 0.45nM filter to remove any cellular debris, assayed for protein concentration using Bradford Assay and prepared for SDS-PAGE as described in Section 2.5.

2.15. Statistics

Experiments were performed at least three times to allow for statistical analysis. Statistical analysis was performed on raw data before negative controls were set to one. The Newman-Keuls multiple range comparison was used to determine statistical differences in data sets with more than two experimental conditions. For pairwise comparisons, the Student's *t* test was used. P values are indicated for each analysis. For each experiment, *Asterisk* indicates pairs in the data set which are statistically different, or populations which do not overlap with any other in the data set, ($p<0.05$).

Chapter 3: Role of Src kinase in MUC1/ICAM-1 signalling

3.1. Introduction

Overexpression of MUC1 and overactivity of the SFK Src have been implicated in breast cancer progression and metastasis [7, 128, 137, 327]. The relationship between MUC1 and Src has been the subject of numerous studies, and Src kinase activity has been implicated in transmission of the MUC1/ICAM-1 signal. Specifically, it has been shown that pre-treatment of human breast cancer or MUC1 expressing HEK 293T cells with the SFK inhibitor PP2 significantly abrogates both the observed ICAM-1 binding induced calcium oscillatory signal [13] and actin cytoskeletal rearrangements [14]. The pathway downstream of Src in the MUC1/ICAM-1 signalling axis leading to actin cytoskeletal rearrangements and motility has been identified as CrkL-Rac1/Cdc42 [14]. A direct association between MUC1 and Src has been demonstrated by multiple groups. Association of both Src SH2 and SH3 domains with MUC1-CD has been reported, and the SH2 domain binding site on MUC1-CD has been identified as $pY^{46}EKV$[267]. Interestingly, Y^{46} is also a reported substrate for Src kinase. Following ICAM-1 binding, increased association of MUC1-CD and Src has been observed, as well as the appearance of tyrosine phosphorylated MUC1-CD. These data can be interpreted to suggest that MUC1 and Src are binding partners, and Src is likely the entity responsible for initiation of the MUC1/ICAM-1 signal through phosphorylation of MUC1-CD and recruitment of downstream signalling mediators such as CrkL, resulting in both CaOs and cell motility. However, due to the non-specificity of the SFK inhibitor PP2 and the circumstantial nature of the MUC1/Src binding data, a definitive role for Src in MUC1/ICAM-1 signalling has not been demonstrated. In this chapter, we will use Src siRNA to specifically decrease the level of Src protein in MUC1-transfected HEK 293T cells and determine the effect on previously described ICAM-1 binding induced signalling events. We hypothesize that reduction of Src kinase levels in MUC1 expressing cells will result

in a significant reduction in ICAM-1 binding induced signalling. Definitive identification of Src as a mediator of the MUC1/ICAM-1 signal is a crucial step in understanding the significance of this binding in cancer metastasis.

3.2. Results

3.2.1. Src protein levels are significantly decreased by siRNA treatment

To determine the optimal dose and incubation period for Src siRNA in order to get the maximum decrease in Src protein levels, we performed a time/dose course on HEK 293T MUC1-CFP cells. In a 24-well format, we varied the concentration of Lipofectamine 2000^{TM} transfection reagent from 0.5µl to 1.5µl per well (manufacturer recommendation 1.0µl per well). We also varied the concentration of siRNA from 10pmol to 40pmol per well (manufacturer recommendation 20pmol per well). For each combination of Lipofectamine and siRNA, we assayed Src protein levels after 24, 48, and 72 hours incubcation, for a total of 30 conditions, and included a control with Lipofectamine reagent only (Fig 3.1). For each condition, lysates were run on SDS-PAGE and probed with anti-Src and anti-tubulin as a loading control. Densitometry on Src bands, normalized to tubulin bands (Image J) was performed, and the results graphed, with the Lipofectamine only control condition set to one (Fig 3.2). Conditions in which Src levels were reduced >50% compared to control are indicated by a hashmark (#).

Based on the time/dose course experiment, we decided that we would use 0.5µl Lipofectamine and 20pmol Src siRNA per well for 48 hours for the remaining experiments. Scramble, non-targeting siRNA was used as a negative control in addition to Lipofectamine only to ensure that siRNA treatment alone does not reduce protein expression levels. Both HEK 293T (parental) and HEK 293T MUC1-CFP cells were assayed for Src and tubulin expression after treatment (Fig 3.3). The level of Src protein

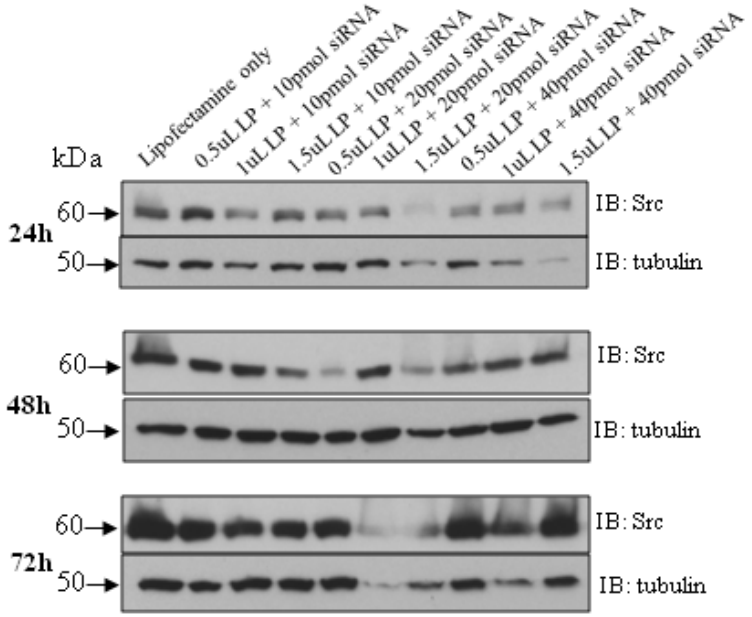


Figure 3.1. Dose and time course analysis of Src siRNA knockdown in HEK293T MUC1-CFP cells. Cells were treated with varying concentrations of Lipofectamide and Src siRNA in a 24-well format for 24, 48 or 72 hours. Cell lysis was followed by SDS-PAGE and probing with anti-Src, followed by membrane stripping and probing with anti-tubulin as a loading control.

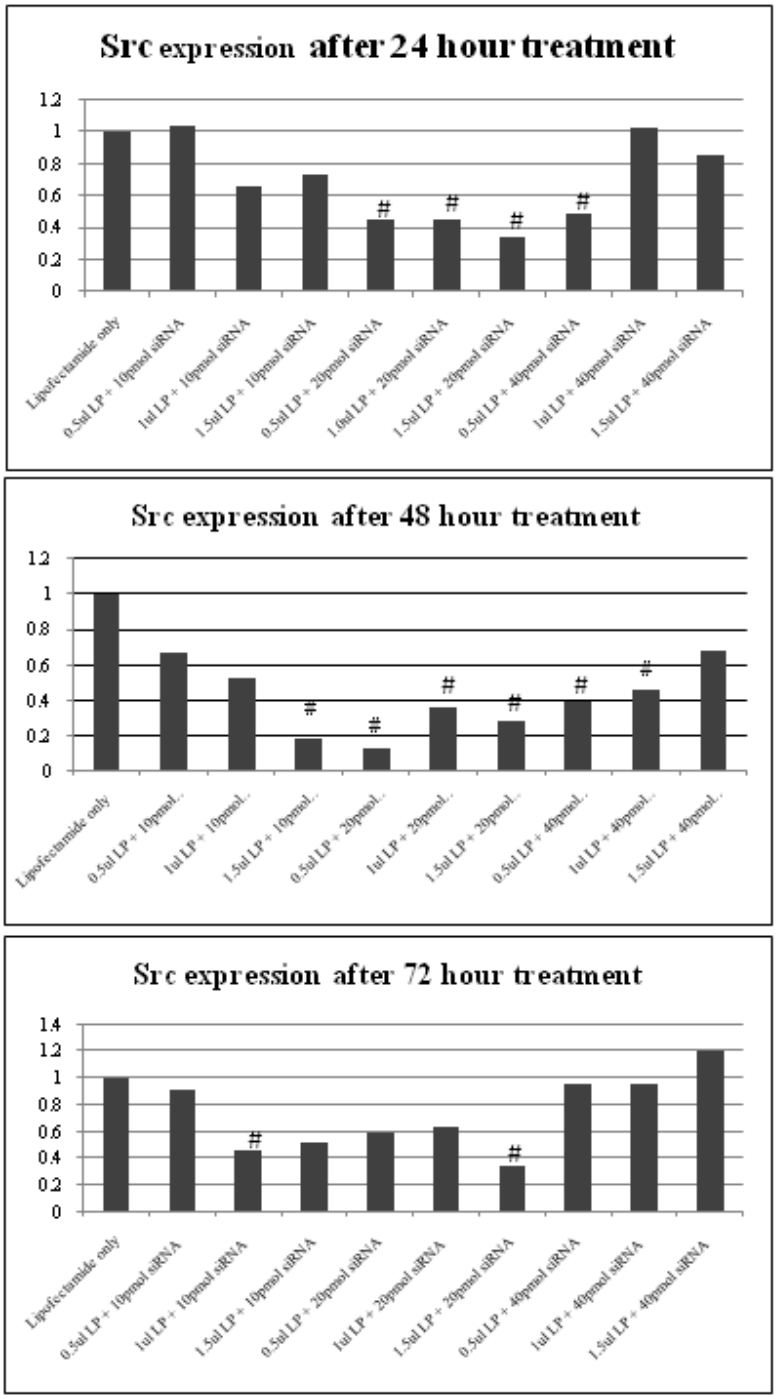


Figure 3.2. Densitometric analysis of Src bands after siRNA knockdown. MUC1-CFP cells were treated with various concentrations of Lipofectamide and Src siRNA for 24, 48 or 72 hours, followed by SDS-PAGE and probing with anti-Src and anti-tubulin. Src bands were analyzed by densitomerty and normalized to tubulin for control of protein loading. Lipofectamide only condition was set to one and each condition presented as a ratio. Hashmarks (#) indicate conditions in which Src protein levels are decreased>50% compared to control. LP = Lipofectamide.

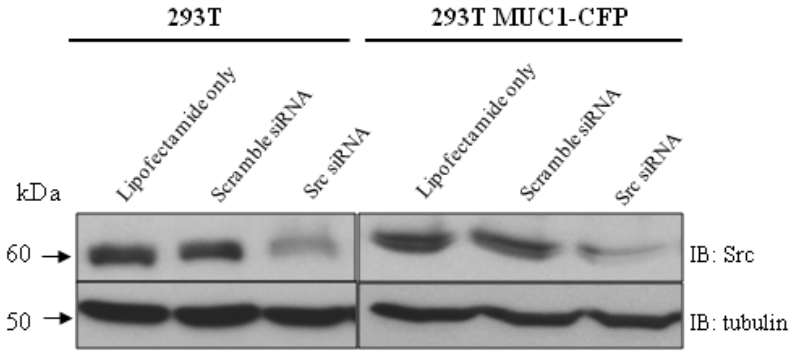


Figure 3.3. Src targeting siRNA results in decreased Src protein levels in HEK 293T and MUC1-CFP transfected cells. HEK 293T and MUC1-CFP transfecants were treated with 0.5µl Lipofectamide and 20pmol Src siRNA for 48 hours, lysed, and ran on SDS-PAGE. Membranes were probed with anti-Src and anti-tubulin as a loading control. Lipofectamide only and Scramble siRNA conditions are included as controls.

was significantly decreased in both cell types after treatment with Src siRNA, while Lipofectamine only and Scramble siRNA had no significant effect. The level of tubulin remained constant for all experimental conditions, demonstrating equal protein loading.

3.2.2. Src is required for MUC1/ICAM-1 binding induced events

Following treatment with Lipofectamine only, Scramble siRNA, or Src siRNA, parental and MUC1-CFP transfectants were assayed for the previously described ICAM-1 binding induced CaOs [13] (Fig 3.4). As parental cells do not express MUC1, the 293T Lipofectamine only condition was set to one as a negative control. All conditions using parental cells resulted in equivalent levels of CaOs, indicating that Src knockdown does not affect the magnitude of CaOs generated in response to ICAM-1 treatment in these cells. In MUC1-transfected cells which were treated with Lipofectamine only, a statistically significant increase in Oscillation Factor after ICAM-1 stimulation was observed compared to parental conditions, in agreement with prior publications describing the MUC1/ICAM-1 binding induced CaOs [13]. Treatment with Scramble siRNA did not result in a significant change in the oscillation factor for MUC1-CFP cells, but treatment with Src siRNA decreased the oscillation factor significantly compared to Lipofectamine only or Scramble siRNA treatments, was still significantly greater than negative control conditions.

Cells were also assayed for transmigration through an ICAM-1 positive monolayer, a previously described phenomenon following MUC1/ICAM-1 binding [15] (Fig 3.5). Again, parental Lipofectamine only treatment condition was set to one and the remaining conditions expressed as a ratio. Parental cell migration was not affected by siRNA treatment, indicating that MUC1 expression is required to elicit ICAM-1 binding mediated CaOs. In MUC1-transfected cells treated with Lipofectamine only, a

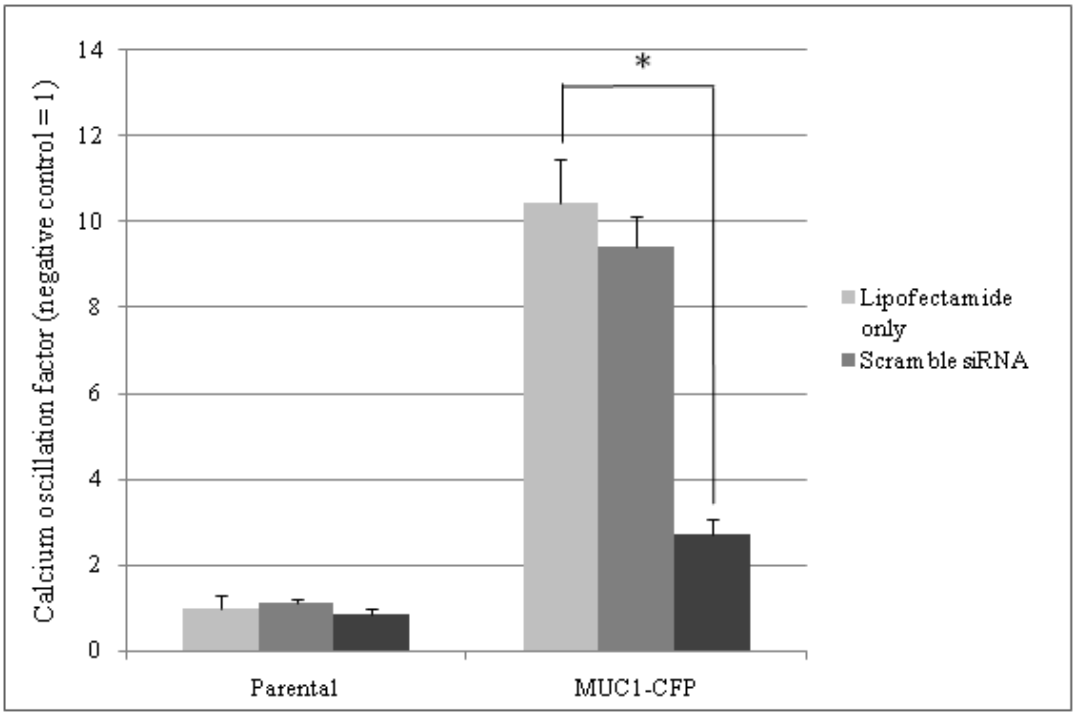


Figure 3.4. siRNA knockdown of Src inhibits ICAM-1 binding induced calcium oscillations in MUC1 expressing cells. HEK 293T (Parental) cells and MUC1-CFP transfectants were treated with NIH ICAM-1 cells and assayed for calcium oscillation factor. Lipofectamide only and Scramble siRNA treatments are included as controls. Parental Lipofectamide only condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average oscillation factors from at least three independent trials; bars, SE. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$).

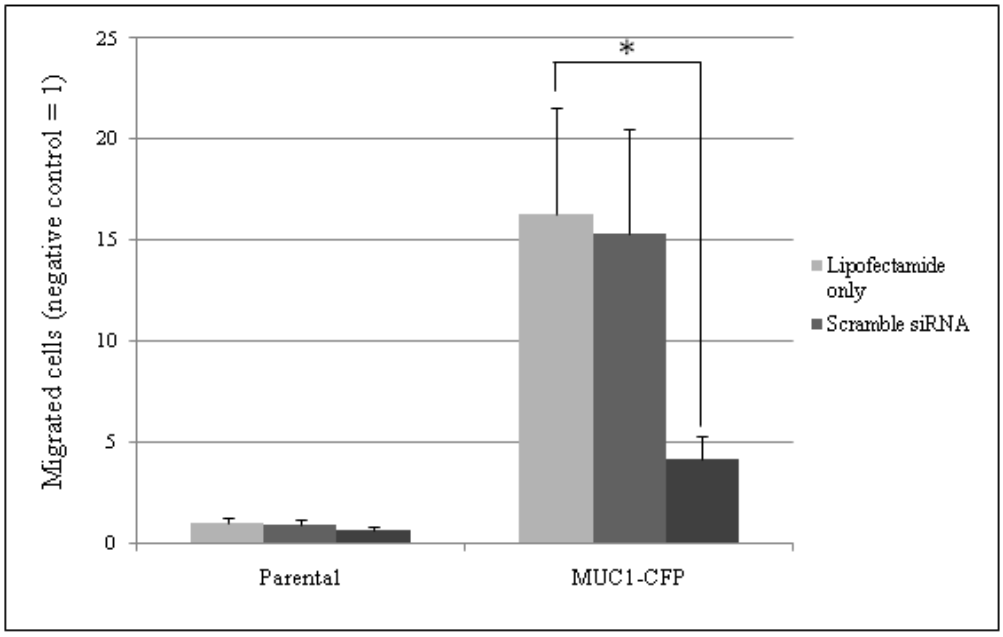


Figure 3.5. siRNA knockdown of Src inhibits ICAM-1 binding induced cell migration in MUC1 expressing cells. . HEK 293T cells and MUC1-CFP transfectants were assayed for migration through an ICAM-1 positive cell monolayer. Lipofectamide only and Scrambled siRNA treatments are included as controls, and 293T Lipofectamide only condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average number of migrated cells per five fields from at least three independent trials; bars, SE. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$).

significantly greater number of cells migrated compared to this control, in agreement with prior data demonstrating that MUC1/ICAM-1 binding results in cell motility and migration [14, 15]. In MUC1-CFP cells, Scramble siRNA did not significantly affect migration levels compared to Lipofectamine only. Treatment of MUC1-CFP cells with Src siRNA significantly reduced the level of cell migration compared to MUC1-CFP Lipofectamine only or Scramble siRNA, but there was still a significantly greater number of cells migrated than parental negative control conditions.

3.3. Conclusion

These data indicate that decreasing the levels of Src protein with siRNA diminish the signalling potential of MUC1/ICAM-1 binding, demonstrating a requirement for Src kinase in effective generation of both CaOs and subsequent cell migration. As siRNA is an effective tool for specific inhibition of protein synthesis, these data demonstrate that Src is a critical component of the MUC1/ICAM-1 signalling axis. As a direct relationship between MUC1 and Src has been demonstrated, investigation of the nature of MUC1/Src binding would reveal information on the mechanism of MUC1/ICAM-1 signalling.

Chapter 4: Dimerization of MUC1-CD and the relationship to Src binding and ICAM-1 induced signalling

4.1. Introduction

Dimerization of a MUC1-CD construct, devoid of a transmembrane domain, has been demonstrated both *in vitro* and *in vivo* [19, 184]. This dimerization has been found to be dependent on the cysteine residues of the membrane-proximal C^1QC motif of the MUC1-CD. This, with the observation that covalently-linked dimers formed *in vitro* suggest that disulfide linkages are responsible for MUC1-CD dimerization. A MUC1-CD peptide containing the C^1QC motif has been demonstrated to disrupt the redox balance of human breast cancer cells, resulting in necrotic cell death in both cellular and animal models [184]. Recently, a small molecule, apigenin, has been identified as an inhibitor of MUC1-CD dimerization and also of cancer cell proliferation [390]. The majority of MUC1 dimerization research has focused on the role of dimerization in redox balance, DNA damage response, and terminal differentiation of cells [183, 184, 391].

Here, we investigated MUC1-CD dimerization in MUC1 constructs containing the TMD, as constitutive dimerization of other cell membrane receptors has been shown to be dependent on the TMD [369, 371, 372]. Using an engineered dimerization domain, we then manipulated MUC1-CD dimerization, and determined if dimerization is necessary for Src recruitment. In Chapter 3, we demonstrated the necessity for Src in ICAM-1 binding induced CaOs and migration. Therefore, we also determined the effect of inhibiting MUC1-CD dimerization (and possibly Src recruitment to MUC1-CD) on ICAM-1 binding induced events. Lastly, we determined if Src binding to MUC1-CD is facilitating MUC1-CD dimerization, as Src contains two potential MUC1-CD binding motifs [267], and it is possible that a single Src molecule can "bridge" a MUC1-CD dimer. We hypothesized that MUC1 exists as a constitutive, covalently linked dimer which is required for Src recruitment and ICAM-1 binding induced signalling; and that Src binding to MUC1-CD SH2/SH3 domains is not required for MUC1-CD dimerization.

Elucidation of the mechanism of MUC1/ICAM-1 binding induced signalling will provide potential targets for anti-metastatic therapies in the future.

4.2. Results

4.2.1. MUC1 forms constitutive cytoplasmic domain dimers in human breast cancer cell lines and transfected HEK 293T cells.

MUC1 positive human breast cancer cell lines MCF-7 and T47D (Fig 4.1A) and HEK 293T cells transfected with MUC1-CFP (Fig 4.1B) or the MUC1 splice variant lacking the tandem repeat domain MUCY-YFP-Fv (Fig 4.1C) were lysed with or without prior treatment with the membrane permeable crosslinker DSS. SDS-PAGE and probing with anti-MUC1-CD revealed the invariable appearance of a new MUC1-CD species at exactly double the molecular weight of the monomeric cytoplasmic domain after treatment with DSS, consistent with the presence of a MUC1-CD homodimer. The appearance of MUC1-CD dimers in MUCY-YFP-Fv transfectants indicates that the tandem repeat domain is not required for dimerization.

We then investigated the contribution of the MUC1 cytoplasmic domain to dimer formation. HEK 293T cells were transfected with MUCY-YFP-Fv and/or CD8/MUC1, described in [380], a chimera of CD8 extracellular and transmembrane domains and MUC1-CD domain, beginning at R^4RK (lacking C^1QC motif). Single transfection of either CD8/MUC1 or MUCY-YFP-Fv and probing with anti-MUC1-CD revealed their molecular weights to be approximately 40 and 75 kDa, respectively (Fig 4.2, lanes 1 and 2). Immunoprecipitation of the CD8/MUC1 + MUCY-YFP-Fv double transfectant (Fig 4.2, lane 3) with anti-CD8 and probing with anti-MUC1-CD (Fig 4.2, lane 4) resulted in the appearance of a 75kDa MUC1-CD species on a Western blot (Fig 4.2, red square), consistent with the molecular weight of MUCY-YFP-Fv. This indicates an association

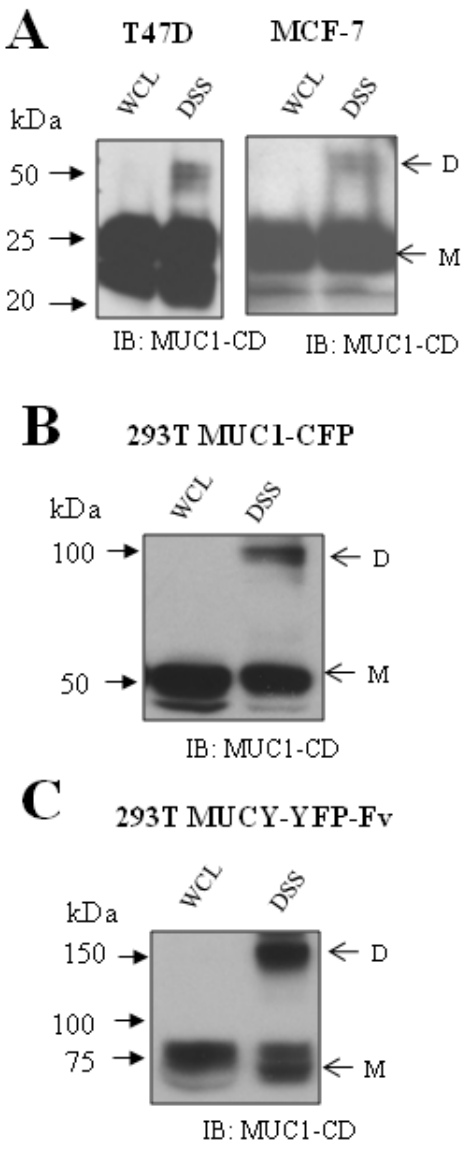


Figure 4.1. MUC1 dimerization in human breast cancer and transfected cell lines. A. Breast cancer cell lines T47D and MCF-7, or HEK 293T cells transfected with MUC1 constructs MUC1-CFP (**B**) or MUCY-YFP-Fv (**C**) were treated with DSS, lysed, ran on SDS-PAGE and probed with anti-MUC1-CD. "D" and "M" indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysate (WCL) lanes are included as controls.

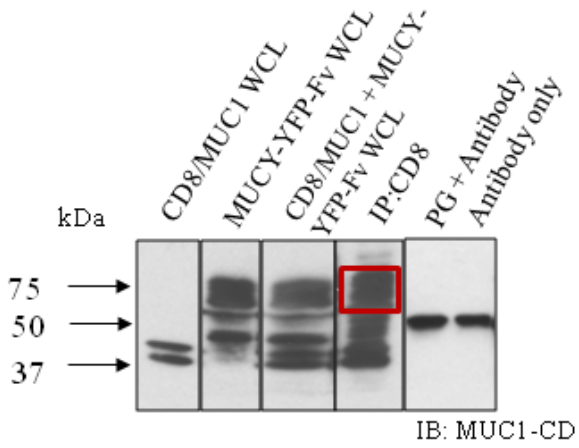
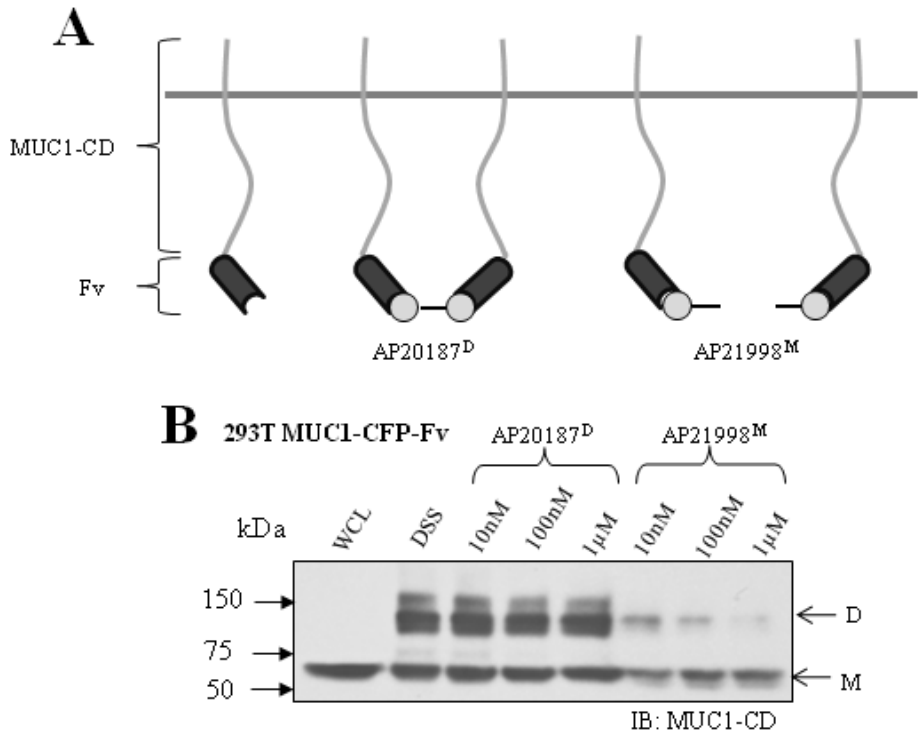


Figure 4.2. The cytoplasmic domain of MUC1 self-associates *in vivo*. HEK 293T cells were transfected with CD8/MUC1 (Lane 1), MUCY-YFP-Fv (Lane 2), or both (Lane 3), lysed, and double transfectants immunoprecipitated with anti-CD8 (Lane 4). Lysates were run on SDS-PAGE and probed with anti-MUC1-CD. PG (Protein G) + Antibody and Antibody only lanes are included as controls. Immunoprecipitation of MUCY-YFP-Fv with anti-CD8 is indicated by the red square.

between CD8/MUC1 and MUCY-YFP-Fv. This association is significant because the CD8/MUC1 construct only contains the cytoplasmic portion of MUC1, beginning at R^4RK, and does not contain the C^1QC motif, fluorescent tags, or the Fv domain. Therefore, association between these two entities must be due to the MUC1 cytoplasmic domain. Taken together, these data show that MUC1-CD forms constitutive cytoplasmic domain dimers which are not dependent on the VNTR domain, TMD, the C^1QC motif, or engineered C-terminal tags.

4.2.2. MUC1 cytoplasmic domain dimerization can be disrupted by addition of an engineered Fv domain and a monomeric Fv domain ligand.

To investigate the importance of MUC1 dimerization in Src association and ICAM-1 induced signalling, we sought to manipulate dimerization using a construct of MUC1 containing a C-terminal Fv domain (ARIAD Pharmaceuticals), and bivalent ($AP20187^D$) or monovalent ($AP21998^M$) ligands. Dimerization of Fv domain containing proteins can be manipulated by addition of Fv domain ligands. Previously, this system has been used to successfully manipulate dimerization of growth factor receptors [392] and G protein-coupled receptors [393]. Mechanistically, the bivalent ligand, which contains two Fv-binding domains, effectively brings two Fv-domain containing proteins within close proximity – "dimerization". The monovalent lingand, which contains one Fv-domain binding domain, is designed to bind to Fv-domain containing proteins and sterically inhibit their interaction with other Fv-domain containing proteins – "disaggregation or "monomerization" (Fig 4.3A). We found that treatment of 293T MUC1-CFP-FvHA cells for one minute with increasing concentrations of $AP20187^D$ did not increase the quantity of MUC1-CD dimers above baseline levels, while $AP21998^M$ treatment resulted in a dose dependant reduction in the level MUC1-CD dimers (Fig


A
MUC1-CD
Fv
AP20187D
AP21998M
B
293T MUC1-CFP-Fv
AP20187D
AP21998M
kDa
WCL
DSS
10nM
100nM
1μM
10nM
100nM
1μM
150
75
50
D
M
IB: MUC1-CD

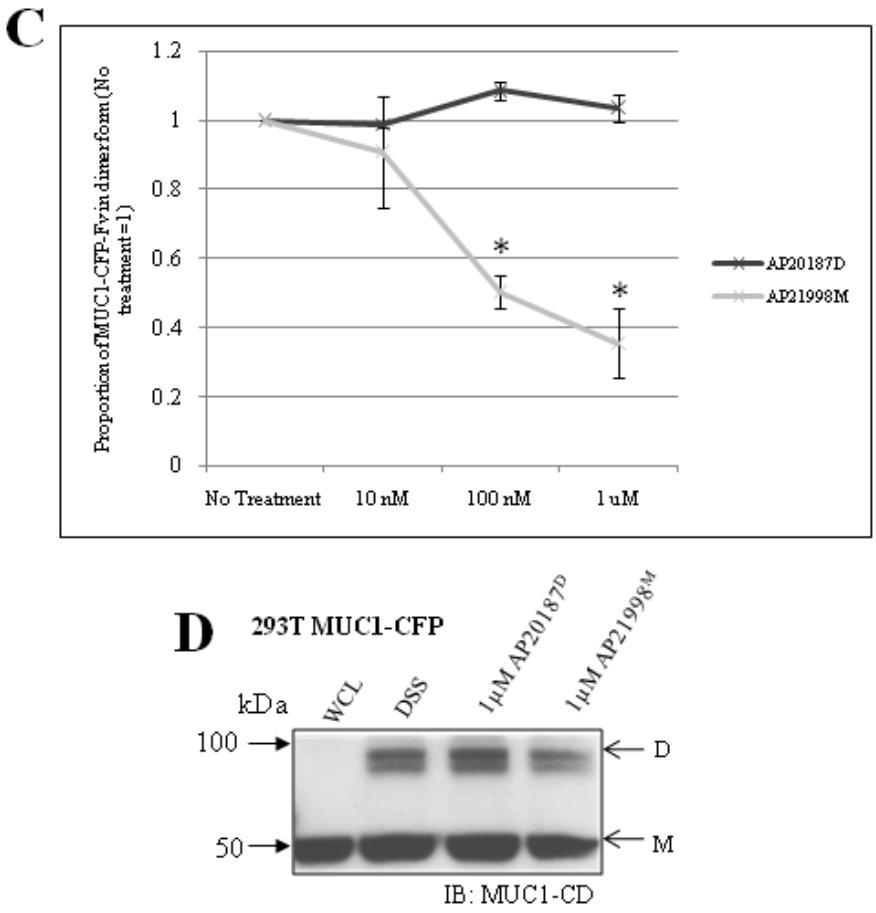


Figure 4.3. Dimerization of MUC1 can be manipulated by addition of an Fv domain and divalent and monovalent Fv domain ligands. **A**. Schematic of the mechanism of dimer formation/disruption by Fv ligands. **B**. Treatment of 293T MUC1-CFP-Fv cells with $AP20187^{D}$/$AP21998^{M}$ and DSS, followed by SDS-PAGE and probing with anti-MUC1-CD. **C**. Densitometric analysis of dimer bands from (**B**) normalized to total MUC1-CD reveal proportion of MUC1-CD in dimer form. "No treatment" condition is set to one and remaining conditions expressed as a ratio. **D**. Treatment of 293T MUC1-CFP cells with $AP20187^{D}$/$AP21998^{M}$ and DSS, followed by SDS-PAGE and probing with anti-MUC1-CD. "D" and "M" indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysates (WCL) are included as controls. *Asterisk* indicates a discrete population that does not overlap with any other population in the data set ($p<0.05$).

4.3B). Densitometric analysis of the dimer bands normalized to total MUC1-CD illustrates the change in proportion of MUC1 in dimer form with $AP21998^{M}$ treatment (Fig 4.3C). As a control, 293T MUC1-CFP cells, which lack the Fv domain, do not show a significant change in dimer quantity following treatment with 1 uM $AP20187^{D}$ or $AP21998^{M}$ (Fig 4.3D).

4.2.3. Disruption of MUC1-CD dimerization does not result in loss of cell viability.

As previous reports [184] have demonstrated that disruption of MUC1-CD dimerization using peptides results in arrest of cell growth and necrotic cell death, we performed a trypan blue exclusion viability assay after treatment with 1uM $AP20187^{D}$ or $AP21998^{M}$ and saw no significant reduction in viability, compared to no treatment control, up to 72 hours exposure (Fig 4.4).

4.2.4. Disruption of MUC1-CD dimerization results in decreased recruitment of total and active Src kinase to MUC1-CD.

To determine the importance of MUC1-CD dimerization in constitutive Src recruitment, 293T MUC1-CFP-Fv (Fig 4.5A), and, as a control, 293T MUC1-CFP (Fig 4.5 B) cells were treated with increasing concentrations of $AP20187^{D}$ or $AP21998^{M}$ for one minute, followed by immunoprecipitation with anti-MUC1-CD. Following separation on SDS-PAGE, blots were probed with anti-Src (total Src) and anti-$Src^{p\text{-}Y416}$ (active Src). In the MUC1-CFP-Fv transfectants, the amount of total and active Src associated with MUC1-CD decreased in a dose-dependent manner with $AP21998^{M}$ treatment (Fig 4.5A, arrows). Treatment with $AP20187^{D}$ did not result in a significant change, and Src recruitment to MUC1-CFP (Fig 4.5B) was not affected by Fv ligand treatment.

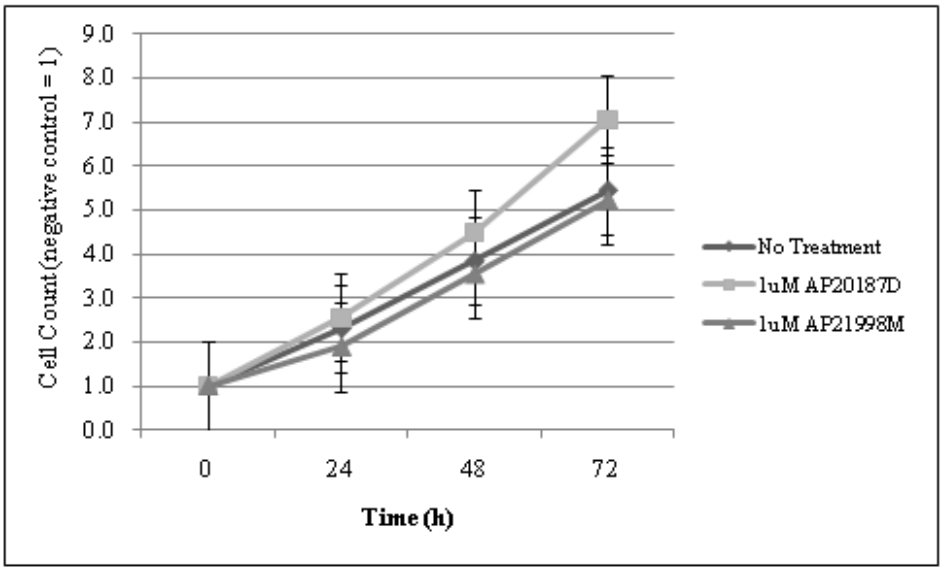


Figure 4.4. Treatment of MUC1-CFP-Fv cells with $AP20187^{D}$ and $AP21998^{M}$ does not affect cell viability. Following treatment for 24, 48, or 72 hours with 1µM $AP20187^{D}$ or $AP21998^{M}$, or no treatment control, cells were assayed for viability using the trypan blue exclusion assay. Cell count prior to experimentation was set to one and the remaining conditions expressed as a ratio. *Asterisk* indicates conditions which do not overlap ($p<0.05$) with any other population in the data set.

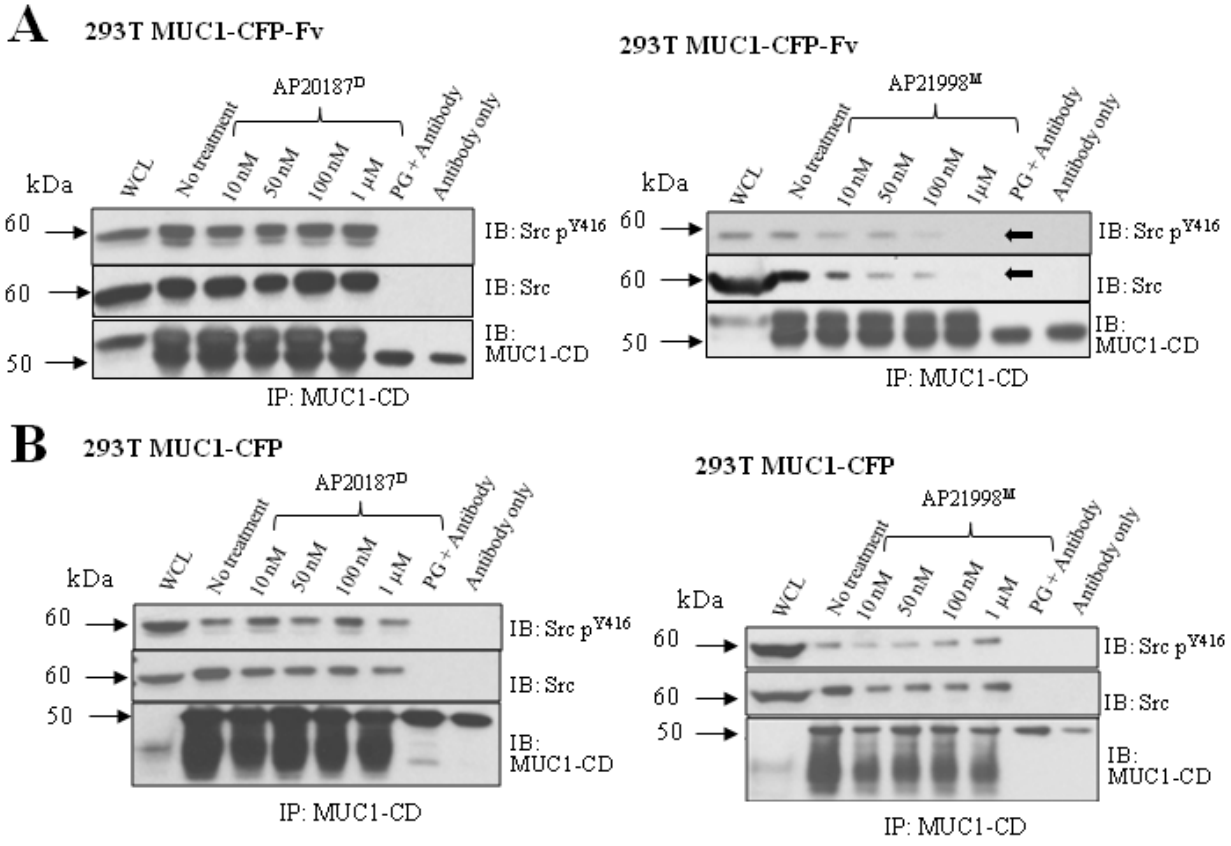


Figure 4.5. Recruitment of Src and Src p^{Y416} to MUC1-CD can be disrupted by inhibition of MUC1-CD dimerization. Co-immunoprecipitation with anti-MUC1-CD was performed on 293T MUC1-CFP-Fv cells **(A)** and MUC1-CFP cells **(B)** after treatment with increasing concentrations of $AP20187^{D}$ **(left panels)** or $AP21998^{M}$ **(right panels)**. Immunoprecipitates were probed for Src p^{Y416}, Src, and MUC1-CD (as a loading control) with stripping of blots between each probe. Whole cell lysate (WCL), PG (Protein G) + Antibody and Antibody only lanes are included as controls.

Densitometric analysis of Src and Src^{P416} normalized to MUC1-CD illustrates these results (Fig 4.6).

4.2.5. Disruption of MUC1-CD dimerization results in decreased ICAM-1 binding induced calcium oscillations and cell migration.

To determine if MUC1-CD dimerization is important in the previously observed ICAM-1 binding induced events, we assayed for ICAM-1 binding induced CaOs and invasion through an ICAM-1 positive monolayer after addition of the Fv ligands 1uM $AP20187^{D}$ or 1uM $AP21998^{M}$ and compared this to a no treatment control. 293T MUC1-CFP-Fv and, as controls, the Fv-domain negative 293T MUC1-CFP cells and the MUC1-negative 293T (parental) cells, were assayed for ICAM-1 binding induced CaOs (Fig 4.7) and invasion through an ICAM-1 monolayer (Fig 4.8). For each experiment, the parental no treatment condition was set to one and the remaining experiments expressed as ratios. MUC1-CFP and MUC1-CFP-Fv transfected cells displayed significant and statistically equivalent increases in CaOs and migration compared to control, in no treatment conditions. This indicates that the presence of the CFP and Fv domains do not interfere with the generation of ICAM-1 binding induced signalling. We found that ICAM-1 binding induced CaOs and invasion in 293T MUC1-CFP-Fv cells was significantly reduced after treatment with $AP21998^{M}$, compared to no treatment control. However, CaOs levels were still significantly greater than those observed in Parental conditions. Treatment with $AP20187^{D}$ resulted in a significant increase in cell migration in 293T MUC1-CFP-Fv cells (Fig 4.8), but did not produce a significant response in the CaOs assay (Fig 4.7). Addition of the Fv domain ligands had no significant effect on the 293T MUC1-CFP transfectants lacking the Fv domain or parental cells lacking MUC1 expression.

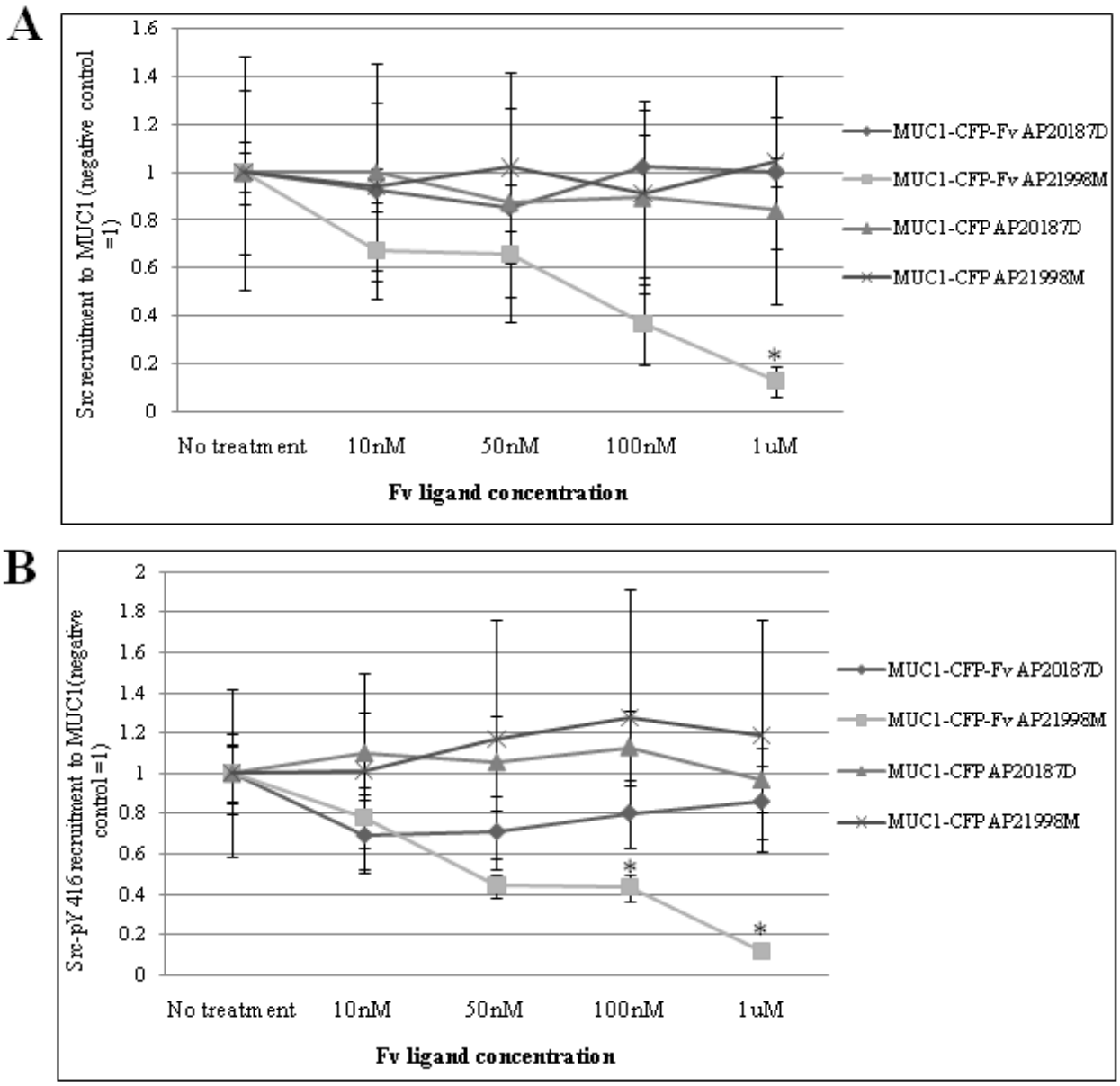


Figure 4.6. Densitometric analysis of Src and Src^{P416} recruitment to MUC1-CFP-Fv and MUC1-CFP after treatment with Fv domain ligands demonstrates significant reduction in Src and Src^{P416} recruitment to MUC1-CFP-Fv after $AP21998^{M}$ treatment. Densitometry was performed on Src (**A**) and $Src^{p\text{-}Y416}$ (**B**) with normalization to MUC1-CD as a loading control. No treatment conditions were set to one for each data set and the remaining conditions expressed as a ratio. *Asterisk* indicates conditions which do not overlap with any others in the data set ($p<0.05$).

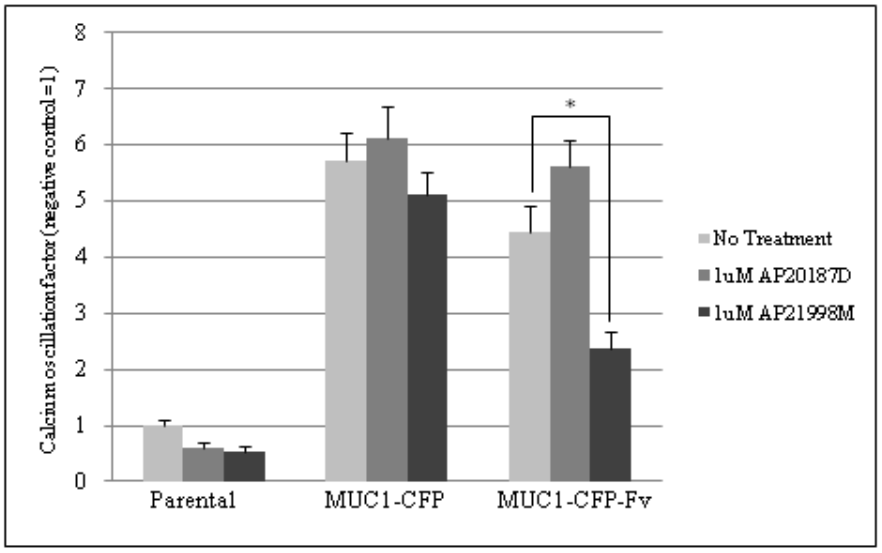


Figure 4.7. Disruption of MUC1-CD dimerization results in inhibition of ICAM-1 binding induced calcium oscillations. HEK 293T (parental), MUC1-CFP, and MUC1-CFP-Fv cells were analyzed for ICAM-1 binding induced calcium oscillations following pre-treatment with 1μM $AP20187^{D}$, 1μM $AP21998^{M}$, or no treatment control. Parental No treatment condition was set to one and the remaining conditions expressed as a ratio. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$).

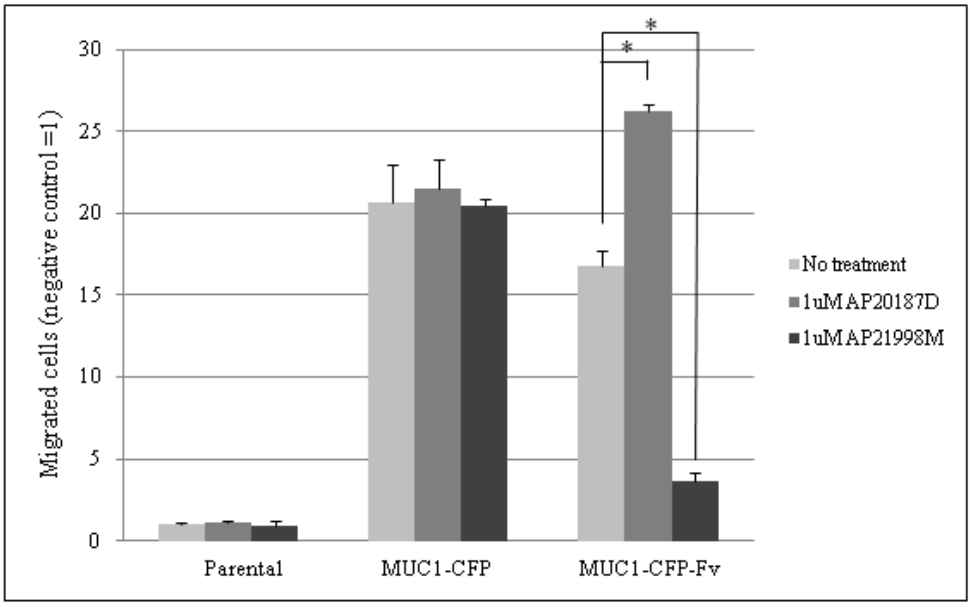


Figure 4.8. Disruption of MUC1-CD dimerization results in inhibition of ICAM-1 binding induced cell migration. HEK 293T (parental), MUC1-CFP, and MUC1-CFP-Fv cells were analyzed for migration through an NIH ICAM-1 positive monolayer following treatment with 1μM $AP20187^{D}$, 1μM $AP21998^{M}$ or no treatment control. Parental No treatment condition was set to one and the remaining conditions expressed as a ratio. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$).

4.2.6. MUC1-CD dimers are not covalently linked

As prior reports [19] have demonstrated that truncated MUC1-CD dimers are covalently linked *in vitro*, we investigated the formation of covalently-linked MUC1-CD dimers *in vivo* before and after ICAM-1 stimulation. By omitting the reducing agent β-mercaptoethanol from the LSB used to prepare lysates for SDS-PAGE, we expected that covalently linked species would remain intact. Reducing (R, + β-mercaptoethanol) and non-reducing (NR, no β-mercaptoethanol) samples were run on separate SDS-PAGE gels as leaching of β-mercaptoethanol can occur. In both human breast cancer MCF-7 cells and 293T MUC1-CFP cells, no evidence of covalently linked MUC1-CD dimerization was observed constitutively (Fig 4.9A) and following stimulation with NIH ICAM-1 cells for 60 seconds (Fig 4.9B) when probed with anti-MUC1-CD. As a control for our technique, 293T CD8/MUC1 transfectants, which are expected to exist as covalently linked dimers via a CD8-ECD bridge [394, 395], were run under reducing and non-reducing conditions, revealing the presence of a covalently linked species at the molecular weight expected for a CD8/MUC1 dimer (Fig 4.9C).

4.2.7. MUC1-CD contains SH2 and SH3 binding domains which act to recruit Src kinase

To further reveal the mechanism of Src binding to MUC1-CD, we mutated the confirmed Src SH2 binding site ($Y^{46}F$; ΔSH2) [267] and/or the putative Src SH3 binding site ($P^{37}A/P^{38}A$; ΔSH3). As discussed in Section 1.2.5., Src SH3 domain binding is ideally suited to the motif "PXXP", with arginine residues lying either N- or C-terminal to the polyproline motif [323], although there are many examples of Src SH3 domain binding motifs lacking this sequence [324]. As Src SH3 domain has been shown to bind MUC1-CD as an undescribed motif [267], the MUC1-CD sequence "R^{34}YVPPSSTDR^{43}"

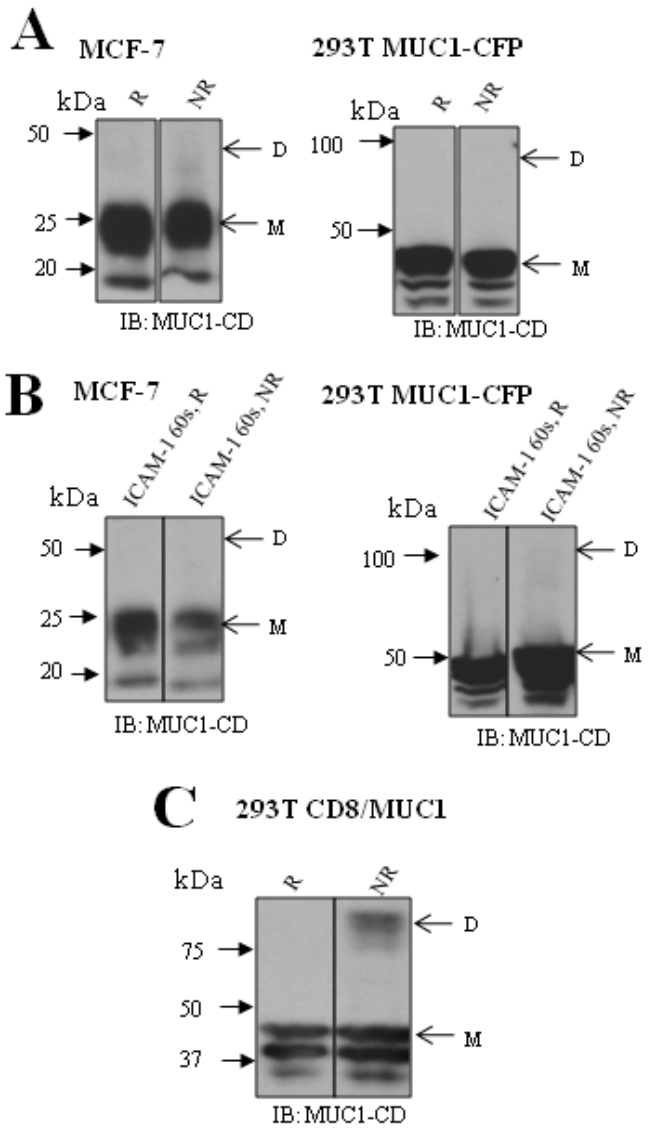


Figure 4.9. MUC1 cytoplasmic domain does not form covalently linked dimers constitutively or following ICAM-1 ligation. Human breast cancer cell line MCF-7 (left panels) and 293T MUC1-CFP transfectants (right panels) were assayed for constiutive (**A**) and 60 second ICAM-1 binding induced (**B**) covalently linked dimers. Reducing (R) and non-reducing (NR) conditions were ran on separate SDS-PAGE gels followed by probing with anti-MUC1-CD. 293T CD8/MUC1 transfectants were included as a positive control (**C**). "D" and "M" indicate expected molecular weights of dimer and monomer, respectively.

is the most likely SH3 binding site, containing both proline and arginine residues. We mutated the SH2 and/or putative SH3 binding domains on the MUC1-CFP-Fv plasmid, transfected HEK 293T cells, and following immunoprecipitation with anti-MUC1-CD, probed for Src and, as a loading control, MUC1-CD (Fig 4.10). We found that mutation of either the SH2 or the SH3 binding domain in MUC1-CD resulted in a decrease in the level of Src recruited to MUC1-CD, although MUC1-CD and Src were still associated. When both the SH2 and SH3 domain were mutated, Src recruitment to MUC1-CD was not detectable. These data indicate the MUC1-CD may recruit Src constitutively by both the SH2 and SH3 binding domains, and when one is mutated recruitment by the other is not affected. When both binding domains are mutated, Src is not recruited to MUC1, indicating that the SH2 and SH3 binding domains are the only Src recruitment motifs present on MUC1-CD.

4.2.8. Src recruitment is not required for MUC1-CD dimerization

To determine if MUC1-CD dimerization is dependent on recruitment of Src kinase, we assayed dimerization in MUC1-CFP-Fv cells with SH2 and/or SH3 domains mutated (Fig 4.11). As described in Section 4.2.6., MUC1-CFP-Fv ΔSH2 and MUC1-CFP-Fv ΔSH3 display reduced recruitment of Src compared to wildtype, while MUC1-CFP-Fv ΔSH2/SH3 does not recruit Src. Following treatment with DSS, cells were run on SDS-PAGE and probed with anti-MUC1-CD. MUC1-CFP-Fv ΔSH2, MUC1-CFP-Fv ΔSH3 and MUC1-CFP-Fv ΔSH2/3 all formed MUC1-CD dimers (Fig 4.11), indicating that Src recruitment to MUC1-CD is not necessary for dimerization.

To further investigate the requirement for Src kinase in MUC1-CD dimerization, we utilized mouse embryonic fibroblasts (MEF) with *Src/Yes/Fyn* triple gene knockout (SYF-/-). Transfection of MUC1-CFP-Fv and treatment with DSS revealed that MUC1-

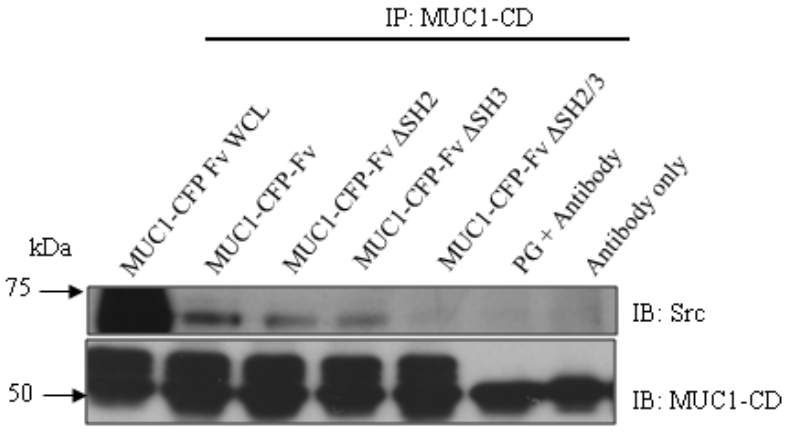


Figure 4.10. MUC1-CD contains SH2 and SH3 binding domains for Src kinase. MUC1-CFP-Fv cells with mutations of the SH2 ($Y^{46}F$; ΔSH2) and/or the putative Src SH3 binding sites ($P^{37}A/P^{38}A$; ΔSH3) were assayed for Src recruitment to MUC1-CD. Immunoprecipiation with anti-MUC1-CD was followed by SDS-PAGE and probing with anti-Src, and anti-MUC1-CD (as a loading control) with stripping of blots between each probe. Whole cell lysates (WCL), PG (Protein G) + Antibody and Antibody only lanes are included as controls.

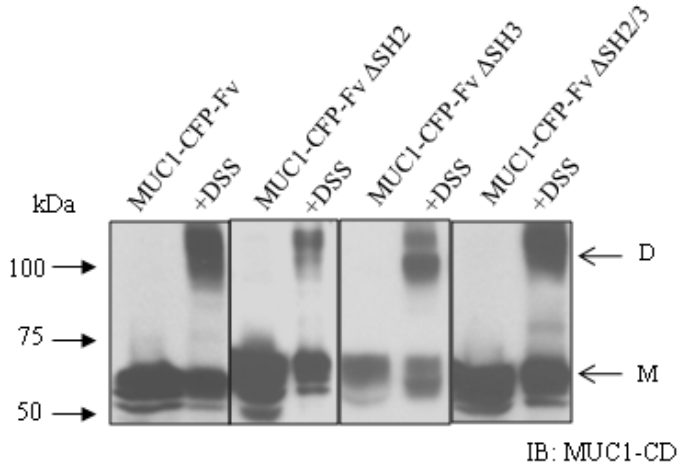


Figure 4.11. MUC1-CFP-Fv SH2 and SH3 binding domain mutants form MUC1-CD dimers. MUC1-CFP-Fv cells with mutations of the SH2 ($Y^{46}F$; ΔSH2) and/or the putative Src SH3 binding sites ($P^{37}A/P^{38}A$; ΔSH3) were assayed for MUC1-CD dimerization by treatment with DSS. Following treatment, cells were ran on SDS-PAGE and probed with anti-MUC1-CD. "D" and "M" indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysates are included as controls.

CD forms dimers in MEF SYF-/- cells lacking SFKs (Fig 4.12). This confirms the results shown in Fig 4.11, demonstrating that MUC1-CD dimerization is not dependent on Src kinase recruitment. Transfection of Src or Y530F Src, a constitutively active mutant, did not significantly affect MUC1-CD dimerization in MEF SYF-/- cells, indicating that the presence of Src does not potentiate MUC1-CD dimerization. Taken together, these data show that MUC1-CD dimerization occurs independently of Src, as Src is not required to be bound to MUC1-CD or present in the cell for dimerization to occur.

4.3. Conclusion

The data presented in this chapter demonstrate the role of MUC1-CD dimerization in ICAM-1 binding induced signalling, a proposed step in breast cancer metastasis, as well as reveal information on the mechanism of MUC1-CD dimerization. MUC1-CD dimerization occurs in both human breast cancer cell lines and transfected HEK 293T cells, indicating that dimerization is not dependent on a malignant phenotype. Further, we demonstrate that MUC1-CD dimerization occurs independent of the tandem repeat domain, and the association of MUC1-Y and CD8/MUC1, which contains only 69aa of the MUC1-CD, indicates that dimerization occurs due to cytoplasmic interactions independent of the C^1QC motif. We also demonstrate that MUC1-CD dimerization in cells is not due to covalent bonding, contrary to other reports [19]. The role of the C^1QC motif in MUC1-CD dimerization and signalling will be investigated further in Chapter 5. Our disruption of MUC1-CD dimerization using the engineered "Fv domain" and monovalent Fv ligands allowed for investigation of the role of dimerization in recruitment of Src kinase and our previously described ICAM-1 binding induced CaOs and cell migration. As we have shown in Chapter 3 that Src kinase is a critical component of the MUC1/ICAM-1 signalling axis, we investigated the effect of MUC1-CD

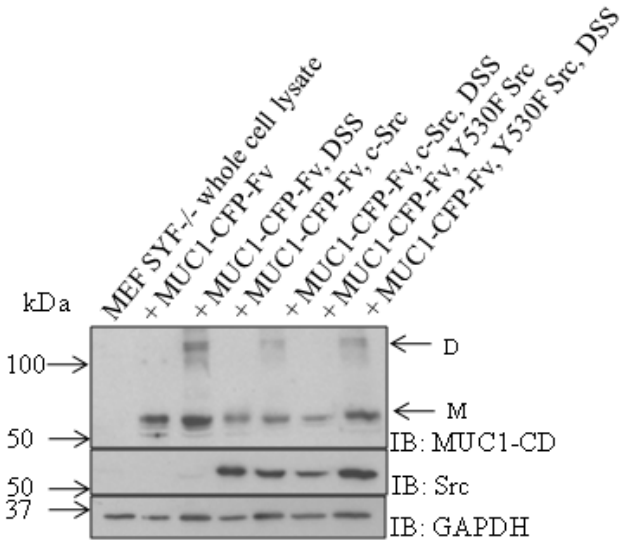


Figure 4.12. MUC1-CFP-Fv forms cytoplasmic domain dimers in MEF SYF-/- cells. MEF SYF-/- cells, lacking Src family kinases, were transfected with MUC1-CFP-Fv alone or in combination with Src or Src Y530F, treated with DSS, and assayed for MUC1-CD dimerization. GAPDH is included as a loading control. "D" and "M" indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysate (WCL) lanes are included as controls.

dimerization on recruitment of Src. We found that disruption of dimerization prevented both Src recruitment and ICAM-1 binding induced events. This finding is novel and significant, as inhibition of ICAM-1 binding induced cell migration represents a potential target in anti-metastatic therapies for breast cancer. Also, our findings showing that disruption of Src binding and ICAM-1 binding induced signalling are both inhibited by interference with MUC1-CD dimerization suggest that Src kinase is a direct modulator of the MUC1/ICAM-1 signal, not an indirect, downstream component. Lastly, we demonstrate that MUC1-CD dimerization occurs independently of Src binding, as MUC1-CD existed as a constitutive dimer in the absence of Src binding, through mutation of Src SH2/SH3 binding domains or knockout of Src. The data presented in this chapter shed light on the mechanism of Src recruitment to MUC1 and ICAM-1 binding induced signalling, representing potential targets for anti-metastatic therapies in the future.

Chapter 5: The role of MUC1 cytoplasmic cysteine residues in dimerization and ICAM-1 binding induced events

5.1. Introduction

It has been reported that MUC1-CD dimerization is dependant on the membrane-proximal cysteine residues of the motif C^1QC [19, 20], resulting in nuclear import. These reports utilized a MUC1-CD construct consisting of 72aa of the CD, without a TMD or ECD. They found that, *in vitro*, MUC1-CD formed covalently linked dimers when separated under non-reducing conditions. In 293T cells, they found that mutation of the cysteine residues of C^1QC to alanine resulted in disruption of dimerization and nuclear localization. Collectively, these results suggested that disulfide linkage was the mechanism of MUC1-CD dimerization.

However, in Chapter 4, we found that, in 293T cells, full-length MUC1-CD dimers were not covalently-linked constitutively or following ICAM-1 treatment. Here, we further investigate this finding, looking specifically at the cysteine residues of the C^1QC motif in MUC1-CD dimerization, Src recruitment, and ICAM-1 binding induced signalling. We hypothesize that mutation of these cysteine residues will not prevent MUC1-CD dimerization or ICAM-1 binding induced signalling, as MUC1-CD dimers are not covalently linked.

5.2. Results

5.2.1. Mutation of MUC1-CD cysteine residues does not prevent cell membrane expression

Prior studies investigating the role of MUC1-CD membrane proximal cysteine residues in membrane localization have yielded contradictory results [179, 180]. Here, we determined the membrane expression levels of a mutant MUC1 construct in which both cysteine residues have been mutated to alanine, MUC1-CFP (AQA) after transfection into HEK 293T cells (Fig 5.1). Parental, non-transfected cells, which express no

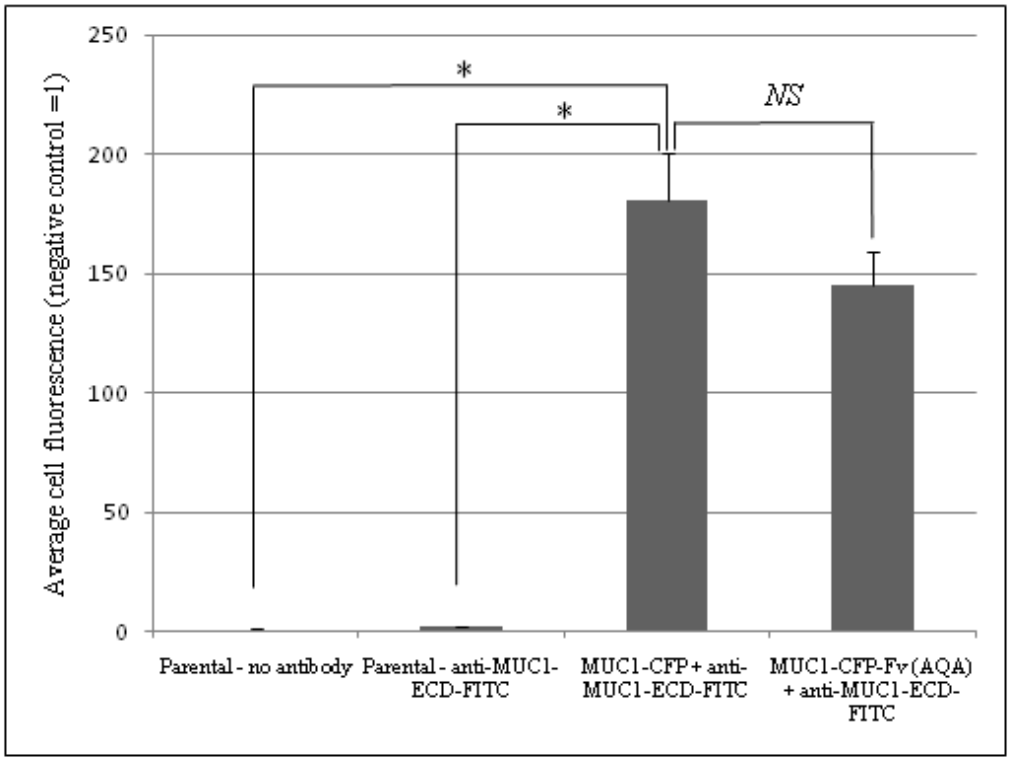


Figure 5.1. MUC1-CFP and MUC1-CFP-Fv (AQA) are expressed on the cell surface of transfected HEK 293T cells. HEK 293T (parental) cells were assayed for auto-fluorescence (no antibody) or non-specifc staining with anti-MUC1-ECD-FITC. Parental cells transfected with MUC1-CFP-Fv (AQA) were assayed for fluorescence after staining with anti-MUC1-ECD-FITC . Bars represent the mean fluorescence for three independent populations of cells. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$). *NS* = not significant ($p<0.05$).

endogenous MUC1, were assayed by flow cytometry, with no staining to control for autofluroescence or after staining with a fluorescently-conjugated anti-MUC1-ECD antibody, anti-MUC1-ECD-FITC, which controls for non-specific binding. For each population of cells, a mean fluorescent intensity was determined, and the average mean from three experiments was plotted. The parental, no staining condition was set to one and the remaining conditions expressed as a ratio. As a positive control, wildtype MUC1-CFP-Fv transfected 293T cells were assayed following staining with anti-MUC1-ECD-FITC, displaying a significant increase in mean cell fluorescence over negative controls. MUC1-CFP-Fv (AQA) transfected 293T cells also displayed a significant increase in mean cell fluorescence compared to negative controls, and these means were not significantly different from wildtype MUC1-CFP-Fv ($p<0.05$) (Fig 5.1). These data indicate that the MUC1-CFP-Fv (AQA) construct is expressed at the cell surface of transfected 293T cells at equivalent levels to wildtype MUC1.

5.2.2. MUC1-CD cysteine residues are not required for MUC1-CD dimerization or Src recruitment

To determine if MUC1-CD membrane proximal cysteine residues are required for our observed constitutive MUC1-CD dimerization, we assayed MUC1-CFP-Fv (AQA) dimerization after DSS treatment (Fig 5.2A). MUC1-CFP-Fv wildtype was used as a positive control. Following treatment with DSS, separation on SDS-PAGE, and probing with anti-MUC1-CD, dimer bands for both MUC1-CFP-Fv and MUC1-CFP-Fv (AQA) were observed, indicating that mutation of MUC1-CD cysteine residues to alanine did not prevent dimer formation.

To determine if the observed MUC1-CFP-Fv (AQA) dimers were functional in the recruitment of Src kinase, we probed for Src after immunoprecipitation with anti-

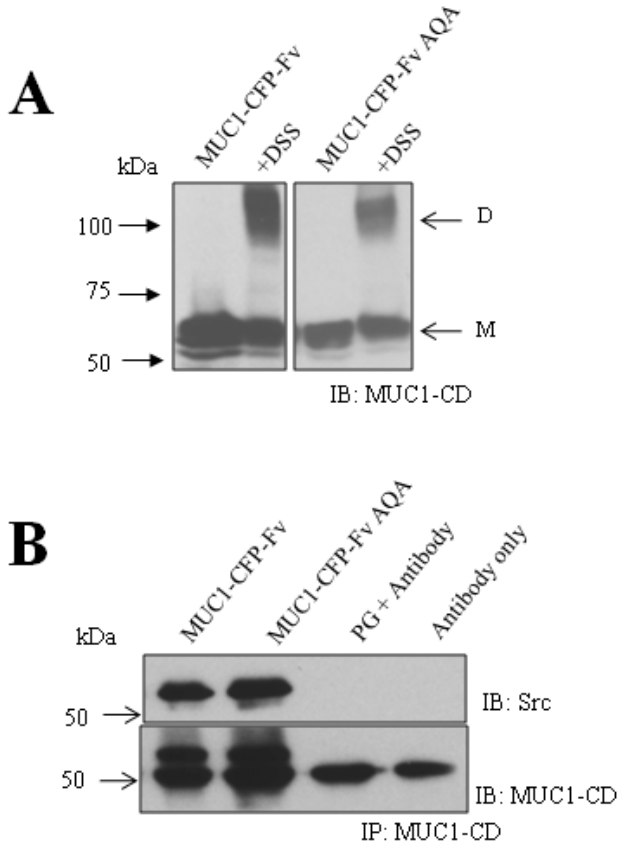


Figure 5.2. MUC1-CFP-Fv (AQA) cytoplasmic domain forms constitutive dimers and recruits Src. A. Following treatment with DSS, cell lysates from 293T cells transfected with MUC1-CFP-Fv and MUC1-CFP-Fv (AQA) were ran on SDS-PAGE and probed with anti-MUC1-CD. **B.** MUC1-CFP-Fv and MUC1-CFP-Fv(AQA) cells were immunoprecipitated with anti-MUC1-CD and probed with anti-Src and anti-MUC1-CD, as a loading control. "D" and "M" indicate expected molecular weights of dimer and monomer, respectively. Whole cell lysate, PG (Protein G) + Antibody and Antibody only lanes are included as controls.

MUC1-CD (Fig 5.2B) in MUC1-CFP-Fv (as a positive control) and MUC1-CFP-Fv (AQA) transfected 293T cells. We found that Src was recruited to MUC1-CFP-Fv (AQA) cells at an equivalent level to MUC1-CFP-Fv wildtype cells, indicating that mutation of membrane proximal cysteine residues does not impair Src recruitment to MUC1-CD dimers.

5.2.3. MUC1-CD cysteine residues are not required for ICAM-1 binding induced calcium oscillations and cell migration

We sought to determine if MUC1-CD membrane proximal cysteine residues are required for the previously observed ICAM-1 binding induced CaOs and cell migration. We assayed MUC1-CFP-Fv (AQA) cells for CaOs following treatment with ICAM-1 or mock transfected cells, as a negative control (Fig 5.3), and migration through an ICAM-1 or mock monolayer (Fig 5.4). Parental 293T cells and MUC1-CFP-Fv wildtype cells were also assayed as negative and positive controls, respectively. Parental, mock treatment condition was set to one and the remaining conditions expressed as a ratio. Parental cells did not display a significant change in CaOs levels when treated with ICAM-1 cells compared to mock transfected cells, indicating that ICAM-1 treatment does not elicit a CaOs response when MUC1 is absent. MUC1-CFP-Fv cells generated a significant increase in CaOs levels when treated with ICAM-1 cells compared to mock treatment and parental conditions, in agreement with previous work investigating MUC1/ICAM-1 binding induced calcium signaling [13]. MUC1-CFP-Fv (AQA) cells also exhibited an increase in CaOs levels in response to ICAM-1 treatment versus mock which was statistically equivalent to that of MUC1-CFP-Fv wildtype. These data indicate that the membrane proximal cysteine residues are not required for generation of the ICAM-1 binding induced CaOs.

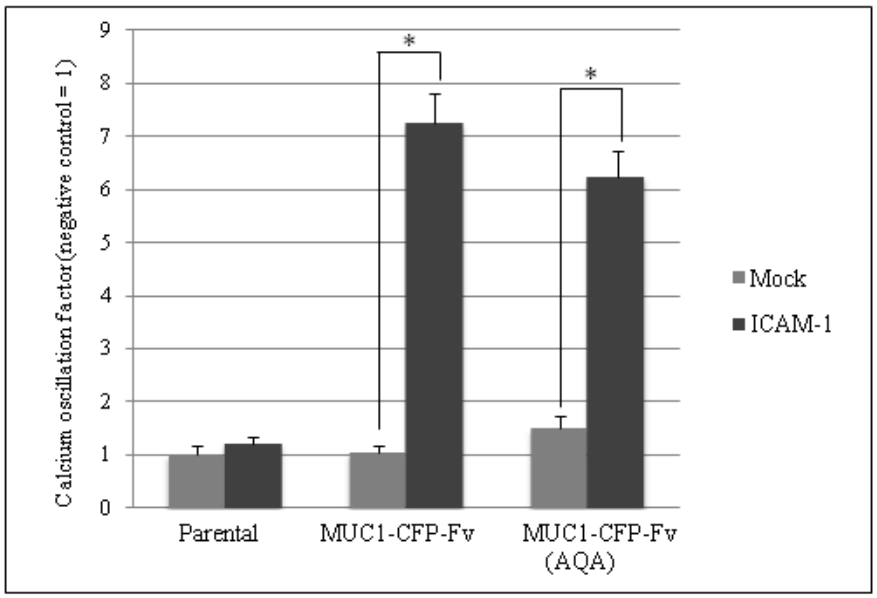


Figure 5.3. MUC1-CFP-Fv (AQA) functions equivalent to MUC1-CFP-Fv in generation of ICAM-1 binding induced calcium oscillations. HEK 293T (parental), MUC1-CFP-Fv and MUC1-CFP-Fv (AQA) cells were assayed for calcium oscillations after treatment with mock or ICAM-1 transfected NIH 3T3 cells. Parental, mock condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average oscillation factors from at least three independent trials; bars, SE. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$).

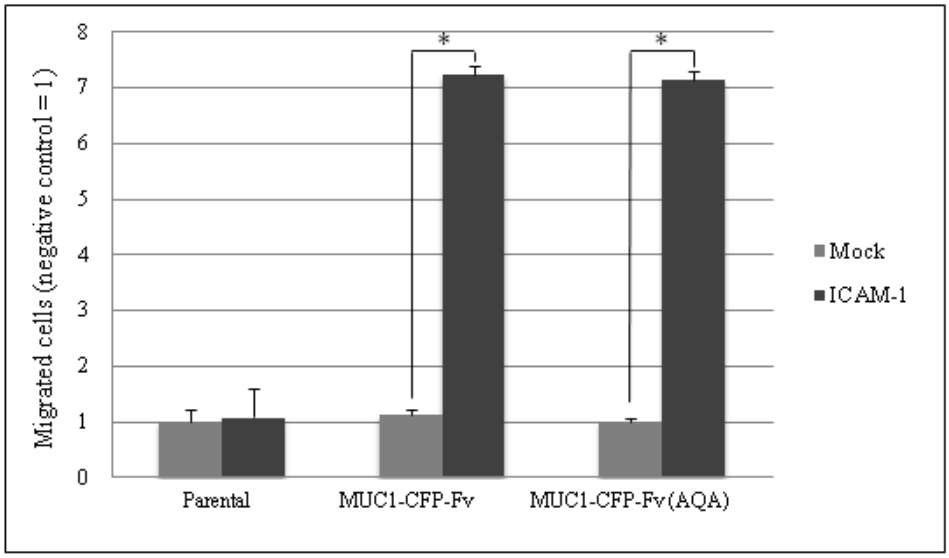


Figure 5.4. MUC1-CFP-Fv (AQA) functions equivalent to MUC1-CFP-Fv in generation of ICAM-1 binding induced cell migration response. HEK 293T (parental), MUC1-CFP-Fv and MUC1-CFP-Fv (AQA) cells were assayed for 24 hour migration through a monolayer of mock or ICAM-1 transfected NIH 3T3 cells. Parental, mock condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average number of migrated cells per five fields from at least three independent trials; bars, SE. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$).

Migration of parental cells was not affected by the presence of ICAM-1 (Fig 5.4), indicating that migration is not stimulated by ICAM-1 in MUC1-negative cells. Migration of MUC1-CFP-Fv wildtype transfectants, however, was significantly increased in the presence of an ICAM-1 monolayer compared to a mock transfected monolayer and parental conditions, in agreement with previous work investigating the MUC1/ICAM-1 binding induced cell migration [15]. MUC1-CFP-Fv (AQA) transfectants responded to the presence of ICAM-1 by increasing cell migration, compared to mock monolayer and parental conditions, to levels statistically equivalent to that of MUC1-CFP-Fv wildtype. These data indicate that the membrane proximal cysteine residues are not required for ICAM-1 binding induced cell motility and invasion.

5.3. Conclusion

The data presented in this chapter identifies a discrepancy in the field of MUC1 research. Contrary to previous reports, we found that the membrane proximal cysteine residues are not required for MUC1-CD dimerization. We also report that the cysteine residues are not required for Src recruitment or ICAM-1 binding induced signalling, in agreement with our results from Chapter 4 demonstrating that MUC1-CD dimers are not covalently linked. We have developed several hypotheses to explain these seemingly conflicting findings, which will be a focus of Section 8.1.2. Our finding that MUC1-CD dimers are not cysteine linked provides information and hypothesis generation on the mechanism of MUC1-CD dimerization, which may be of clinical importance if inhibitors to MUC1-CD dimerization are to be investigated as an anti-metastatic therapy in the future.

Chapter 6: Investigating the role of MUC1 extracellular domain shedding in dimerization and ICAM-1 binding induced signalling

6.1. Introduction

MUC1 is a reported substrate for the proteases ADAM17 and MT1-MMP [24, 25], both of which act to remove the majority of the large, heavily glycosylated ECD, leaving a fragment of MUC1 containing a short extracellular portion, the TMD, and CD. Cleavage by ADAM17 occurs constitutively while cleavage by MT1-MMP must be stimulated by treatment with pervanadate [25]. The exact physiological and/or pathological roles of MUC1 S2 cleavage have yet to be investigated, but several studies have implicated ADAM17 mediated MUC1-ECD cleavage in embryo implantation, with cleavage occurring locally at the site of blastocyst attachment [210, 396]. Also, the observation that increased levels of MUC1-ECD in human serum is correlated with a poor prognosis in breast cancer suggests that this cleavage event could be of clinical importance [27, 28].

In this chapter, we investigate the role of MUC1-ECD removal in dimerization, Src recruitment and ICAM-1 binding induced CaOs and cell invasion. Due to the large, heavily glycosylated nature of the ECD, we originally hypothesized that it would sterically inhibit dimerization of the MUC1-CD and its removal must proceed it. However, as ICAM-1 binds to the VNTR domain of MUC1, the ECD must be intact in order for ICAM-1 binding to induce cytoplasmic signalling cascades. In light of our findings in Chapter 4 demonstrating that ICAM-1 induced signalling requires MUC1-CD dimerization, we conclude that dimerization must occur with the ECD intact in at least one dimer partner to allow for ICAM-1 ligation and signal transmission. Due to the dimeric nature of ICAM-1 [302], it is most likely that ICAM-1 binds to a MUC1 dimer with both dimer partners having the ECD intact.

However, in order for cell migration to occur following MUC1/ICAM-1 binding, this interaction must be disrupted to allow the cell to move forward, which could be

achieved by MUC1-ECD cleavage. Therefore, we hypothesize that cleavage of MUC1-ECD is not required for MUC1-CD dimerization, but follows ICAM-1 binding and is required for ICAM-1 binding induced CaOs and cell migration. To test our hypothesis, we used a broad spectrum inhibitor of ADAMs, TAPI-0, to inhibit ADAM17 activity, followed by assaying for dimerization, Src recruitment, and ICAM-1 binding induced events. We carried out all experiments in both MUC1-transfected HEK 293T cells and human breast cancer cell line T47D in order to account for cell-type differences and allow for conclusions to be drawn regarding the role of MUC1-ECD cleavage in breast cancer progression.

6.2. Results

6.2.1. TAPI-0 inhibits MUC1-ECD cleavage in both MUC1-transfected cells and human breast cancer cells

HEK 293T cells transfected with MUC1-CFP (Fig 6.1A) and the human breast cancer cell line T47D (Fig 6.1B) were treated withwith 100μM of TAPI-0 for 24, 30, 40 or 48 hours. Both cell lysate and conditioned media was collected and run on SDS-PAGE followed by probing for MUC1-ECD and MUC1-CD. MUC1-CFP cell lysate and conditioned media were also assayed for E-cadherin, a substrate of ADAM10 [397], to determine if TAPI-0 inhibited this enzyme as well. In 'No treatment' control of both MUC1-CFP transfected and T47D human breast cancer cells, both MUC1-ECD and CD were present in WCL, while only MUC1-ECD was present in 24 hour conditioned media. This is consistent with constitutve cleavage of MUC1 which releases MUC1-ECD. E-cadherin-ECD was present in both WCL and conditioned media of MUC1-CFP transfected cells, although the species present in WCL ran at approximately 120kDa while the species present in the conditioned media ran at approximately 80kDa. This

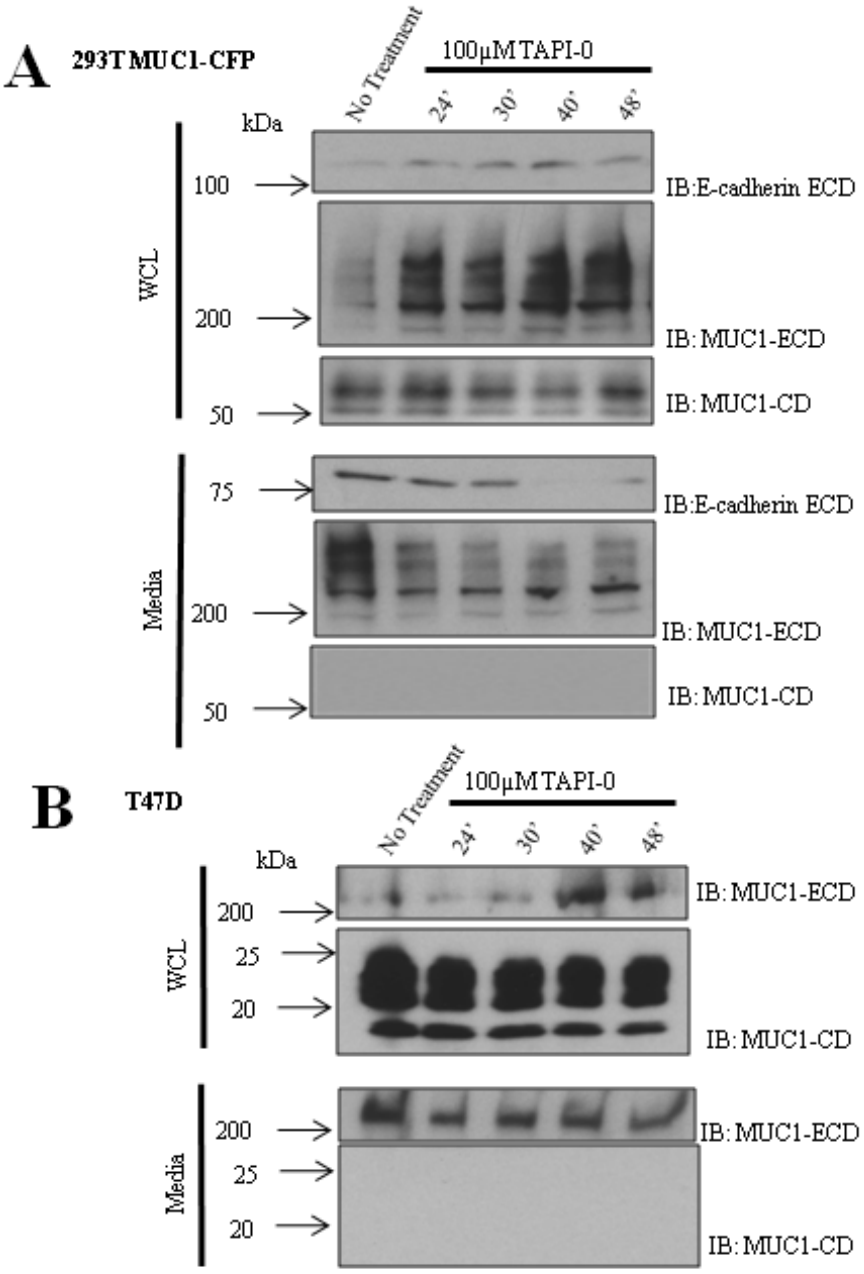


Figure 6.1. MUC1-ECD shedding in MUC1-CFP and T47D cells can be inhibited with TAPI-0, a metalloprotease inhibitor. Following treatment with 100uM TAPI-0 for various time periods (24-48 hours), 293T MUC1-CFP cells (**A**) and human breast cancer T47D cells (**B**) were lysed (WCL) and 24 hour-conditioned media collected. Following assay for protein concentration, samples containing equal protein concentration were ran on SDS-PAGE. Lysates were probed with anti-MUC1-ECD, anti-MUC1-CD, and in (**A**), anti-E-cadherin-ECD.

difference in molecular weights can be interpreted to indicate that the smaller species present in the conditioned media is a product of a cleavage event. In the case of constitutive cleavage of MUC1, the molecular weights of the MUC1-ECD species present in WCL and conditioned media do not appear to be of distinct molecular weight.

In MUC1-CFP transfected 293T cells (Fig 6.1A), treatment with TAPI-0 for 24 hours resulted in an increase in the amount of MUC1-ECD in the WCL, consistent with a reduction in ECD cleavage. In agreement with this, the amount of MUC1-ECD in the conditioned media after 24 hour TAPI-0 treatment was reduced. As a control, the level of MUC1-CD in the whole cell lysate remained constant. Increasing treatment time did not result in a significant change from the 24 hour treatment condition in MUC1-CFP cells. These data indicate that TAPI-0 treatment inhibits MUC1-ECD cleavage in MUC1-CFP transfected cells, with maximum inhibition occurring at 24 hours treatment.

Levels of E-cadherin-ECD in whole cell lysate (Fig 6.1A) increased with TAPI-0 treatment, with maximum levels appearing after 40 hours of treatment. Also, a reduction of the level of E-cadherin-ECD in the conditioned media at 40 hours of treatment was observed. These data indicate that TAPI-0 treatment also inhibits cleavage of E-cadherin, although cleavage of MUC1 and E-cadherin are maximally inhibited at different time points.

In human breast cancer T47D cells (Fig 6.1B), treatment with TAPI-0 for 40 hours resulted in a maximal increase in the level of MUC1-ECD present in WCL, compared to no treatment control levels. As a control, the level of MUC1-CD in WCL remained constant for all time points. The level of MUC1-ECD in the conditioned media decreased after 24 hour treatment and this decrease was constant for all treatment time points, compared to no treatment control. These data indicate that TAPI-0 treatment

inhibits MUC1-ECD cleavage in T47D cells, with maximum inhibition occurring at 40 hours treatment.

6.2.2. Inhibition of MUC1-ECD cleavage does not inhibit MUC1-CD dimerization

To determine if MUC1-ECD cleavage is required for the observed constitutive MUC1-CD dimerization, we treated MUC1-CFP-Fv transfected 293T cells and human breast cancer T47D cells with 100μM TAPI-0 for 24 or 40 hours, respectively, then assayed for dimerization of MUC1-CD by DSS treatment (Fig 6.2). As a control, a "No treatment" condition was also assayed for MUC1-CD dimerization. Both 293T MUC1-CFP-Fv (Fig 6.2A) and T47D cells (Fig 6.2B) displayed equivalent levels of MUC1-CD dimer compared to no treatment control when MUC1-ECD cleavage was inhibited, indicating that MUC1-ECD cleavage is not required for MUC1-CD dimerization.

6.2.3. Inhibition of MUC1-ECD cleavage does not inhibit MUC1-CD Src recruitment

To determine if inhibition of MUC1-ECD cleavage affects recruitment of Src to MUC1-CD, we treated MUC1-CFP-Fv transfected HEK 293T cells (Fig 6.3A) and human breast cancer T47D cells (Fig 6.3B) with 100μM of TAPI-0 for 24 or 40 hours, respectively. We then immunoprecipitated with anti-MUC1-CD and assayed for Src by SDS-PAGE. In both MUC1-CFP-Fv and T47D cells, Src was recruited to MUC1-CD after treatment with TAPI-0 at levels equivalent to "No treatment" control. This indicates that MUC1-ECD cleavage is not required for Src recruitment to MUC1-CD in MUC1 transfected or human breast cancer cells.

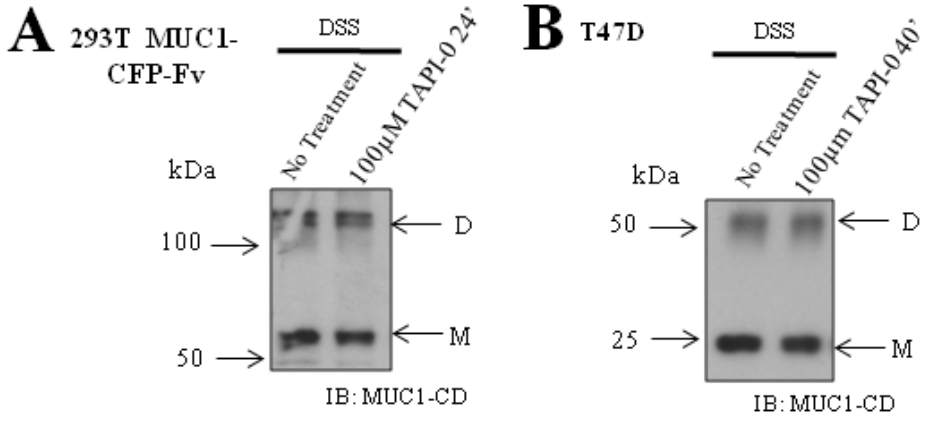


Figure 6.2. Inhibition of MUC1 extracellular domain cleavage in MUC1-CFP-Fv and T47D cells does not inhibit MUC1-CD dimerization. MUC1-CFP-Fv transfected HEK 293T cells (**A**) and human breast cancer T47D cells (**B**) were treated with 100μM TAPI-0 for 24 or 40 hours, respectively. Following treatment with the crosslinker DSS, Cell lysates were run on SDS-PAGE and probed with anti-MUC1-CD. "D" and "M" indicate expected molecular weights of dimer and monomer, respectively.

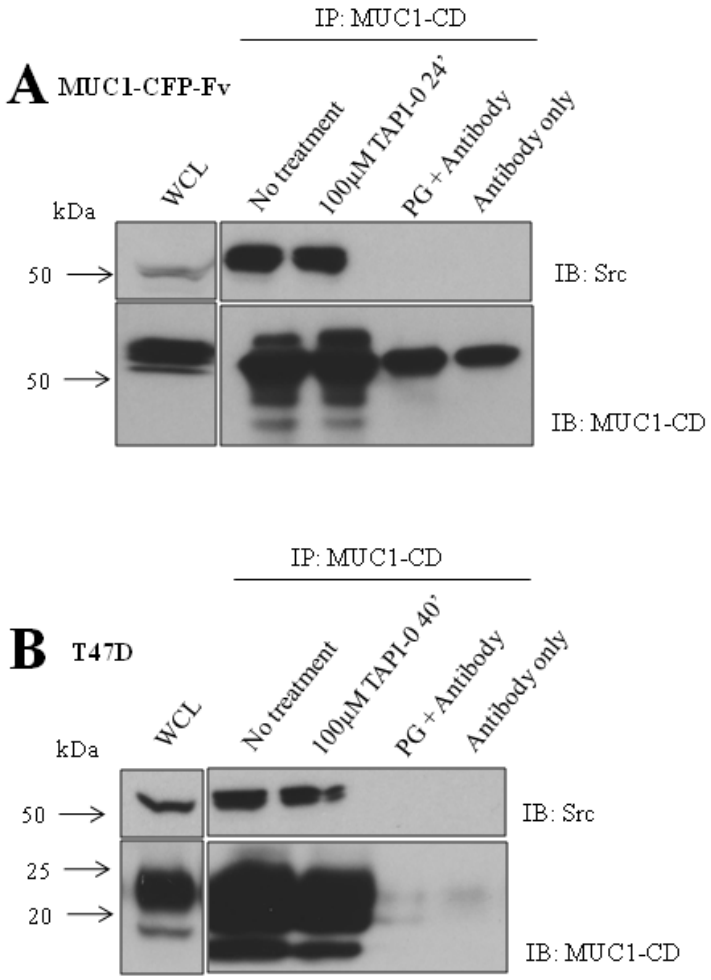


Figure 6.3. Inhibition of MUC1 extracellular domain cleavage in MUC1-CFP-Fv and T47D cells does not inhibit Src recruitment to MUC1 cytoplasmic domain. MUC1-CFP-Fv transfected HEK 293T cells (**A**) and human breast cancre T47D cells (**B**) were treated with 100uM TAPI-0 for 24 or 40 hours, respectively. Cell lysates were then immunoprecipitated with anti-MUC1-CD, followed by SDS-PAGE and probing with anti-Src and anti-MUC1-CD as a loading control. Whole cell lysate (WCL), Protein G (PG) + antibody, and antibody only lanes are included as controls.

6.2.4. Inhibition of MUC1-ECD cleavage does not inhibit ICAM-1 binding induced calcium oscillations

To determine if MUC1-ECD cleavage is required to induce ICAM-1 binding induced CaOs, we pre-treated MUC1-CFP transfected 293T cells and human breast cancer T47D cells with 100μM TAPI-0 for 24 or 40 hours, respectively, then assayed for the level of ICAM-1 induced CaOs signalling (Fig 6.4). As controls, parental (HEK 293T) cells, which do not express MUC1, were also assayed; and NIH Mock cells, which do not express ICAM-1, were used as a negative control treatment. In parental cells, the "No treatment" condition treated with Mock cells was set to one and the remaining conditions expressed as a ratio. Parental cells did not display any significant increase in CaOs level in any treatment condition, indicating that MUC1 expression is necessary for induction of ICAM-1 binding induced CaOs. MUC1-CFP transfected cells displayed significantly greater levels of CaOs when treated with mock cells compared to parental, and T47D cells treated with mock cells did not display significantly different levels compared to parental. The increased response of MUC1-CFP transfected cells to mock cells could be due to cell type differences or experimental variables such as Fluo-3 loading.

We found that in both MUC1-CFP transfected cells and T47D cells, the level of CaOs induced by ICAM-1 binding was not significantly affected by TAPI-0 treatment, compared to no treatment controls. The levels of CaOs generated was significantly greater in MUC1-CFP transfected cells compared to T47D cells, and may be attributed to cell type specific differences in protein expression and phenotype. These data indicate that MUC1-ECD cleavage is not required for generation of ICAM-1 binding induced CaOs in MUC1 transfected cells and human breast cancer cells.

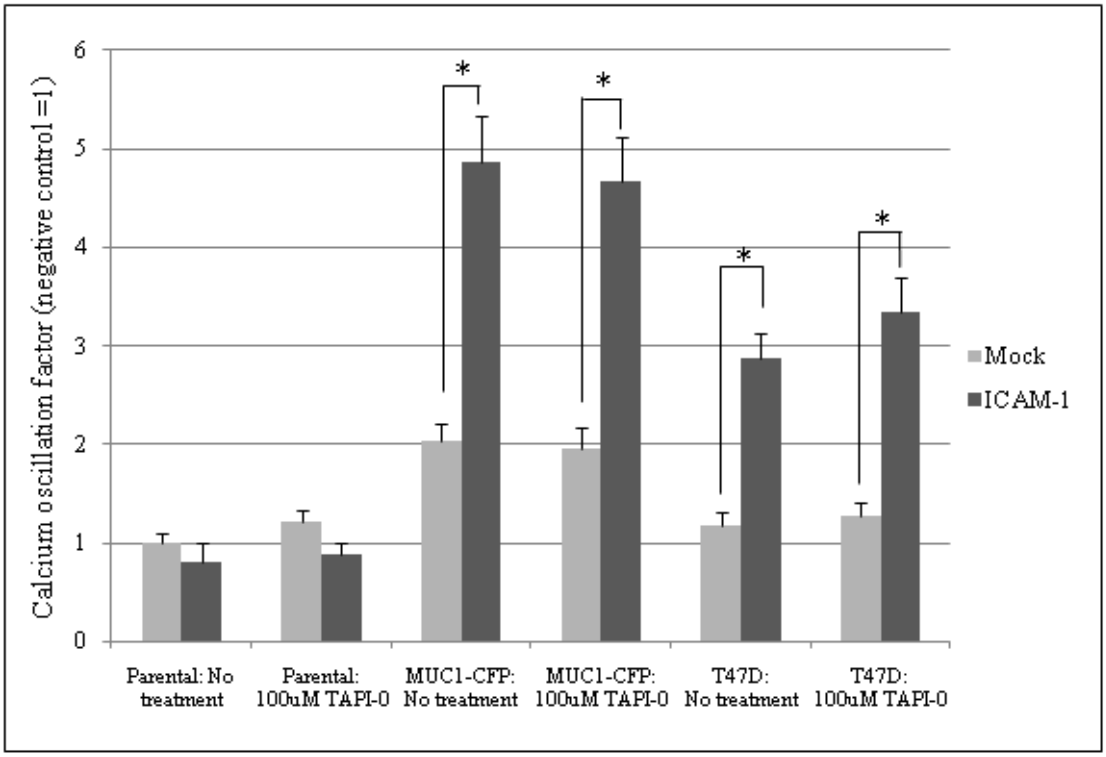


Figure 6.4. Inhibition of MUC1 extracellular domain cleavage in MUC1-CFP and T47D cells does not result in inhibition of ICAM-1 binding induced calcium oscillations. HEK 293T (parental), MUC1-CFP transfected 293T cells and human breast cancer T47D cells were assayed for calcium oscillations after treatment with mock or ICAM-1 transfected NIH 3T3 cells. Where indicated, cells were pre-treated with 100uM of TAPI for 24 hours (parental and MUC1-CFP cells) or 40 hours (T47D cells). Parental, mock condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average oscillation factors from at least three independent trials; bars, SE. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$).

6.2.5. Inhibition of MUC1-ECD cleavage results in inhibition of cell migration through an ICAM-1 positive monolayer

To determine if MUC1-ECD cleavage is necessary for ICAM-1 binding induced cell migration, we pre-treated MUC1-CFP transfected HEK 293T cells and human breast cancer T47D cells with 100μM of TAPI-0 for 24 or 40 hours, respectively (Fig 6.5). This was followed by a 24 hour Transwell migration assay, in which 100μM TAPI-0 was also present, through a monolayer of ICAM-1 or Mock transfected NIH 3T3 cells. As a control, HEK 293T parental cells, which do not express MUC1, were assayed. The parental, no treatment condition assayed for migration through a Mock monolayer was set to one and the remaining conditions expressed as a ratio. Parental cells did not display a significant increase in cell migration in any treatment condition, indicating that MUC1 expression is necessary for induction of ICAM-1 binding induced cell migration.

Both MUC1-CFP and T47D cells exhibited a significant increase in cell migration when exposed to an ICAM-1 monolayer as compared to a Mock monolayer and parental conditions, indicating that both MUC1 and ICAM-1 expression are necessary to induce cell migration. In all cell types treated with Mock treatment cells, the level of migration was not significantly different from the parental negative control treatment condition. T47D cell migration levels in response to ICAM-1 were significantly less than that of MUC1-CFP cells, which may be attributed to cell type specific differences in protein expression and phenotype.

When treated with 100μM TAPI-0 and assayed for migration through an ICAM-1 monolayer, both MUC1-CFP and T47D cells exhibited significant decreases in the level of cell migration compared to No treatment control, although migration levels were still significantly greater than the parental negative control group. These data indicate that

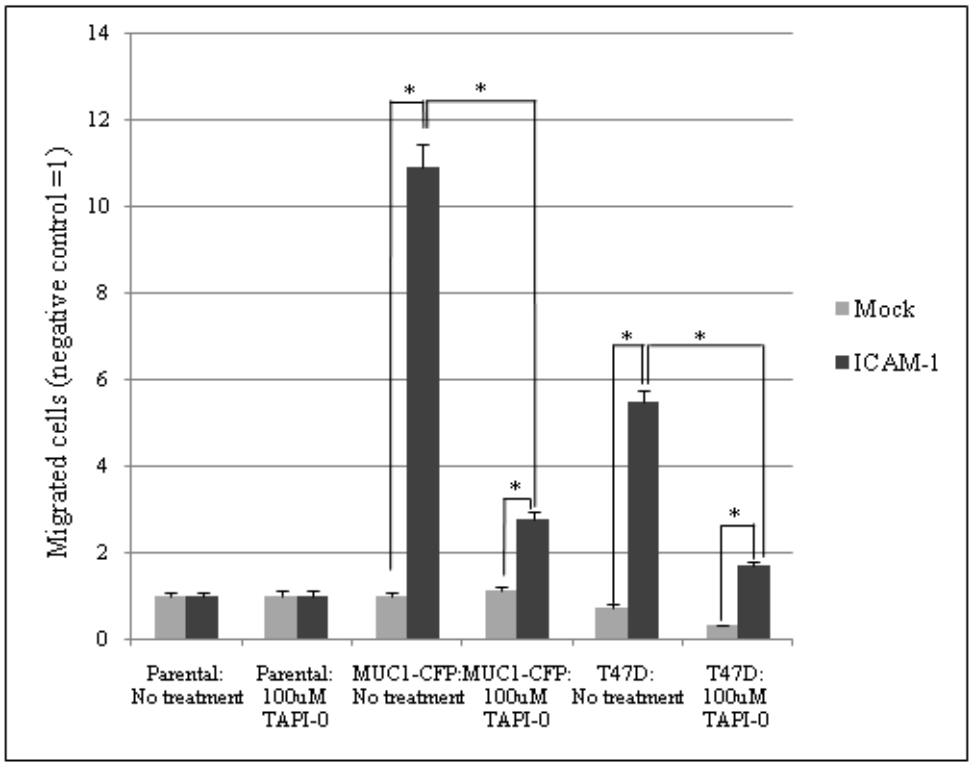


Figure 6.5. Inhibition of MUC1 extracellular domain cleavage in MUC1-CFP and T47D cells results in inhibition of ICAM-1 binding induced cell migration. HEK 293T (parental), MUC1-CFP transfected 293T cells and human breast cancer T47D cells were assayed for cell migration through a monolayer of mock or ICAM-1 transfected NIH 3T3 cells. Where indicated, cells were treated with 100μM of TAPI for 24 hours (parental and MUC1-CFP cells) or 40 hours (T47D cells) prior to treatment, and during 24 hour migration assay. Parental, mock condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average number of migrated cells per five fields from at least three independent trials; bars, SE. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$).

inhibition of MUC1-ECD cleavage results in a decrease in ICAM-1 binding induced cell migration.

6.2.6. Inhibition of MUC1-CD dimerization does not affect MUC1-ECD cleavage

To determine if MUC1-CD dimerization is required for MUC1-ECD cleavage, we inhibited MUC1-CD dimerization by addition of 1μM $AP21998^{M}$ to 293T MUC1-CFP-Fv cells. As controls, a no treatment condition and a 1 μM $AP20187^{D}$ treatment condition were included. After 24 hours treatment, we assayed the levels of MUC1-CD and MUC1-ECD in whole cell lysate (Fig 6.6A) and 24 hour conditioned media (Fig 6.6B). We found that treatment with $AP21998^{M}$ did not significantly affect the levels of MUC1-ECD present in conditioned media or whole cell lysate.

6.3. Conclusion

In this chapter we investigated the role of MUC1-ECD cleavage in MUC1-CD dimerization, Src recruitment, and ICAM-1 binding induced CaOs and migration. We first determined that MUC1-ECD cleavage in MUC1-CFP transfected 293T cells and human breast cancer cells could be maximally inhibited by treatment with the broad spectrum metalloprotease inhibitor TAPI-0 for 24 or 40 hours, respectively. TAPI-0 is a known inhibitor of the reported constitutive MUC1 protease ADAM17, and has also been shown to inhibit PMA-stimulated MUC1 shedding, indicating it inhibits activity of MT1-MMP as well [25]. Our observation that E-cadherin-ECD cleavage was also inhibited by TAPI-0 treatment indicates that ADAM10, the reported E-cadherin protease, is another target of TAPI-0.

We found that inhibition of MUC1-ECD cleavage with TAPI-0 did not affect MUC1-CD dimerization or Src recruitment. With respect to ICAM-1 binding induced

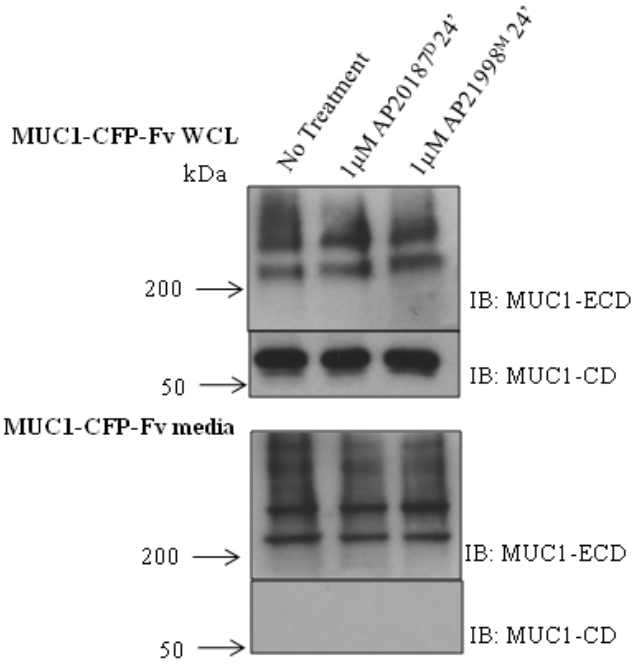


Figure 6.6. Inhibition of MUC1-CD dimerization does not affect MUC1-ECD cleavage. Following treatment with 1μM AP20187^{D} or AP21998^{M} for 24 hours, 293T MUC1-CFP-Fv cells were lysed (WCL) and 24 hour-conditioned media collected. Following assay for protein concentration, samples containing equal protein concentration were ran on SDS-PAGE. Lysates were probed with anti-MUC1-ECD, anti-MUC1-CD as a loading control. No treatment condition is presented as a control.

events, we found that MUC1-ECD cleavage was not necessary for the generation of CaOs, but was required for cell migration. Lastly, as we reported in Chapter 4 that MUC1-CD dimerization is required for ICAM-1 induced motility, and we reported here that MUC1-ECD cleavage is also required for ICAM-1 induced motility, we determined if MUC1-CD dimerization is a requirement for MUC1-ECD cleavage. We found that MUC1-ECD cleavage is not dependent on MUC1-CD dimerization. The finding that inhibition of MUC1-ECD cleavage results in decreased cell migration is significant, as it identifies a novel target for potential anti-metastatic therapies.

Chapter 7. Investigating the role of MUC1 autoproteolytic cleavage in ICAM-1 binding induced signalling

7.1. Introduction

Immediately following translation, the MUC1 protein undergoes an autoproteolytic S1 cleavage, resulting in two fragments which reassociate and are exported to the cell membrane [132]. This cleavage is termed "autoproteolytic" because it is not enzymatically catalyzed, rather, it occurs due to high conformational stress at the site of cleavage, G/SVVV. This motif is located within the conserved SEA domain, and several membrane-tethered mucins undergo this cleavage event (Fig 1.7) [133]. The resulting MUC1 protein, consisting of a large VNTR-containing N-terminal portion and a smaller, membrane-spanning C-terminal portion (Fig 1.8), exists as a stable heterodimer, but dissociates upon treatment with strong detergents. The physiological role, if any, of this cleavage event is not known, but mutational studies investigating this cleavage have raised some interesting possibilities. Mutation of the glycine and/or serine residue(s) at the site of S1 cleavage prevents autoproteolysis, resulting in a MUC1 protein that is made of a single continuous polypeptide. Functional studies on these mutants have found that MUC1-ECD shedding, possibly mediated by the MUC1 proteases ADAM17 or MT1-MMP [24, 25] does not occur, although the mutant proteins are expressed at the cell surface [26]. An additional report found that relief of conformational stress by insertion of several glycines residues immediately preceding the cleavage site (G_nGSVVV) also blocks S1 cleavage, but functional studies were not performed [200]. These data suggest two possibilities: that MUC1 dissociates at the S1 cleavage site, requiring prior autoproteolytic cleavage and releasing the ECD; or, that a second, enzymatically catalyzed S2 cleavage event, requires MUC1 to be in heterodimeric form.

As high levels of serum MUC1-ECD have been reported to correlate with a poor prognosis in breast cancer patients [27, 208], the mechanism, physiological stimuli, and potential downstream cell signalling of MUC1-ECD shedding are of clinical significance.

As we reported in Chapter 6, MUC1-ECD shedding by S2 cleavage is not required for dimerization, Src recruitment, or ICAM-1 binding induced CaOs. However, inhibiting MUC1-ECD shedding significantly decreased the level of migration through an ICAM-1 monolayer in MUC1-transfected cells and MUC1-expressing human breast cancer cells. As cell migration is a crucial step in metastasis, the prevention of MUC1-ECD S2 cleavage has potential as a novel therapeautic target.

Here, we investigated the importance of MUC1 S1 cleavage on MUC1-ECD S2 cleavage and ICAM-1 induced signalling events. By utilizing previously described direct (G/SVVV to VPVVV) and indirect (G_4GSVVV) mutations of the S1 cleavage site, we aimed to prevent S2 cleavage and assay for ICAM-1 binding induced CaOs and cell migration. We hypothesized that mutation of the S1 cleavage site would result in disruption of S2 cleavage and therefore MUC1-ECD shedding. Building from our results from Chapter 6, we further hypothesize that S1 cleavage mutants will be functional in generation of ICAM-1 binding induced CaOs but will be deficient in ICAM-1 binding induced cell motility. These investigations will add to the field of MUC1 research as the physiological and pathological functions of S1 cleavage are not yet understood.

7.2. Results

7.2.1. Direct and indirect mutation of the MUC1 S1 cleavage site does not prevent cell membrane expression

As prior studies on heterodimeric proteins have indicated that cell membrane export can be affected by mutation of the S1 cleavage site [203], we sought to determine if our mutant MUC1 proteins were expressed at the cell surface. We determined the membrane expression levels of mutant MUC1 constructs transfected into HEK 293T cells in which the S1 cleavage site was mutated directly, to VPVVV (MUC1 (VP)); or

indirectly, in which glycine residues were inserted prior to the cleavage site to relieve conformational strain – G_4GSVVV (MUC1-CFP (G4)) (Fig 2.1; Fig 7.1). Parental, non-transfected cells were assayed by flow cytometry, with no staining to control for autofluorescence or after staining with a fluorescently-conjugated anti-MUC1-ECD antibody, anti-MUC1-ECD-FITC, which controls for non-specific binding. For each population of cells, a mean fluorescent intensity was determined, and the average mean from three experiments was plotted. The parental, no staining condition was set to one and the remaining conditions expressed as a ratio. As a positive control, wildtype MUC1-CFP transfected 293T cells were assayed following staining with anti-MUC1-ECD-FITC, displaying a significant increase in mean cell fluorescence over negative controls (Fig 7.1). MUC1 (VP) and MUC1-CFP (G4) transfected 293T cells also displayed a significant increase in mean cell fluorescence compared to negative controls, and these means were not significantly different from wildtype MUC1-CFP ($p<0.05$) (Fig 7.1). These data indicate that MUC1 (VP) and MUC1 (G4) constructs are expressed at the cell surface of transfected 293T cells at equivalent levels to wildtype MUC1.

7.2.2. MUC1 (G4)-ECD is constitutively shed from the cell surface while MUC1 (VP)-ECD is not

To determine if MUC1 S1 cleavage is required for constitutive MUC1-ECD shedding, we assayed levels of MUC1-ECD in both whole cell lysate (Fig 7.2A) amd 24-hour conditioned media (Fig 7.2B) for MUC1 (VP) and MUC1-CFP (G4) transfected HEK 293T cells. As a positive control, wildtype MUC1-CFP transfected 293T cells were also assayed. In whole cell lysates, MUC1-ECD was present for MUC1-CFP, MUC1 (VP), and MUC1-CFP (G4) transfected cells (Fig 7.2A). Expectedly, MUC1-CD staining also revealed high levels of MUC1-CD in all cell lines tested. In MUC1 (VP) and MUC1-

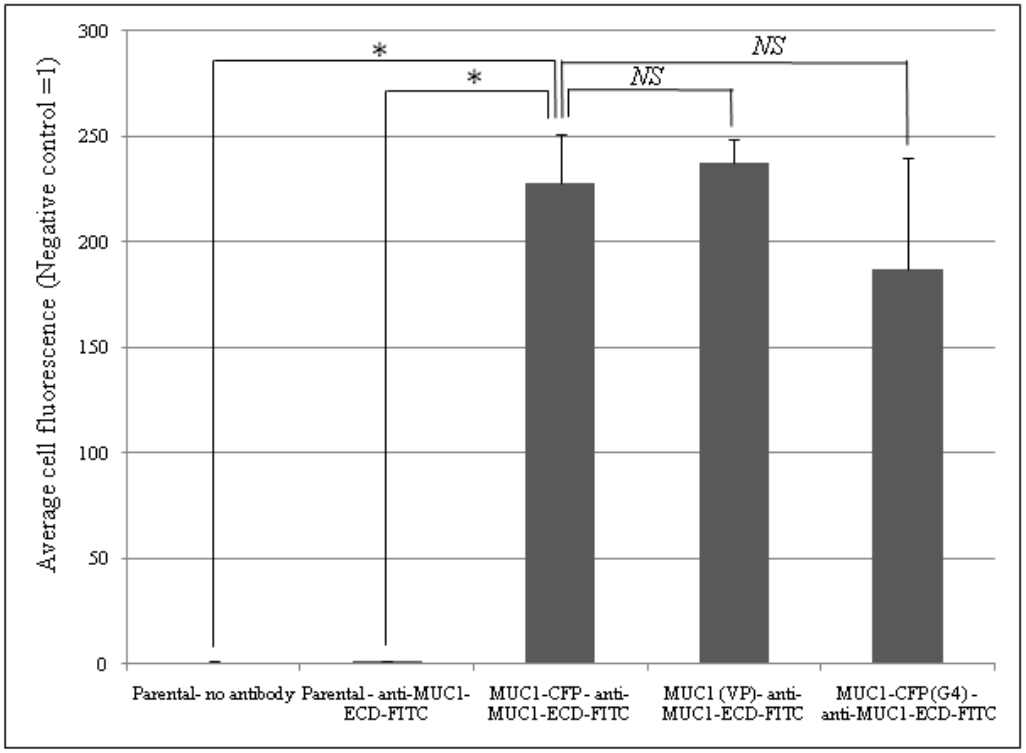


Figure 7.1. MUC1-CFP, MUC1 (VP), and MUC1-CFP (G4) are expressed on the cell surface of transfected HEK 293T cells. HEK 293T (parental) cells were assayed for auto-fluorescence (no antibody) or non-specific staining with anti-MUC1-ECD-FITC. Parental cells transfected with MUC1-CFP, MUC1 (VP) or MUC1-CFP (G4) were assayed for fluorescence after staining with anti-MUC1-ECD-FITC . Parental condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average cell fluorescence from at least three independent trials; bars, SE. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$); *NS* indicates non-significance ($p<0.05$).

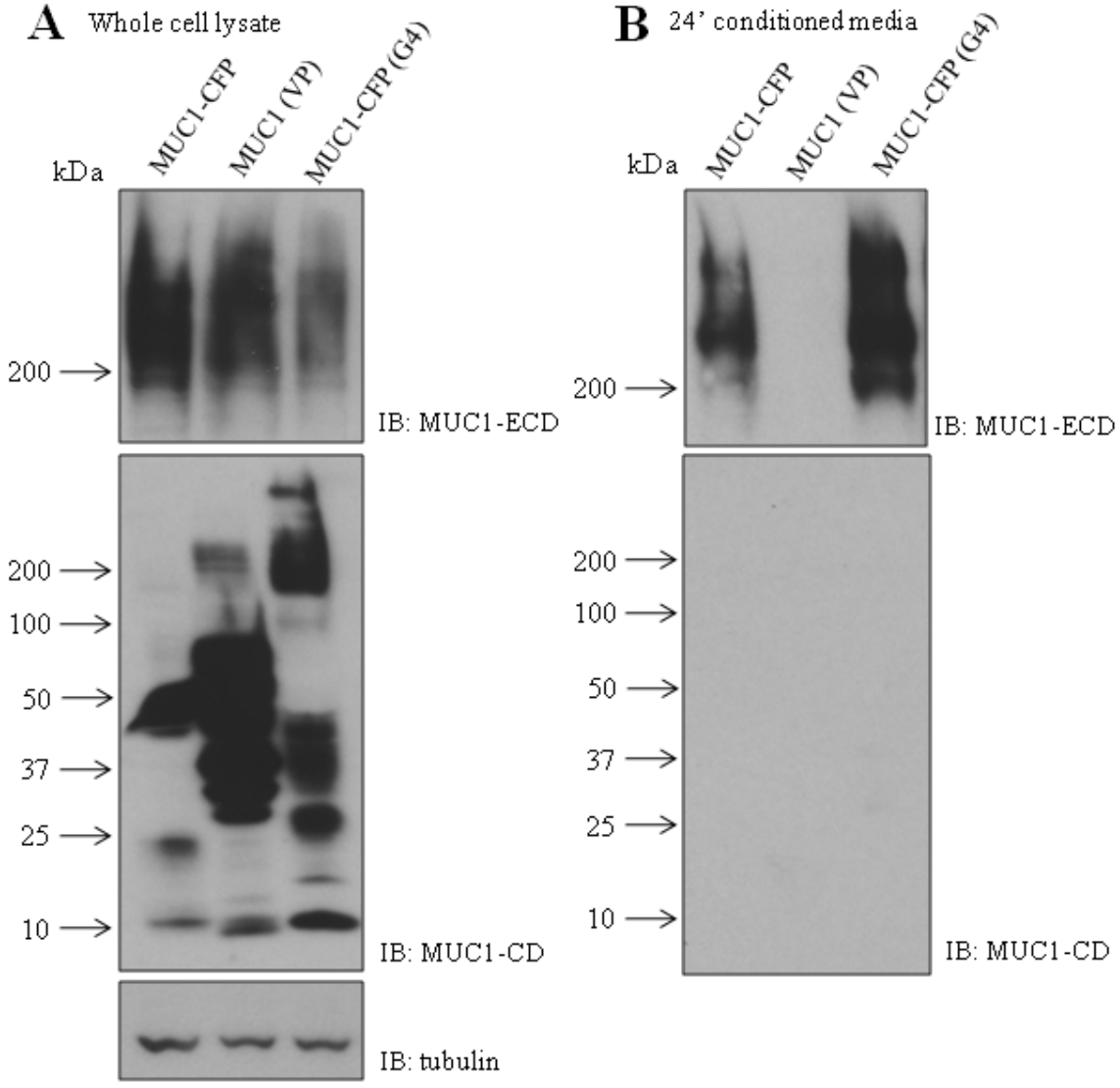


Figure 7.2. MUC1-CFP and MUC1-CFP (G4) undergo extracellular domain shedding while MUC1 (VP) does not. Whole cell lysate (A) and 24 hour conditioned media (B) were collected from HEK 293T cells transfected with wildtype MUC1-CFP, MUC1 (VP), or MUC1-CFP (G4), assayed for protein concentration, and equal protein amounts run on SDS-PAGE. Blots were probed with anti-MUC1-ECD, anti-MUC1-CD, and anti-tubulin as a loading control in whole cell lysate.

CFP (G4) transfected cells, the presence of MUC1-CD bands at high molecular weights (>200kDa) confirms that at least a portion of MUC1 in these cells exists as a full-length, high molecular weight species. As a loading control, WCLs were also assayed for tubulin, with constant levels confirming equal protein loading. Taken together, these data indicate that wildtype MUC1 is constitutively shed from the cell surface, confirming the studies of others [24, 25]. MUC1 (VP), which contains a mutation at the site of S1 cleavage and does not exist as a heterodimer at the cell surface, does not undergo S2 cleavage and MUC1-ECD release. MUC1-CFP (G4), which contains an insertion of four glysine residues prior to the wildtype S1 cleavage site, also does not exist as a heterodimer at the cell surface, but does undergo constitutive S2 cleavage, releasing MUC1-ECD into the environment. These data demonstrate that although S1 cleavage is not required for constitutive MUC1-ECD shedding, the nature of the mutation preventing S1 cleavage is a determining factor in S2 cleavage susceptibility.

In conditioned media of both wildtype MUC1-CFP and MUC1-CFP (G4) transfected cells, MUC1-ECD was detectable; while in MUC1 (VP) conditioned media, no MUC1-ECD was present (Fig 7.2B). To control for presence of full-length MUC1 from cell debris, we also assayed media for MUC1-CD, and it was absent in MUC1-CFP, MUC1 (VP) and MUC1-CFP (G4) conditioned media.

7.2.3. Autoproteolytic cleavage is not required for ICAM-1 binding induced calcium oscillations

We sought to determine if MUC1 S1 cleavage is necessary for the previously described ICAM-1 binding induced cytoplasmic CaOs. We assayed ICAM-1 binding induced CaOs in MUC1-CFP, MUC1 (VP) and MUC1-CFP (G4) transfected HEK 293T

(parental) cells (Fig 7.3). Although both MUC1 (VP) and MUC1-CFP (G4) do not undergo S1 cleavage, we reported in Section 7.2.2. that MUC1-CFP (G4) transfected cells undergo MUC1-ECD cleavage while MUC1 (VP) transfected cells do not. As negative controls, we assayed parental cells, which do not express MUC1, and we treated with Mock NIH 3T3 cells, which do not express ICAM-1. The parental, Mock treatment condition was set to one and the remaining conditions expressed as a ratio. As a positive control, we assayed ICAM-1 binding induced CaOs in wildtype MUC1-CFP transfected 293T cells, a previously described event [13].

Parental cells did not display any significant increase in CaOs level in any treatment condition, indicating that MUC1 expression is necessary for induction of ICAM-1 binding induced CaOs. In all cells treated with Mock transfected cells, the level of CaOs was not significantly different from the parental negative control treatment condition. Wildtype MUC1 transfected cells displayed a significant increase in ICAM-1 binding induced CaOs compared to negative controls, confirming the previously described event [13]. MUC1-CFP (G4) transfected cells also displayed a significant increase in ICAM-1 binding induced CaOs compared to negative controls, which was statistically equivalent to that of wildtype MUC1 transfected cells. MUC1 (VP) transfected cells generated ICAM-1 binding induced CaOs which was significantly decreased compared to MUC1-CFP cells. However, MUC1 (VP) cells did display a significant increased in CaOs when treated with ICAM-1 cells compared to Mock cells. These data demonstrate that S1 cleavage of MUC1 is not required for induction of ICAM-1 binding induced CaOs. However, the nature of the mutation conferring S1 cleavage resistance is a determinant of the response elicited upon ICAM-1 stimulation.

7.2.4. Autoproteolytic cleavage is not required for ICAM-1 binding induced cell invasion

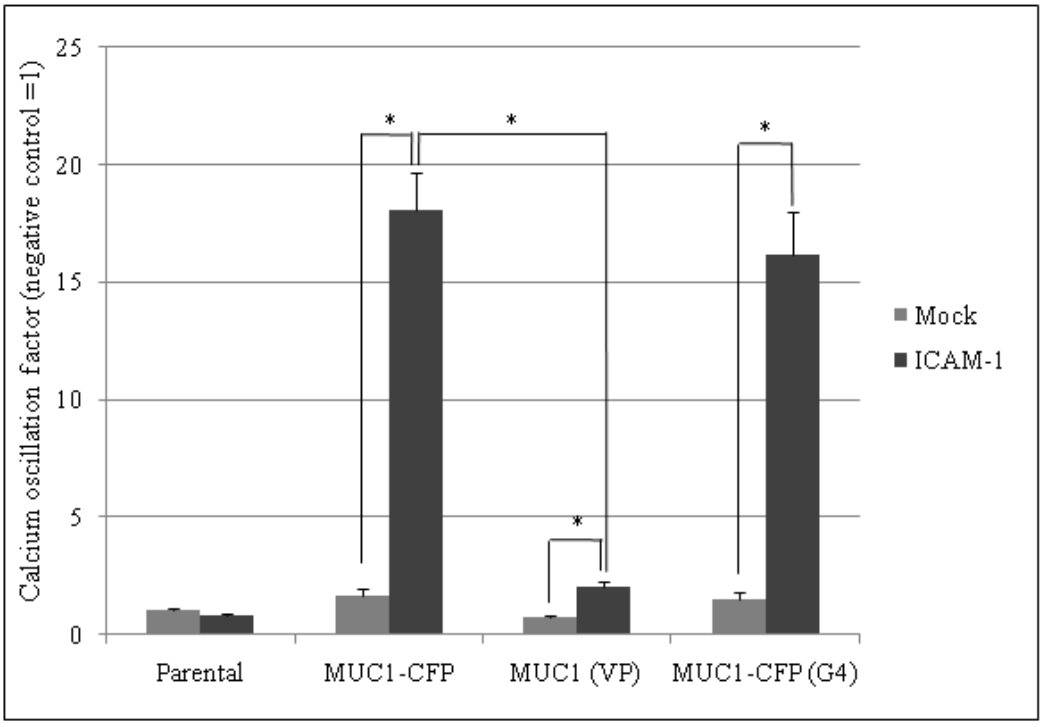


Figure 7.3. MUC1-CFP and MUC1-CFP (G4) transfected cells display equivalent levels of ICAM-1 binding induced calcium oscillations, while MUC1 (VP) transfected cells display significantly reduced levels. HEK 293T (parental), MUC1-CFP, MUC1 (VP) and MUC1-CFP (G4) cells were assayed for calcium oscillations after treatment with mock or ICAM-1 transfected NIH 3T3 cells. Parental, mock condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average oscillation factors from at least three independent trials; bars, SE. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$).

To determine if MUC1 S1 cleavage is necessary for the previously described ICAM-1 binding induced cell invasion, we assayed cell migration through an ICAM-1 positive monolayuer for MUC1 (VP) and MUC1-CFP (G4) transfected HEK 293T (parental) cells (Fig 7.4). Although both MUC1 (VP) and MUC1-CFP (G4) do not undergo S1 cleavage, we reported in Section 7.2.2. that MUC1-CFP (G4) transfected cells undergo MUC1-ECD cleavage while MUC1 (VP) transfected cells do not. As negative controls, we assayed migration of parental cells, which do not express MUC1; and we assayed for migration through a monolayer of Mock transfected NIH 3T3 cells, which do not express ICAM-1. The parental, mock monolayer condition was set to one and the remaining conditions expressed as a ratio. As a positive control, we assayed ICAM-1 binding induced migration in wildtype MUC1 transfected 293T cells, a previously described event [15]. Parental cells did not display a significant increase in migration levels in any treatment condition, indicating that MUC1 expression is necessary for induction of ICAM-1 binding induced cell migration. In all cells assayed for invasion through a Mock transfected monolayer, the level of cell migration was not significantly different from the parental negative control treatment condition. Wildtype MUC1 transfected cells displayed a significant increase in migration through an ICAM-1 monolayer compared to negative controls, confirming the previously described event [15]. MUC1-CFP (G4) transfected cells also displayed a significant increase in migration through an ICAM-1 monolayer compared to negative controls, which was statistically equivalent to that of wildtype MUC1 transfected cells. However, levels of migration through an ICAM-1 monolayer in MUC1 (VP) transfected cells were significantly reduced compared to wildtype MUC1-CFP cells, although migration was significantly increased above that observed in Mock monolayer condition. These data demonstrate that S1 cleavage of MUC1 is not required ICAM-1 binding induced cell migration. However,

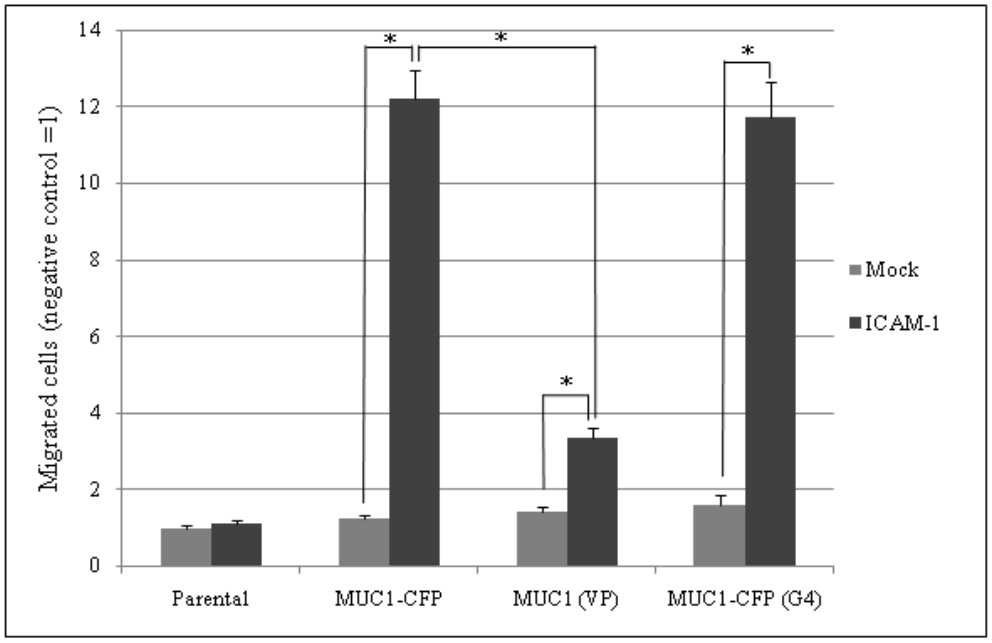


Figure 7.4. MUC1-CFP and MUC1-CFP (G4) transfected cells display equivalent levels of ICAM-1 binding induced cell migration, while MUC1 (VP) transfected cells display significantly reduced levels. MUC1-CFP, MUC1 (VP) and MUC1-CFP (G4) transfected 293T (parental) cells were assayed for 24 hour migration through a monolayer of mock or ICAM-1 transfected NIH 3T3 cells. Parental, mock condition is set to one with the remaining experimental conditions expressed as a ratio. Columns represent average number of migrated cells per five fields from at least three independent trials; bars, SE. *Asterisk* indicates pairs in the data set which are statistically different ($p<0.05$).

the nature of the mutation conferring S1 cleavage resistance is a determinant of the response elicited upon ICAM-1 stimulation.

7.3. Conclusion

In this chapter, we investigated the role of MUC1 autoproteolytic (S1) cleavage in MUC1-ECD shedding and previously described ICAM-1 binding induced events. In Chapter 6, we reported that MUC1-ECD S2 cleavage is required for ICAM-1 binding induced cell migration, although it was not required for ICAM-1 binding induced CaOs. Here, we investigated the role of S1 cleavage on MUC1-ECD shedding from the cell surface and ICAM-1 binding induced events. We found that a mutation which directly targeted the MUC1 S1 cleavage site, G/SVVV to VPVVV, resulted in an S1 cleavage deficient mutant which did not release MUC1-ECD constitutively. This mutant was also deficient in ICAM-1 binding induced CaOs; and although it did display a significantly increase in cell migration when exposed to ICAM-1 expressing cells when compared to Mock treatment cells, the increase was still significantly below the level of wildtype MUC1.

Another mutation indirectly prevented MUC1 S1 cleavage by inserting four glycine residues directly before the S1 cleavage site, G/SVVV to G_4GSVVV. This mutant, when transfected into HEK 293T cells, demonstrated constitutive MUC1-ECD shedding, showing that S1 cleavage is not a requirement for MUC1-ECD release. MUC1-CFP (G4) transfectants responded to ICAM-1 stimulation by generating CaOs and cell migration levels statistically equivalent to wildtype MUC1 transfectants. Taken together, these data demonstrate that S1 cleavage is not required for generation of ICAM-1 binding induced CaOs and cell invasion. However, in light of the different levels of ECD shedding and responses to ICAM-1 stimulation in the S1 cleavage deficient mutants, we

conclude that the nature of the mutation conferring resistance to autoproteolysis is a determinant of MUC1 protein function.

Chapter 8: Discussion

8.1. Interpretation of results

8.1.1. Src in MUC1/ICAM-1 signalling

Src has been implicated in MUC1/ICAM-1 binding induced signalling in several studies. For example, prior investigations on the MUC1/ICAM-1 binding induced signal have demonstrated that inhibition of Src with the broad-spectrum SFK inhibitor PP2 prevented ICAM-1 binding induced CrkL recruitment to MUC1, CaOs, and cell migration [13, 14]. PP2 is not a specific inhibitor of c-Src, and also inhibits activity of other SFKs such as Lck, Fyn, and Hck; as well as non-SFKs such as EGFR [349, 398-400]. As EGFR has been shown to phosphorylate MUC1-CD on Y^{46}, the same site that serves as a substrate and binding site for Src, it is possible that PP2 inhibits EGFR-mediated phosphorylation of MUC1-CD, and is at least partially responsible for the observed effect [361]. Also, as Src is involved in many processes critical for cell motility (described in Section 1.2.5.4), the lack of cell motility after Src inhibition is not surprising. To demonstrate a direct and critical role for Src in MUC1/ICAM-1 signalling, specific inhibition of Src, and of the MUC1/Src interaction, was required.

In addition, careful analysis of the available literature on MUC1 and Src activity in breast cancer demonstrates the likelihood of a cooperative relationship in cancer progression. Src inhibition, at the genetic level, in Luminal B and Tamoxifen-resistant Luminal B breast cancer cells has been shown to inhibit cell migration [128, 401]. Src inhibition has also been shown to be synergistic with concomitant Tamoxifen in Luminal B breast cancer cells in inhibiting invasion and preventing resistance to anti-hormone therapies, along with a decrease in FAK activity [402]. Src inhibition has also been shown to increase E-cadherin/β-catenin mediated cellular adhesion and decrease phosphorylation of β-catenin, which is necessary for localization to the nucleus [359]. We propose that these observations can be explained by an association between MUC1 and

Src in breast cancer cell adhesion and motility. Tamoxifen treatment decreases MUC1 expression [403, 404], and down-regulation of MUC1 has been shown to increase association between E-cadherin and β-catenin and cell adhesion [256]. Also, MUC1 has been shown to bind to FAK [268], a Src substrate. A MUC1/Src/FAK complex could promote membrane localization and activity of FAK, increasing cell migration. Thus the additive effect of Tamoxifen, the stabilization of intercellular adhesions and decreased FAK activity are logical consequences of the dual inhibition of MUC1 and Src in the same pathway. Taken together, these data provide support for the hypothesis that MUC1 and Src cooperate in promotion of cell migration and breast cancer metastasis, and dual inhibition of the activity of these proteins, along with inhibition of the MUC1/Src association, is an attractive possibility for anti-metastatic therapies.

In Chapter 3, we demonstrated that knockdown of Src with siRNA in MUC1 expressing cells results in disruption of ICAM-1 binding induced events. The use of siRNA provides a more specific inhibition of c-Src than PP2, although we cannot exclude the possibility that other SFKs in HEK 293T cells were silenced as well due to non-specificity of our anti-Src antibody. However, the siRNA used in Src knockdown was modified to reduce off-target inhibition and also run through a BLAST search to determine levels of non-specific binding prior to use [405]. The observed decreases in ICAM-1 binding induced CaOs and cell invasion demonstrate that Src is a critical component of the ICAM-1/MUC1 signalling axis. Direct interaction between MUC1 and Src have been previously observed, and increased MUC1/Src binding has been demonstrated following ICAM-1 binding, suggesting that Src plays an early and direct role in initiation of MUC1/ICAM-1 signalling. However, based on the evidence presented in Chapter 3, we cannot exclude the possibility that MUC1/Src binding is not directly involved in transmission of the ICAM-1 induced signal, but rather Src plays a

critical role downstream of MUC1. Therefore, further studies aiming to specifically inhibit the MUC1/Src interaction were performed to investigate this possibility and will be discussed below.

8.1.2. MUC1 dimerization

Several studies have indicated a role for MUC1-CD dimerization in cellular signalling cascades implicated in progression of pathologies. Experiments using a CD8/MUC1 chimera containing 69-aa of MUC1-CD, identical to the construct used in these studies, demonstrated that treatment of CD8/MUC1 transfected cells with anti-CD8 antibody resulted in phosphorylation of the MUC1 portion of the chimera [380]. Further studies by this group demonstrated that anti-CD8 treatment of the chimera also resulted in activation of the Ras-MEK-ERK2 pathway and phosphorylation of ERK1/2, which were dependent on tyrosine phosphorylation of MUC1-CD [381, 406]. Also, pERK1/2 was localized to the nucleus following anti-CD8 treatment. Although these studies did not investigate the mechanism of anti-CD8 induced activation of MUC1, the bivalent nature of antibodies makes dimerization a likely possibility. Simultaneous binding of an antibody to two identical epitopes on distinct proteins would result in spatial and temporal co-localization of the proteins in live cells, akin to dimerization. This method has been utilized previously to induce dimerization of other cell membrane receptors [382, 384]. Therefore, these studies provided preliminary evidence that dimerization of a portion of MUC1-CD, as the chimera did not contain the ECD, TMD or $C^{1}QC$ motif of MUC1, could induce phosphorylation of MUC1 and activation of downstream signalling cascades.

Further evidence that MUC1-CD dimerization is important in cellular signalling came several years later from a different research group. In 2007, a study was published

which demonstrated that nuclear localization of MUC1-CD, a truncated construct lacking MUC1 ECD and TMD, was dependent on the C^1QC motif of MUC1-CD [19]. Further, they showed that MUC1-CD formed covalently linked dimers *in vitro*, and C^1QC-dependent dimers *in vivo*. They concluded that MUC1-CD forms oligomers *in vivo* which are dependent on the cysteines of the C^1QC motif. However, although the evidence presented may suggest that *in vivo* dimers are the result of disulfide linkage of cysteine residues, they did not investigate this possibility. Subsequent publications from this group characterized a peptide inhibitor, containing the MUC1-CD C^1QC motif, as an inhibitor of MUC1-CD oligomerization. Administration of this peptide in cellular models resulted in attenuation of MUC1-CD nuclear targeting, disruption of redox balance, growth arrest and necrotic death of human breast cancer cells [21]. Administration of this compound to mice bearing xenografts of breast, prostate, multiple myeloma, and chronic mylogenous leukemia invariably resulted in cancer regression [20-22, 183]. Although this body of work demonstrates that oligomerization of MUC1-CD is important in tumor progression, the evidence presented on the mechanism of MUC1-CD oligomerization is inconclusive. The use of a truncated MUC1-CD construct *in vivo* lacking the MUC1 ECD and TM domain would result in a MUC1 species which is not present at the cell surface. The mechanism of dimerization of full-length, membrane-tethered MUC1 and truncated, cytoplasmic MUC1 may be different, and therefore conclusions drawn using this construct cannot necessarily be applied to full-length or naturally occurring MUC1 existing at the cell membrane. Also, interaction between MUC1 and other cell membrane proteins is a critical step in most MUC1 functions, as MUC1 does not have tyrosine kinase activity and most MUC1 kinases are localized to the cell membrane (Sections 1.2.2.3., 1.2.3.1.). Without proper assessment of the function of this cytoplasmic MUC1

construct, the observation that it forms covalent dimers *in vitro* and cysteine-dependent oligomers *in vivo* does not necessarily apply to wildtype, transmembrane MUC1.

The observation of covalently linked MUC1-CD dimers *in vitro* is not surprising due to the oxidizing nature of extracellular environments. However, in cells, the reducing environment of the cytosol is unfavourable for the formation and maintenance of disulfide unless the redox balance is disrupted [407]. However, the formation of cytoplasmic disulfide bonds under conditions of oxidative stress could act as a "sensor" of redox disruption and trigger the expression and/or activation of antioxidant proteins [408]. As MUC1 has been implicated in maintenance of cellular redox balance [294, 295, 409], and disruption of MUC1-CD oligomerization has been shown to disrupt the redox balance in cancer cells [20, 21], we propose that the $C^{1}QC$ motif of MUC1-CD may facilitate MUC1-CD dimerization in situations of oxidative stress. This could result in MUC1-CD nuclear localization, initiating a transcription cascade resulting in upregulation of antioxidant enzymes as well as MUC1 itself [294, 295].

We interpret our results presented in Chapters 4 and 5 with reference to the studies described above. Our initial observation that MUC1-CD forms constitutive dimers *in vivo* is in agreement with the literature. However, we made a novel finding in that we observed dimer formation in cells transfected with full-length MUC1 and human breast cancer cells expressing MUC1 at the cell surface (Fig 4.1). The molecular weight of our dimeric species indicate that, at a minimum, the full, membrane tethered portion of the MUC1 protein is participating in dimerization. Additionally, our work using dual-transfection of MUCY-YFP-Fv and CD8/MUC1 (Fig 4.2) demonstrates a direct interaction between the cytoplasmic domains of MUC1. The CD8/MUC1 construct used in these experiments contains 69-aa of the MUC1-CD (no $C^{1}QC$ motif), indicating that the "MUC1 dimerization motif" is present in this sequence. Taken together, these

observations demonstrate for the first time that MUC1-CD, containing the TMD and a portion of the ECD, forms constitutive dimers. This finding is significant as the mechanism of MUC1 function in human cancers, particularly breast cancers, has been the subject of intense research over the last decade. The discovery that a portion of MUC1-CD exists as a dimer opens up many interesting lines of research, as parallels can be made between potential function of MUC1 and other receptors that exist as constitutive cell membrane dimers. For example, the GHR has been found to exist as a constitutive dimer which becomes extracellularly disulfide linked, relocates to DRMs, and becomes resistant to cleavage following ligand binding facilitating cellular signalling [373-375]. As MUC1 has been found to be covalently linked *in vitro*, reside in DRMs, and is subject to cleavage, investigation of these possibilities is appealing in light of evidence presented here [19, 24, 410, 411].

Next, we manipulated MUC1-CD dimerization using an engineered "Fv dimerization domain" which was designed to facilitate or inhibit dimerization of proteins fused to it [388, 389]. Treatment of MUC1-CFP-Fv with the bivalent compound $AP20187^{D}$, designed to facilitate dimerization, did not result in a significant increase in MUC1-CD dimerization (Fig 4.3). We have considered several possibilities to explain this unexpected result. MUC1-CFP-Fv monomers may be spatially segregated in DRMs, endocytotic vesicles, mitochondria and/or the nucleus of cells, preventing $AP20187^{D}$ interactions. Another possibility is that MUC1 dimerization is a transient event, with the equilibrium of MUC1-CD dimer formation and dissociation favouring dissociation. It is also plausible that artificial dimers are created by $AP20187^{D}$ treatment, but the MUC1-CD species are not brought within sufficient proximity by $AP20187^{D}$ to allow for DSS crosslinking and dimer detection and/or dimer functionality.

However, our treatment of MUC1-CFP-Fv cells with the monovalent compound $AP21998^{M}$ resulted in a dose-dependent and significant reduction in MUC1-CD dimerization (Fig 4.3). This increased efficacy, compared to $AP20187^{D}$, may be due to increased availability of MUC1-CD dimers to $AP21998^{M}$ binding, increased propensity for MUC1-CD dimers to dissociate, rather than form, artificially, and/or $AP21998^{M}$ being of sufficient size to disrupt MUC1-CD dimers to an extent that DSS crosslinking and dimer function is impaired. In any case, we observed a significant reduction in MUC1-CD dimerization following treatment of MUC1-CFP-Fv transfected cells with 1μM $AP21998^{M}$ for one minute, providing a method for investigation of the role of MUC1-CD dimerization in Src recruitment and ICAM-1 binding induced signalling. Importantly, treatment with $AP21087^{D}$ and $AP21998^{M}$ did not result in a significant loss of cell viability up to 72 hours of treatment (Fig 4.4), as other studies inhibiting MUC1 dimerization have demonstrated that cell death is a result [22, 184]. Our results may differ from these due to differences in cell types and mechanism of dimerization inhibition.

Next, we demonstrated that treatment of MUC1-CFP-Fv transfected cells with increasing concentrations of $AP21998^{M}$ resulted in a dose dependent decrease in the level of Src (total) and Src^{pY416} (active) associated with MUC1 in co-immunoprecipitation experiments (Fig 4.5). These results demonstrate that the MUC1-CD dimer is the conformation which is competent to bind and possibly activate Src. This could be due to several, non-mutually exclusive possibilities. As Src contains both SH2 and SH3 domains capable of binding to SH2 and SH3 binding domains, respectively, it is possible that Src binds to each member of a MUC1-CD dimer at either a SH2 or SH3 binding domain. These dual interactions could be dependent, as binding of the Src SH3 or SH2 domain to a ligand increases the propensity for binding of the other to a ligand [331]. Also, the

proximity of SH2 and SH3 binding domains in a single MUC1 species (7aa) is not favourable for the simultaneous binding of the large, bulky SH2 and SH3 domains of Src [412]. However, this same report found that short linker sequences between SH2 and SH3 binding domains can facilitate dimerization of both SH2/3 domain containing proteins and SH2/3 binding domain containing proteins. They propose a model in which a single Src molecule can simultaneously bind two peptides via its SH2 and SH3 domains; or in which two Src molecules can simultaneously bind one peptide containing SH2 and SH3 binding domains [412]. Therefore, it is possible that a MUC1 dimer binds a single Src molecule via both SH2 and SH3 interactions; and the MUC1 monomer does not bind Src with high affinity. As the affinity for Src SH3 binding sites is generally lower than for SH2 binding sites, we propose that transient binding may occur between MUC1 monomers and Src SH3 domain, resulting in partial activation of Src (Section 1.2.5.2). However, in MUC1 dimers, the MUC1-CD Src substrate Y^{46} may be in favourable proximity for phosphorylation, generating an SH2 binding domain and resulting in both stable binding and activation of Src. Therefore, our observation that disruption of MUC1 dimers results in decreased recruitment of total and active Src would be explained by the absence of a stable recruitment and activation site for Src in MUC1 monomers.

Our subsequent experiments investigated the effect of MUC1 dimer disruption, and therefore Src recruitment, on the previously observed ICAM-1 binding induced CaOs and cell migration events [13, 15]. We demonstrated in Chapter 3 that Src is a critical component of the MUC1/ICAM-1 binding induced signalling cascade, yet we could not exclude the possibility that Src was acting far downstream of MUC1, as Src is a well known component of cell migratory machinery (Section 1.2.5.4). Here, we show that inhibition of the MUC1/Src interaction results in significant decreases in ICAM-1

induced CaOs and cell migration (Fig 4.7, Fig 4.8), demonstrating a correlation between MUC1/Src binding and ICAM-1 binding induced signalling. We cannot exclude the possibility that disruption of MUC1 dimerization inhibits its interaction with another critical downstream signalling partner and disruption of Src binding is not the cause of loss of signalling capacity. However, the body of evidence presented in this thesis and in other published works supports the hypothesis that MUC1/ICAM-1 binding induced signalling is mediated by Src.

Our observation that MUC1-CFP-Fv transfected cells display increased migration through an ICAM-1 monolayer when treated with $AP20187^{D}$ compared to no treatment control is in conflict with our observation that treatment with this compound does not induce increased dimerization or increased Src recruitment. However, the dimerization experiments were performed after treatment with $AP21087^{D}$ for one minute, while the migration assay occurs over 24 hours. Therefore, it is possible that long-term treatment of MUC1-CFP-Fv transfected cells with $AP20187^{D}$ does increase MUC1-CD dimerization. This possibility was examined, but results were inconsistent and no conclusions could be drawn (data not shown).

In light of the previously published work claiming that MUC1-CD dimerization is dependent on the cysteines of the $C^{1}QC$ motif [19], we hypothesized that MUC1-CD dimers were covalently linked via disulfide bonds. If MUC1-CD dimers were bound by covalent bonds, we would expect that in the absence of a reducing agent they would remain intact and be detectable on SDS-PAGE. However, no constitutive MUC1-CD dimers were detected under non-reducing conditions (Fig 4.9). We also assayed for covalently linked MUC1-CD dimers following ICAM-1 stimulation for one minute, as in GHR, which also exists as a constitutive dimer, ligand binding induces extracellular disulfide bridge formation and stimulates signalling [373]. These data indicate that

MUC1-CD dimers are not disulfide-linked constitutively or following ICAM-1 binding. Due to the reducing environment of the cytoplasm, however, this is not surprising. Formation and maintenance of disulfide bonds is highly unfavourable in cells in a balanced redox state [407]. However, MUC1 does contain a cysteine residue in the TMD, which could also facilitate disulfide bridge formation, although the data presented here does not support this possibility. MUC1 does not contain any extracellular cysteine residues, which would be the most likely site of disulfide bridge formation. Therefore, we conclude that the mechanism of MUC1-CD dimerization does not involve disulfide bonds.

To further investigate this hypothesis, in Chapter 5, we mutated the cysteines of the C^1QC motif to alanines, abolishing the potential for disulfide bond formation. Cells transfected with the MUC1-CFP-Fv (AQA) mutant were functional in terms of dimerization, Src recruitment, and ICAM-1 binding induced signalling (Fig 5.2, Fig 5.3, Fig 5.4). As we have previously determined that dimerization is necessary for Src recruitment and ICAM-1 induced signalling, we conclude that the cysteines of the C^1QC motif are not required for dimerization or these dimerization dependent events. This confirms our conclusions from Chapter 4 that MUC1 dimers are not covalently linked via disulfide bridges. However, previous work has also demonstrated involvement of the C^1QC motif in palmitoylation [180]. As MUC1 has been localized to DRMs, and palmitoylation of proteins has been shown to increase the affinity of proteins for DRMs, it is possible that the C^1QC motif is important in MUC1 DRM residence [410, 411, 413]. However, as DRM disruption has been shown to inhibit ICAM-1 binding induced CaOs, our observation that MUC1-CFP-Fv (AQA) transfected cells exhibited ICAM-1 binding induced CaOs at levels similar to wildtype conflicts with this hypothesis. It is possible that there are several mechanisms of MUC1 DRM residence, and that association of

MUC1 with different DRM subtypes, defined by lipid and protein composition, have different effects on MUC1 functionality [414]. However, investigation of this speculation would require greater understanding of both MUC1 and DRM function.

We also investigated the importance of Src binding in MUC1 dimerization. As Src is known to bind to MUC1 at SH2 and SH3 domains, it is possible that Src binding and MUC1 dimer formation are co-dependent events, with Src effectively "bridging" a MUC1 dimer. First, we further investigated the mechanism of Src recruitment to MUC1 by mutating key residues of the MUC1 reported SH2 ($Y^{46}F$) and proposed SH3 ($P^{37}A$, $P^{38}A$) binding domains, either individually or together. We found that Src recruitment to MUC1 was reduced compared to wildtype in the single ΔSH2 and ΔSH3 mutants, and was undetectable in the dual ΔSH2/3 mutant (Fig 4.10). We concluded that when one of the Src binding domains is mutated, Src is still able to bind to the intact domain. However, binding to the intact domain is impaired, possibly because stable binding of Src requires the presence of accesible SH2 and SH3 binding domains, such as that proposed for a MUC1 dimer. When both domains are absent, MUC1 is incapable of binding Src. Therefore we assayed MUC1 dimerization in these mutants to determine if impaired Src binding was correlated with impaired dimerization. We found that MUC1-CD formed dimers as effectively in ΔSH2, ΔSH3 and ΔSH2/3 binding domain mutants as in wildtype (Fig 4.11), demonstrating that impaired Src binding does not affect constitutive MUC1-CD dimerization. To test this hypothesis in another way, we transfected MEF SYF-/- (Src, Yes, Fyn knockout) with MUC1-CFP-Fv and assayed for MUC1-CD dimerization. MUC1-CD formed constitutive dimers in these cells in the absence of Src, and no significant change in MUC1-CD dimerization was observed following transfection of Src or Src $Y^{530}F$, a constitutively active mutant (Fig 4.12). We conclude that MUC1-CD dimerization is independent of Src and SH2 and SH3 binding domain interactions, and

Src binding to MUC1 does not potentiate dimerization. Therefore, inhibition of Src binding to MUC1 through peptides or small molecule inhibitors would not be expected to disrupt MUC1-CD dimerization. It is possible that ICAM-1 binding induces additional signalling cascades distinct from those mediated by Src, but these pathways have not been discovered nor do we know if MUC1 dimerization is required for all ICAM-1 binding induced functions.

However, our observation that disruption of MUC1 dimerization inhibited ICAM-1 binding induced cell migration offers an appealing target for anti-metastatic therapies. The most effective way to attempt to disrupt ICAM-1 binding induced signalling may be to target MUC1 dimerization, which would disrupt Src-mediated signalling as well as other potential pathways involving MUC1 dimerization. A possible model for inhibition of MUC1 dimerization is found in ErbB2, which is overexpressed in many breast cancers and is the target for numerous therapeutic compounds [415]. A first-line treatment for patients with ErbB2 positive breast cancer is treatment with an anti-ErbB2 antibody, Trastuzumab. Several studies have indicated that one of the effects of Trastuzumab on ErbB2 may be to prevent dimerization [377, 416]. Using this as a model, an anti-MUC1 antibody which targets the extracellular portion of the membrane tethered subunit could be used to sterically inhibit MUC1-CD dimerization, and therefore Src recruitment. Also, elucidation of the mechanism of MUC1-CD dimerization would provide additional targets for anti-metastatic therapies. Other groups have used peptides composed of portions of MUC1-CD to disrupt MUC1 dimerization and/or binding to downstream effectors [20, 21, 273, 280]. However, the mechanism of action of these peptides and the effect on cellular growth and invasion is unclear. One study reported an increase in cellular invasion following treatment with a MUC1-CD peptide, M1, encompassing the Src SH2 binding site, the potential CrkL recruitment site, and the β-

catenin binding site [273]. However, a separate study utilized a peptide consisting of a portion the M1 peptide, containing the Src and β-catenin binding sites, but not the potential CrkL site, found that this peptide inhibited invasion [280]. It is not known whether the binding of these peptides to MUC1 effectors activates or inhibits their signalling capacities, or if they form functional or non-functional MUC1-peptide psuedodimers. One possible explanation for the discrepancy in the studies described above could be the presence of both Src and CrkL recruitment sites in the invasion promoting peptide, possibly acting as a scaffold to induce pro-migratory signalling. Also, the effect of these peptides on MUC1-CD dimerization has not been tested, and could shed light on their mechanism of action. MUC1-CD peptides show therapeutic promise regardless of the lack of understanding of their mechanism, and should be pursued clinically. Recently, a small molecule has been reported to disrupt MUC1 dimerization [390], offering an alternative to the use of MUC1-CD peptides in inhibition of MUC1 dimerization.

In summary, we have demonstrated that dimerization of MUC1-CD occurs constitutively, does not require cysteine residues of the C^1QC motif, and is required for Src recruitment and ICAM-1 binding induced events. We propose that inhibition of MUC1-CD dimerization, by anti-MUC1 antibodies, peptides, or small molecule inhibitors, could disrupt migration of circulating breast cancer cells overexpressing MUC1 by blocking ICAM-1 binding induced signalling cascades.

8.1.3. MUC1 cleavage

In chapter 6, we investigated the role of MUC1-ECD shedding in ICAM-1 binding induced signaling. As prior reports had already identified MUC1 as a constitutive substrate of ADAM17, acting to release the heavily glycosylated ECD, we hypothesized

that use of a broad spectrum metalloprotease inhibitor, TAPI-0, known to target ADAM17 would result in disruption of ICAM-1 binding induced signalling. The concentration of TAPI-0 used in these experiments (100μM) was chosen because other reports [24, 25, 210] demonstrated that this concentration inhibited constitutive, PMA-induced, and TNF-α induced MUC1 shedding. We carried out these experiments in both MUC1 transfected cells and human breast cancer T47D cells in order to apply our results to the study and treatment of MUC1 overexpressing Luminal B breast cancers. As ICAM-1 binding and signal transmission requires an intact MUC1-ECD, we hypothesized that cleavage is required following ICAM-1 binding to a MUC1 dimer, resulting in generation of CaOs and cell migration. Therefore, cleavage would not be necessary for dimerization and Src recruitment. We found that treatment with TAPI-0 resulted in decreased MUC1-ECD cleavage, confirming our method of assaying the role of MUC1-ECD cleavage in MUC1 function (Fig 6.1). Next, we determined the effect of TAPI-0 treatment on MUC1-CD dimerization. We found that in both MUC1 transfected and human breast cancer T47D cells, inhibition of MUC1-ECD cleavage did not affect MUC1-CD dimerization levels (Fig 6.2). We interpret this to indicate that MUC1-CD dimers form with the MUC1-ECD intact in both dimer partners. This is surprising, as the MUC1-ECD would be expected to repel other large, negatively charged entities. However, it is possible that although the MUC1-CD dimers exist within 11.4Å (demonstrated by DSS reactivity), the MUC1-ECDs of the dimer partners may exist at a greater distance from eachother. It is not clear whether ICAM-1 binds to MUC1 as a dimer, which could facilitate clustering of MUC1 dimers. This possibility is appealing, as clustering of MUC1 dimers could facilitate trans-autophosphorylation of associated Src molecules at Y^{416}, resulting in full activation and initiation of tyrosine phosphorylation cascasdes. Although the dimeric form of ICAM-1 has demonstrated increased binding to

its ligand LFA-1 [417], more recent studies have indicated that ICAM-1 dimerization may not be required for LFA-1 binding, and the structure of native ICAM-1 at the cell surface is still under debate [299, 301]. If ICAM-1 binding to MUC1 requires MUC1-ECD dimerization, it is possible that our observation that MUC1 dimer disruption inhibits ICAM-1 binding induced signalling (Chapter 4) may be due in part to impaired ICAM-1 ligation to MUC1-ECD. Further studies investigating the role of both MUC1 and ICAM-1 dimerization in the binding and signal initiation would provide information on the potential of targeting MUC1-ECD and/or ICAM-1 dimerization in a clinical setting.

Additional data presented in Chapter 6 demonstrates that inhibition of MUC1-ECD cleavage by TAPI-0 does not inhibit Src recruitment (Fig 6.3) or ICAM-1 binding induced CaOs (Fig 6.4) in MUC1 transfected or human breast cancer T47D cells. However, TAPI-0 treatment did significantly inhibit ICAM-1 binding induced cell migration (Fig 6.5). As we previously observed that TAPI-0 treatment did not inhibit dimer formation, the observation TAPI-0 treatment does not inhibit Src recruitment, a dimer dependant event, was expected. However, the different response to TAPI-0 treatment in generation of ICAM-1 binding induced CaOs and cell migration was surprising. As the time courses for initiation of CaOs (~30 seconds) [13] and cytoskeletal rearrangements leading to migration (4 minutes) [14], are not drastically different, it is plausible that ICAM-1 binding could lead to initiation of parallel pathways, both beginning with activation of Src and tyrosine phosphorylation events. However, cell migration assays were performed 24 hours following MUC1/ICAM-1 ligation, indicating long-term implications of MUC1/ICAM-1 binding beyond the characterized, early signalling pathways. ICAM-1 binding induced CaOS signal involves Src, PI3K, and PLC as mediators [13]; ICAM-1 binding induced cell migration involves Src, CrkL, Rac/Cdc42 [14] as mediators. Therefore, we propose that ICAM-1 binding to MUC1

initiates distinct signalling pathways mediated by Src, with MUC1-ECD cleavage not being important in the pathways leading to CaOs while being a critical component of the pathway leading to cell migration.

However, a major limitation of our interpretation of the effect of TAPI-0 treatment on MUC1/ICAM-1 binding induced migration is due to the non-specificity of our inhibition strategy. TAPI-0 is present during the duration of the migration assay (24 hours) to ensure continued inhibition of MUC1 S2 cleavage, and it is absent during the CaOs assay (3 minutes). Therefore, in the migration assay, metalloproteases present in the cell monolayer (ICAM-1 cells) would be subjected to inhibition as well. TAPI-0 treatment would be expected to inhibit the activity of several metalloproteases, such as ADAM10 and ADAM17, in both MUC1 and ICAM-1 expressing cells present in a migration assay. This would result in inhibition of cleavage of ADAM10/ADAM17 substrates distinct from MUC1, some of which may be involved in MUC1/ICAM-1 mediated cell migration. Therefore, we cannot exclude the possibility that TAPI-0 treatment inhibits cell migration in several, non-mutually exclusive ways, with inhibition of MUC1-ECD cleavage being one possible target. For example, it has been reported that ICAM-1 is a substrate of ADAM17 [418]. Therefore it is possible that the different effects of TAPI-0 treatment on ICAM-1 binding induced CaOs and cell migration is due to inhibition of ICAM-1 cleavage in the migration assay. To investigate this possibility, ADAM17 activity would have to be specifically inhibited in MUC1 and ICAM-1 cells, using knockouts or siRNA, to determine the role of ADAM17 cleavage of MUC1 and ICAM-1 in transmission of the MUC1/ICAM-1 signal. Also, investigation of the effect of TAPI-0 treatment on the described MUC1/ICAM-1 binding induced CrkL/Rac/Cdc42 pathway in MUC1 expressing cells would shed light in the requirement of MUC1 cleavage on induction of this signalling pathway. In addition to ICAM-1, TAPI-0 would

also be expected to inhibit cleavage of several other cell membrane proteins with known roles in migration. For example, L-1 cell adhesion molecule (L1-CAM) has been demonstrated as a substrate for ADAM10 in carcinoma cells [419]. Following cleavage, the membrane-bound portion is a substrate for γ-secretase, resulting in nuclear translocation and gene transcription [419]. Also, the soluble portion released following cleavage has been shown to promote migration of breast cancer cells [420]. Therefore the inhibition of L1-CAM cleavage by TAPI-0 in our migration assays could be at least partially responsible for our observations. In addition to L1-CAM, other ADAM10/ADAM17 substrates with demonstrated roles in progression of carcinomas include ErbB2, activated leukocyte cell adhesion molecule (ALCAM), IL6-R, epigen, E-cadherin, and C4.4A [227, 397, 421-427]. Therefore it is likely that inhibition of cleavage of proteins distinct from MUC1 are contributing to our observations. Although this complicates interpretation of our data, it also demonstrates that targeting ADAM10/ADAM17 activity would likely result in inhibition of several pathways involved in cancer progression, and is therefore appealing in a clinical context. It is clear that more research is needed to fully appreciate the role S2 cleavage of MUC1 in MUC1/ICAM-1 binding induced signalling, although in our interpretation of our results we will assume that TAPI-0 inhibition of MUC1-ECD cleavage is at least partially responsible for our observations.

In light of our observations that ICAM-1 binding induced motility requires both MUC1-CD dimerization (Chapter 4) and MUC1-ECD cleavage (Chapter 6), we sought to determine if MUC1-ECD cleavage is dependent on MUC1-CD dimerization. Using MUC1-CFP-Fv constructs, we inhibited MUC1-CD dimerization using AP21998^{M} and assayed for MUC1-ECD cleavage. We found no significant change in MUC1-ECD shedding following inhibition of MUC1-CD dimerization, indicating that the MUC1

protease is not specific to MUC1 dimers. Therefore, we propose that cleavage of MUC1-ECD occurs both constitutively and following ICAM-1 ligation for both MUC1 monomers and dimers. However, the consequence of each of these cleavage events on cellular behaviour is not known. The fates these MUC1 cleavage products may be distinct, and the role of each of these cleavage events in cellular transcription, migration, and survival may be of clinical importance.

Cytosolic CaOs have been shown to be important in cell migration, although the mechanism of calcium-induced cell motility is not fully understood [121]. As MUC1 cleavage is required for ICAM-1 binding induced migration, which occurs after CaOs, it is possible that the CaOs induced by ICAM-1 binding acts to stimulate cleavage of MUC1, triggering a signalling pathway leading to migration. This hypothesis would account for the observation that cleavage inhibition prevents migration and not CaOs in ICAM-1 stimulated cells. The constitutive MUC1 protease, ADAM17, has been shown to be activated by calcium influx [428]. However, several studies show that in response to calcium influx, the major metalloprotease activated is ADAM10 [429-431]. As we observed inhibition of cleavage of the ADAM10 substrate E-cadherin in response to TAPI-0, and TAPI-0 inhibits ICAM-1 binding induced cell migration, it is possible that the MUC1 protease involved in ICAM-1 induced cell migration is ADAM10. Migration of leukocytes has been shown to be dependent on cleavage of type 1 transmembrane proteins by calcium influx induced activation of ADAM10 [432]. However, it has also been shown that ADAM17 activity can be stimulated by activation of Src kinase [433]. This possibility is not only appealing in light of the observations that MUC1/ICAM-1 binding triggers Src-mediated pathways (Chapter 3) [13, 14], but also in light of the observation that Src activity is increased in many human cancers [127, 128, 325]. It is possible that Src activation through pathways specific to, or upregulated in, cancer cells

results in increased ADAM17 activity. This could result in cleavage of MUC1 as well as other ADAM17 substrates such as TNF-α and TGF-α, with potential consequences for both the substrate cell and surrounding stromal tissue through paracrine interactions. We hypothesize that both ICAM-1 induced Src activation and CaOs may play a role in stimulation of ADAM10/ADAM17 activity, resulting in MUC1-ECD cleavage and cell migration, although other downstream effects are a likely possibility. Full investigation of these possibilities would require specific inhibition of ADAM10 and ADAM17 activity, which could be achieved through the use of siRNA.

As recruitment of CrkL and Rac/Cdc42 activation occur within 10 seconds and 4 minutes, respectively, of ICAM-1 ligation, we expect that recruitment of CrkL occurs upon initial ICAM-1 binding and Src activation. Downstream activation of Rac/Cdc42 would lead to initiation of membrane ruffling and lamellipodial/filopodial protrusions. However, if CrkL recruitment and Rac/Cdc42 activation occur following initial ICAM-1 binding, the question remains as to the purpose of MUC1 cleavage in ICAM-1 induced cell migration. It is possible that MUC1-ECD S2 cleavage could result in additional cleavage events and/or nuclear localization of MUC1-CD in a manner similar to NOTCH (Section 1.2.2.4, Fig 1.12). As MUC1-CD is a reported substrate for γ-secretase and has been localized to the nucleus, these possibilities are likely [19, 178, 212]. As the MUC1 induced gene signature includes proteins involved in motility [434], S2 cleavage induced nuclear localization and transcriptional activity of MUC1 could contribute to cell migration. Also, cleavage of MUC1-ECD would release the MUC1 expressing cell from its "tether" to the ICAM-1 expressing cell, which would be required for effective cell migration. This can be seen as a parallel to cleavage of focal contact proteins by calpain, another Ca^{2+} activated protease, also a critical component of cell motility (Section 1.1.3.2.). Cleavage of ICAM-1 bound MUC1-ECD by ADAM17 or ADAM10 would

contribute to detachment of migrating breast cancer cells from endothelial cells, facilitating extravasation. Without cleavage of MUC1-ECD, the interaction between ICAM-1 and MUC1-ECD could prevent MUC1 expressing cells from invading into stromal tissues. Also, as we demonstrated that dimerization is not required for S2 cleavage (Fig 6.2), ICAM-1 bound to MUC1 monomers would be susceptible to cleavage as well, which would be required for motility. Finally, the observation that increased levels of MUC1-ECD in the serum of breast cancer patients is correlated with a poor prognosis adds further weight to the notion that MUC1 cleavage is involved in cancer metastasis *in vivo* [27, 28]. Collectively, the literature and data presented in this thesis suggest a functional role for MUC1-ECD cleavage in breast cancer progression and that targeting MUC1-ECD cleavage may represent a target for anti-metastatic therapy.

In summary, we conclude that MUC1-ECD cleavage is required for ICAM-1 binding induced cell motility. However, MUC1-ECD cleavage is not required for dimerization, Src recruitment, or ICAM-1 binding induced CaOs. We hypothesize that ICAM-1 binding leads to Src phosphorylation of MUC1, recruitment of downstream signalling mediators leading to CaOs and cytoskeletal rearrangement. Cytosolic CaOs induced by ICAM-1 binding activate ADAM 17 and/or ADAM10, resulting in MUC1-ECD cleavage. MUC1 cleavage then initiates MUC1 nuclear localization, MUC1 induced transcription, and detachment of MUC1 expressing cells from ICAM-1 expressing cells, collectively resulting in cell migration.

In our final Chapter, we investigated the role of MUC1 autoproteolytic cleavage on MUC1-ECD cleavage and ICAM-1 binding induced events. Two cleavage site mutants were employed to test our hypothesis. We found that S1 cleavage is not required for S2 cleavage or ICAM-1 induced events, as demonstrated by the MUC1-CFP (G4) mutant. However, the nature of the mutation conferring S1 cleavage resistance was an

important determinant in the functional status of the MUC1 protein, as we observed that the MUC1 (VP) mutant was not fully functional in ICAM-1 binding induced events.

One approach we took in inhibition of MUC1 autoproteolytic cleavage was to directly mutate the residues involved in S1 cleavage. This method was previously described [26], with the authors reporting that impaired S1 cleavage led to a reduction in MUC1-ECD S2 cleavage from the cell surface, which we confirmed in our study (Fig 6.2). The reported constitutive MUC1-ECD protease, ADAM17, cleaves at a number of reported seqeuences and is sensitive to conformational changes in its protein substrates [435-437]. The mutation implemented (G/SVVV to VPVVV) inhibits MUC1 autoproteolytic cleavage by replacing the functional groups involved in the chemical reaction leading to cleavage, we can hypothesize that the conformational strain leading to cleavage in wildtype MUC1 is not relieved in this mutant. Therefore, cleavage by ADAM17 at a site C-terminal to this mutant may be impaired due to global change in protein structure. Furthermore, we observed a significant reduction in ICAM-1 binding induced CaOs and cell migration in this mutant compared to wildtype MUC1, indicating that ICAM-1 binding induced signalling is also impaired. It is possible that ICAM-1 binding itself is disrupted in these cells, although the positive binding of anti-MUC1-ECD antibodies to MUC1 (VP) (Fig 6.2) suggests that a native conformation is adopted in the VNTR domain. However, the disruption in S2 cleavage suggests a disruption in protein conformation at other regions of the protein. Abnormal conformation of the MUC1 (VP) mutant could result in impaired protein dimerization, membrane localization, binding to signalling mediators, or cleavage by γ-secretase. Although investigation of these possibilities is interesting, the cytoplasmic domain banding pattern of MUC1 (VP) makes interpretation of these experiments problematic. Determination of MUC1 (VP) dimerization is difficult, as appearance of any new bands after DSS

treatment would be obscured by existing bands. Also, immunoprecipitation experiments, to determine interaction with signalling mediators such as Src, cannot be normalized between MUC1 (VP) and wildtype MUC1, again due to abnormal banding patterns. However, careful analysis of experiments investigating ICAM-1 binding induced signalling does reveal some information on the mechanism of MUC1 (VP) impairment. MUC1 (VP) elicits significantly greater ICAM-1 binding induced CaOs and cell migration when compared to Mock treatment condition (Fig 7.3, Fig 7.4), indicating a response to ICAM-1 binding. However, this response is significantly reduced compared to wildtype MUC1. Therefore we hypothesize that MUC1 (VP) does bind to ICAM-1, and the signal is transmitted into the cytoplasm resulting in cell signalling leading to CaOs and cell migration. However, the reduction in CaOs and migration compared to wildtype MUC1 suggests that critical component(s) of the signalling pathway(s) is/are dysfunctional.

The second approach we took to investigating the importance of MUC1 autoproteolytic cleavage in MUC1-ECD shedding and ICAM-1 binding induced events was relief of the conformational strain which results in autoproteolytic cleavage by insertion of four glycine residues immediately N-terminal to the cleavage site. This mutant was also previous described [200], with authors reporting that it did not undergo autoproteolytic cleavage. However, *in vivo* studies were not performed. Here, we report that this mutant, MUC1 (G4), does not undergo autoproteolytic cleavage *in vivo*, yet it is subject to MUC1-ECD shedding (Fig 6.2). Based on our rationale that ADAM17 is conformationally sensitive, we can hypothesize that the conformational stress resulting in autoproteolytic cleavage in wildtype MUC1 is relived in this mutant, and it adopts a native conformation at the cell surface. The observations that MUC1-CFP (G4) elicits ICAM-1 binding induced CaOs and cell migration at levels equivalent to wildtype

supports this hypothesis. These observations also demonstrate that S1 cleavage is not required for ICAM-1 binding induced signalling, a novel finding. However, again we cannot appropriately assay dimerization or association with signalling mediators in this mutant as the cytoplasmic banding pattern is problematic.

In summary, we effectively disrupted MUC1 autoproteolytic cleavage by two distinct methods and found that the MUC1 (VP) mutant was impaired in both MUC1-ECD shedding and ICAM-1 binding induced signalling. The MUC1 (G4) mutant functioned normally both in MUC1-ECD shedding and ICAM-1 binding induced signalling. We propose that the different functionalities of these mutants can be explained by the effect of the mutation on the conformation of the protein. Unfortunately, the ability of these proteins to dimerize and recruit downstream signalling mediators such as Src could not be properly determined due to the abnormal presentation of the mutants on SDS-PAGE. However, this study does reveal information on the role of MUC1 autoproteolytic cleavage in receptor function, which could possibly be applied to other proteins which undergo S1 cleavage.

8.2. Comprehensive model of MUC1/ICAM-1 signalling

Based on the available body of literature and data presented in this thesis, we propose the following working model of MUC1/ICAM-1 binding induced signalling (Fig 8.1). In resting cells, MUC1 exists as both full-length monomeric and dimeric species. Src is recruited, via MUC1-CD SH3 binding domain, to both MUC1-CD monomers and dimers, acting to partially unfold and activate Src. However, only in MUC1 dimers is Src able to access Y^{46} of MUC1-CD, phosphorylate it, and bind to this SH2 binding domain. This binding results in stable association between Src and MUC1 dimers, while the association between MUC1-CD monomers and Src is transient.

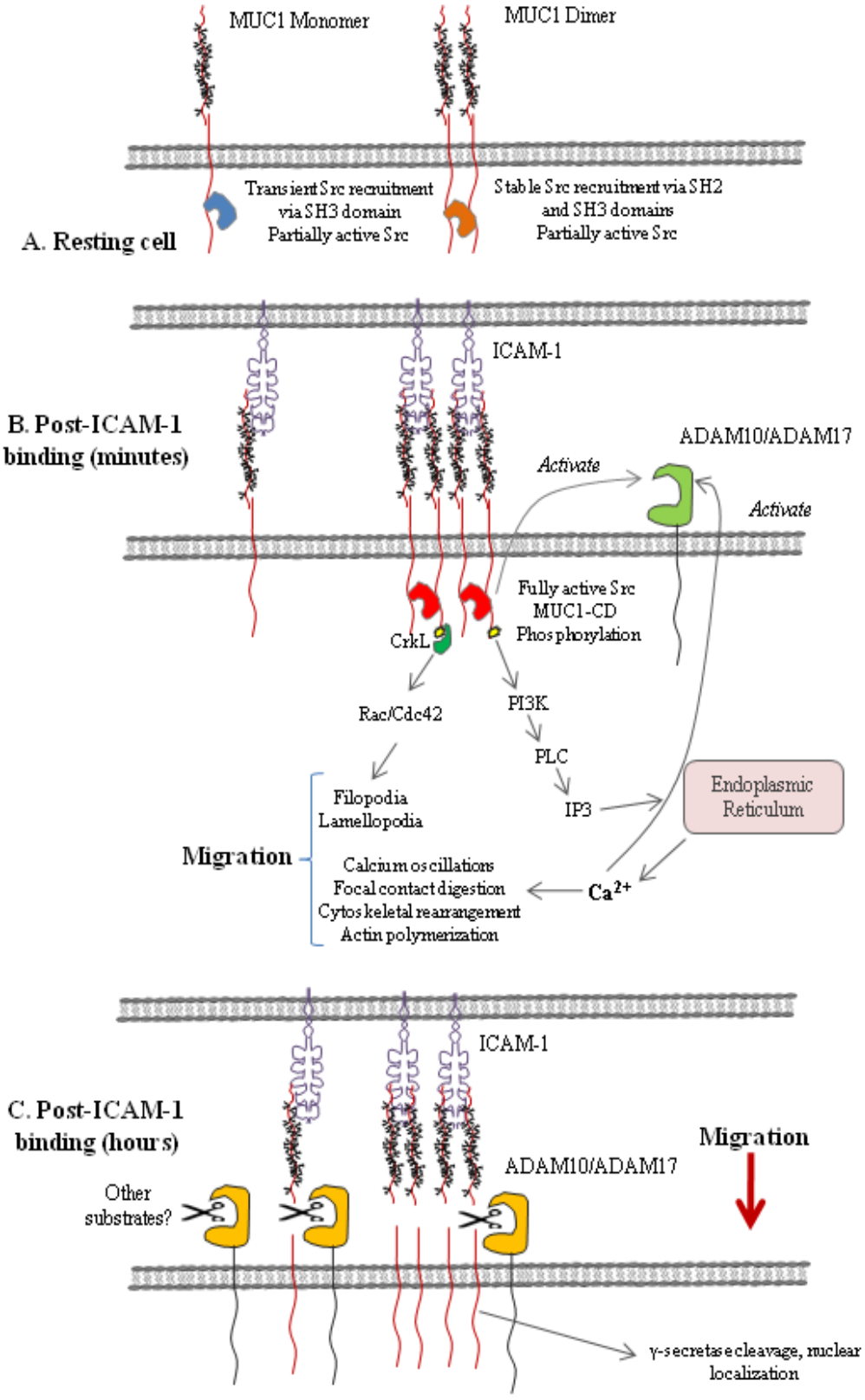


Figure 8.1. Schematic of proposed MUC1/ICAM-1 binding induced signaling.

Following binding of ICAM-1 to MUC1 monomers and dimers, the presence of activated Src bound to MUC1 dimers allows for initiation of signalling, while MUC1 monomers are not competent to elicit signalling cascades. We propose that ICAM-1 binding induced clustering of MUC1/Src complexes may faciliatate *trans*-autophosphorylation of Src species at Y^{416}, resulting in full activation. Src is then able to phosphorylate MUC1-CD tyrosine residues, such as Y^{20} and Y^{60}, which recruit and activate downstream signalling mediators such as PI3K and CrkL, respectively. This point in MUC1/ICAM-1 signalling represents the divergence of signalling pathways leading to CaOs and cytoskeletal rearrangement, which culminate with cell migration. The CrkL mediated pathway continues with activation of Rac/Cdc42, leading to lamellipodial and filipodial protrusions, a key step in cell migration. Activation of PI3K leads to a PLC mediated pathway, leading to generation of IP3 and release of calcium from the endoplasmic reticulum, resulting in the observed CaOs signal. This calcium signal acts to activate proteins involved in focal contact digestion, cytoskeletal rearrangement, and actin polymerization, key steps in cell migration. This calcium signal, as well as activated Src, can also activate ADAM10 and/or ADAM17. As the MUC1-expressing cell begins to move in response to the signalling cascades initiated by ICAM-1 ligation, ADAM10 and/or ADAM17 act to cleave MUC1-ECD, releasing the MUC1/ICAM-1 interaction and allowing the cell to move forward. Also, activated ADAM10 and/or ADAM17 would likely cleave other cell membrane substrates, which would further contribute to the metastatic phenotype and facilitate cell movement.

In summary, we propose that ICAM-1 ligation to MUC1 dimers acts to initate signalling cascades which result in cell movement. Interaction between circulating breast cancer cells, which often overexpress MUC1, and endothelial cells, which express ICAM-1, would therefore result in movement of breast cancer cells through the

endothelial cell layer and into the stromal tissue, where a metastatic tumor could form. Inhibition of critical steps in this pathway, such as MUC1 dimerization, Src recruitment, or cleavage, could therefore be targeted clinically to reduce metastasis, and therefore mortality, in breast cancer patients.

8.3. Clinical implications

It is well established that MUC1 is overexpressed in several different types of cancer (Section 1.2.2.6). In this study, we utilized both MUC1 transfectants and human breast cancer cell lines which overexpress MUC1, T47D and MCF-7. T47D and MCF-7 are Luminal B subtype breast cancer, which are characterized by ER and/or PR positivity, a higher proliferation rate, and a slow, steady recurrence trend with a poor prognosis [53]. Treatment of ER positive breast tumors involves administration of Tamoxifen, an ER antagonist, although resistance to Tamoxifen occurs frequently [438]. Interestingly, treatment of breast cancer cells with Tamoxifen is correlated with a decrease in MUC1 expression, which may be explained by the presence of an estrogen response element in the promoter of *MUC1* which would be susceptible to Tamoxifen treatment [403, 404]. However, MUC1 expression and the MUC1 induced gene signature are upregulated in ER positive, Tamoxifen resistant breast cancers [434]. Therefore we can hypothesize that the upregulation of MUC1 may be involved in the acquisition of Tamoxifen resistance in Luminal B breast cancers. As we propose here that MUC1 dimerization and cleavage may play a role in nuclear localization and gene transcription of MUC1, it is possible that ICAM-1 binding induced signalling results in MUC1 nuclear localization and upregulation of the *MUC1* gene (as MUC1 can be auto-inductive [439]), contributing to Tamoxifen resistance. Therefore future investigations should examine the relationship between ICAM-1 binding to MUC1 and MUC1-induced gene expression potentially

leading to Tamoxifen resistance. It is possible that combinations of drugs targeting MUC1 cleavage and/or dimerization could be combined with Tamoxifen to increase its efficacy and decrease resistance, which would lead to decreased patient mortality.

Extensive studies have been performed investigating dimerization of members of the Erb family of proteins. Inhibition of Erb receptor homo/heterodimerization with antibodies, peptides, and small molecule inhibitors in the treatment of breast cancer have been described [440-442]. However, acquired resistance to these therapies, specifically to Trastuzumab in ErbB2 overexpressing cancers, has been observed [443]. It has been proposed that Trastuzumab acts by inhibition of both ErbB2 cleavage and dimerization [377, 444, 445]. Our work investigating MUC1 dimerization has revealed that non-cysteine mediated MUC1-CD dimerization is required for Src recruitment and ICAM-1 binding induced cell signalling, resulting in cell motility. We also report that MUC1 ECD-shedding, by ADAM10 and/or ADAM17, is involved in ICAM-1 binding induced cell migration. Interestingly, antagonists of a cleaved fragment of MUC1, possibly the product of ECD-shedding, have been shown to work in combination with Trastuzumab to overcome Trastuzumab resistance in breast cancer cells. As ADAM10 has been identified as an ErbB2 activating protease [426], it is possible that in cells expressing both MUC1 and ErbB2, ICAM-1 binding induced CaOs could result in activation of ADAM10 and cleavage of both substrates. Activation of both MUC1 and ErbB2 signalling cascades could contribute to tumor metastasis, growth and survival. The possibility that these receptors work synergistically in breast cancer progression provides an intriguing target for future studies and potential therapies. Specifically, dual inhibition of MUC1 and ErbB2 signalling could be achieved through inhibition of ADAM17 and/or ADAM10. Synergistic inhibition of cancer progression could also be achieved through combination of a metalloprotease inhibitor with specific inhibitors of MUC1 and/or ErbB2 signalling.

A study combining an ADAM17/ADAM10 inhibitor with an inhibitor or ErbB2 kinase activity resulted in synergistic inhibition of breast cancer cells in a mouse model [446]. Compounds inhibiting MUC1-CD dimerization, such as those described in [21] and [390], could be used in combination with these therapies to specifically inhibit both MUC1 and ErbB2 function in breast cancer, potentially leading to decreased patient mortality.

The data presented in this thesis also reveals information on the relationship between MUC1 and Src in breast cancer. Src overactivity is implicated in breast cancer progression [17], and we demonstrate here that ICAM-1/MUC1 signaling requires Src activity. Therefore, increased Src activity in MUC1 expressing breast cancers could result in potentiation of the ICAM-1 binding induced signal, facilitating metastasis. As we found that MUC1 dimerization is required for effective recruitment of Src and ICAM-1 binding induced signalling, we would hypothesize that inhibition of MUC1 dimerization would prevent Src binding and downstream signalling leading to cell migration. The rational combination of drugs inhibiting several components of a single pathway or parallel pathways is of clinical interest [18], as it is believed this could lead to both greater efficacy and specificity. Therefore, a combination of drugs targeting MUC1 dimerization, Src recruitment to MUC1, Src activity and/or MUC1 cleavage could potentially specifically inhibit ICAM-1 binding induced signalling in metastatic breast cancer cells and should be the focus of basic and clinical research in the future.

In conclusion, analysis of data presented here in the context of current clinical strategies employed in breast cancer treatment reveals potential for development of combinational therapy regimes involving MUC1 dimerization and/or cleavage. However, additional research on the mechanism of ICAM-1/MUC1 signalling and the relationship between MUC1 and other pathways involved in breast cancer progression is needed.

8.4. Limitations of study

We recognize several limitations of the experiments presented in this thesis and here we discuss the implications of these limitations on our interpretation of the data. In Chapter 3, we successfully utilized siRNA to knockdown Src to determine the necessity for Src in the ICAM-1/MUC1 signalling pathway. We proposed that this method was more specific than using a broad-spectrum kinase inhibitor such as PP2 which inhibits several SFKs as well as EGFR. However, we cannot exclude the possibility that other SFKs in HEK 293T cells were silenced as well due to non-specificity of our anti-Src antibody. The siRNA used in Src knockdown was modified to reduce off-target inhibition and also run through a BLAST search to determine levels of non-specific binding prior to use [405], and candidate siRNA sequences indicating homology to off-target genes were eliminated. Therefore, although we did not empirically demonstrate that the siRNA used in this study only targeted c-Src, we have confidence in the methods utilized by our supplier. Additional investigations would be needed to identify c-Src as the only SFK involved in ICAM-1/MUC1 signalling, although the data presented here indicate that c-Src is likely the primary mediator.

Our investigations on MUC1 dimerization reveal that MUC1-CD dimerization is required for Src recruitment and ICAM-1 binding induced events. However, we fail to identify the mechanism of MUC1-CD dimerization. We do eliminate several possibilities through the use of splice variants, mutants, and chimeric proteins. Dimerization of MUC1-Y indicates that the VNTR domain is not required for dimerization; interaction between MUC1-Y and the CD8/MUC1 chimera indicate that the ECD, TM and C^1QC motif are not required for dimerization; and dimerization and functionality of the MUC1-CFP-Fv (AQA) mutant indicates that the cysteine residues of the C^1QC motif are not required for dimerization and ICAM-1 binding induced signalling. Therefore we propose

that interactions in the last 69aa of the MUC1-CD (beginning R^4RK) are responsible for dimerization. This hypothesis is problematic as there are no known dimerization motifs in this portion of MUC1, although this does not rule out the possibility of a novel dimerization domain. Our investigations on the requirement of Src kinase binding to MUC1-CD in MUC1-CD dimerization demonstrate that Src kinase is not responsible for facilitating MUC1-CD dimerization, however, it is possible that other MUC1-CD interacting protein fulfills this role. In addition, our work investigating the role of MUC1 dimerization *in vivo* employs an engineered Fv domain and Fv ligands. This system is artificial, and use of these constructs may not necessarily be representative of MUC1 signalling in a natural state. For example, addition of $AP21998^M$, in addition to disrupting MUC1 dimerization, could interfere with MUC1 binding to signalling partners directly by changing the conformation of MUC1-CD. Also, the long term effects of Fv ligands on dimerization of MUC1-CFP-Fv were not investigated. As cell migration assays were performed over a 24-hour period, the state of MUC1 dimerization at this time point is relevant. However, we were not able to obtain consistent results when investigating this. If animal studies were to be performed in the future using Fv domain containing MUC1, investigations on the long term effects of Fv ligands on MUC1 dimerization would need to be established. These possibilities and proposed future experiments are of clinical interest as inhibition of MUC1-CD dimerization represents a potential target for anti-metastatic therapy, although additional research is required before this possibility can be fully investigated.

Our investigations of the role of MUC1-ECD shedding in MUC1 function rely on the use of TAPI-0, a broad spectrum metalloprotease inhibitor. This inhibitor has activity against ADAM17, ADAM10, and several other metalloproteases [24, 447, 448]. Therefore we cannot exclude the possibility that our use of TAPI-0 resulted in inhibition

of another, unidentified MUC1 sheddase. We propose that the action of TAPI-0 against ADAM10 may be an alternative explanation for our observations, as ADAM10 is known to be activated by calcium influx [429]. Targeting of ADAM17 and/or ADAM10 with siRNA would be a potential method to investigate the role of each protease specifically in MUC1/ICAM-1 function. However, as both ADAM10 and ADAM17 have been demonstrated to have roles in breast cancer progression, an inhibitor targeting function of both metalloproteases may have the greatest clinical potential [421, 426, 446], and therefore it may not be necessary to identify the protease responsible for MUC1 shedding in ICAM-1 induced migration.

In our ICAM-1 binding induced migration assays, TAPI-0 was present throughout the duration of the 24-hour experiment to ensure continued inhibition of MUC1 S2 cleavage, and therefore our inhibition of MUC1-ECD cleavage was not specific. As we propose that MUC1 S2 cleavage must occur to "release" the MUC1/ICAM-1 tether, it is also possible that cleavage of ICAM-1 occurs with the same result. ICAM-1 is a reported substrate of ADAM17 [418], and is reported to occur constitutively in NIH 3T3 cells [449], although the effect of TAPI-0 on ICAM-1 S2 cleavage has not been determined. In addition to ICAM-1, inhibition of cleavage of other ADAM10/ADAM17 substrates likely contributed to our observations. Further investigation of the effect of TAPI-0 on cleavage of ICAM-1 and other relevant ADAM10/ADAM17 substrates is required to determine the mechanism of TAPI-0 inhibition of MUC1/ICAM-1 binding induced migration. To fully appreciate the clinical applicability of these metalloprotease inhibitors in treatment of MUC1-overexpressing breast cancer cells, additional work is needed. This would include, but is not limited to, specific inhibition of MUC1 and ICAM-1 shedding, and investigation of potential cross-talk between the MUC1/ICAM-1 pathway and cleavage of other ADAM10/ADAM17

substrates. This work would shed light on the potential clinical benefits and side-effects of these inhibitors.

In this study we focused on transmission of the MUC1/ICAM-1 signal through activation of Src kinase. However, it is possible that distinct pathways are activated in response to MUC1/ICAM-1 binding which could confound our results. For example, MUC1 is known to bind EGFR, and EGFR can phosphorylate MUC1 on Y^{46}. As this is the residue that we considered a substrate and binding site for Src kinase, we cannot rule out the possibility that EGFR is involved in transmission of the MUC1/ICAM-1 signal through phosphorylation of Y^{46}, creating a binding and activation motif for Src. In addition, we have not investigated the role of β-catenin, a well-described MUC1 binding partner, in the MUC1/ICAM-1 signaling cascade. Phosphorylation of MUC1 at Y^{46} has been shown to result in increased binding between MUC1 and β-catenin, and therefore activation of Src upon ICAM-1 ligation would be expected to facilitate β-catenin/MUC1 binding and β-catenin signalling. Full understanding of the mechanism of MUC1/ICAM-1 signalling would require investigation of these potentially interacting components.

In summary, although several major limitations of this study have been identified, we feel that our work has contributed significantly to the study of MUC1 in breast cancer progression and has provided many exciting new hypotheses for future studies.

8.5. Overall conclusions and future directions

Although previous publications from our laboratory have identified signalling mediators involved in transmission of the ICAM-1/MUC1 signal, events occurring at the level of MUC1 have not been identified prior to this work [13, 14]. Elucidation of the mechanism of ICAM-1 induced signalling provides targets for MUC1-specific therapies

which have clinical potential in the treatment of breast cancer progression. We show here that MUC1 dimerization is required for constitutive Src recruitment and ICAM-1 binding induced CaOs and cell invasion, a critical step in breast cancer metastasis. Investigation of the mechanism of MUC1 dimerization reveals that, contrary to other reports investigating MUC1dimerization [19], MUC1 does not exist as a covalently linked dimer, and is independent of cytoplasmic cysteine residues. Additional research is needed to positively identifify the entity or domain responsible for MUC1 dimerization. This information will contribute to the potential development of MUC1 dimerization inhibitors which have significant clinical potential, alone and in combination with drugs targeting other aspects of MUC1/ICAM-1 signalling or other proteins contributing to breast cancer progression.

We also investigated the role of MUC1-ECD shedding, a previously described event, in ICAM-1 binding induced signalling. Our results demonstrate that although MUC1-ECD shedding is not required for dimerization, Src recruitment, or ICAM-1 binding induced CaOs, it is necessary for ICAM-1 binding induced cell migration. This data provides an additional MUC1 target in breast cancer therapies, as inhibition of MUC1-ECD cleavage, by direct inhibition of the cleavage site or by inhibition of enzymes responsible for MUC1-ECD shedding, would be expected to result in decreased migration. We have also demonstrated that S1 cleavage of MUC1 is not required for S2 cleavage or ICAM-1 binding induced events, suggesting that S1 cleavage may not have a physiological purpose. In summary, we propose that rational combination of inhibitors of MUC1 dimerization, Src recruitment to MUC1, and MUC1 cleavage could synergistically inhibit metastasis of MUC1 overexpressing breast cancers. However, further investigations, particularly on the mechanism of MUC1 dimerization and Src

recruitment, as well as the fate of MUC1 cleavage products, are required before this hypothesis can be properly tested clinically.

References

1. **Canadian Cancer Society's Steering Committee: Canadian Cancer Statistics 2010.** Toronto; 2010.
2. Patanaphan V, Salazar OM, Risco R: **Breast cancer: metastatic patterns and their prognosis.** *Southern Medical Journal* 1988, **81:**1109-1112.
3. Steeg PS: **Tumor metastasis: mechanistic insights and clinical challenges.** *Nature Medicine* 2006, **12:**895-904.
4. Mareel M, Leroy A: **Clinical, cellular, and molecular aspects of cancer invasion.** *Physiological Reviews* 2003, **83:**337-376.
5. Rahn JJ, Dabbagh L, Pasdar M, Hugh JC: **The importance of MUC1 cellular localization in patients with breast carcinoma: an immunohistologic study of 71 patients and review of the literature.** *Cancer* 2001, **91:**1973-1982.
6. Brockhausen I, Yang JM, Burchell J, Whitehouse C, Taylor-Papadimitriou J: **Mechanisms underlying aberrant glycosylation of MUC1 mucin in breast cancer cells.** *European Journal of Biochemistry / FEBS* 1995, **233:**607-617.
7. Croce MV, Isla-Larrain MT, Demichelis SO, Gori JR, Price MR, Segal-Eiras A: **Tissue and serum MUC1 mucin detection in breast cancer patients.** *Breast Cancer Research and Treatment* 2003, **81:**195-207.
8. Gendler SJ: **MUC1, the renaissance molecule.** *Journal of Mammary Gland Biology and Neoplasia* 2001, **6:**339-353.
9. Park HK, Seo UH: **MUC1 from the Mucin Family as Potential Tools in Breast Cancer Immunotherapy.** *Journal of Breast Cancer* 2009, **12:**125-133.
10. Mall AS: **Analysis of mucins: role in laboratory diagnosis.** *Journal of Clinical Pathology* 2008, **61:**1018-1024.
11. Vogetseder W, Feichtinger H, Schulz TF, Schwaeble W, Tabaczewski P, Mitterer M, Bock G, Marth C, Dapunt O, Mikuz G: **Expression of 7F7-antigen, a human adhesion molecule identical to intercellular adhesion molecule-1 (ICAM-1) in human carcinomas and their stromal fibroblasts.** *International Journal of Cancer* 1989, **43:**768-773.
12. Regimbald LH, Pilarski LM, Longenecker BM, Reddish MA, Zimmermann G, Hugh JC: **The breast mucin MUCI as a novel adhesion ligand for endothelial intercellular adhesion molecule 1 in breast cancer.** *Cancer Research* 1996, **56:**4244-4249.
13. Rahn JJ, Shen Q, Mah BK, Hugh JC: **MUC1 initiates a calcium signal after ligation by intercellular adhesion molecule-1.** *Journal of Biological Chemistry* 2004, **279:**29386-29390.
14. Shen Q, Rahn JJ, Shaw A, Hendzel M, Sun X, Hoffman P, Zhang J, Gunesekera N, Bernier A, Hugh J: **MUC1 initiates Src-CrkL-Rac1/Cdc42-mediated actin cytoskeletal protrusive motility after ligating ICAM-1.** *Molecular Cancer Research* 2008.
15. Rahn JJ, Chow JW, Horne GJ, Mah BK, Emerman JT, Hoffman P, Hugh JC: **MUC1 mediates transendothelial migration in vitro by ligating endothelial cell ICAM-1.** *Clinical & Experimental Metastasis* 2005, **22:**475-483.
16. Hiscox S, Morgan L, Green T, Nicholson RI: **Src as a therapeutic target in anti-hormone/anti-growth factor-resistant breast cancer.** *Endocrine-related Cancer* 2006, **13 Suppl 1:**S53-59.
17. Aleshin A, Finn RS: **SRC: A Century of Science Brought to the Clinic.** *Neoplasia* 2010, **12:**599-607.
18. Dent P, Curiel DT, Fisher PB, Grant S: **Synergistic combinations of signaling pathway inhibitors: Mechanisms for improved cancer therapy.** *Drug Resistance Updates* 2009, **12:**65-73.

19. Leng YM, Cao C, Ren J, Huang L, Chen DS, Ito M, Kufe D: **Nuclear import of the MUC1-C oncoprotein is mediated by nucleoporin Nup62.** *Journal of Biological Chemistry* 2007, **282:**19321-19330.
20. Joshi MD, Ahmad R, Yin L, Raina D, Rajabi H, Bubley G, Kharbanda S, Kufe D: **MUC1 oncoprotein is a druggable target in human prostate cancer cells.** *Molecular Cancer Therapeutics* 2009, **8:**3056-3065.
21. Raina D, Ahmad R, Joshi MD, Yin L, Wu ZK, Kawano T, Vasir B, Avigan D, Kharbanda S, Kufe D: **Direct Targeting of the Mucin 1 Oncoprotein Blocks Survival and Tumorigenicity of Human Breast Carcinoma Cells.** *Cancer Research* 2009, **69:**5133-5141.
22. Yin L, Ahmad R, Kosugi M, Kufe T, Vasir B, Avigan D, Kharbanda S, Kufe D: **Survival of human multiple myeloma cells is dependent on MUC1 C-terminal transmembrane subunit oncoprotein function.** *Molecular Pharmacology* 2010, **78:**166-174.
23. Parry S, Silverman HS, McDermott K, Willis A, Hollingsworth MA, Harris A: **Identification of MUC1 proteolytic cleavage sites in vivo.** *Biochemical & Biophysical Research Communications* 2001, **283:**715-720.
24. Thathiah A, Blobel CP, Carson DD: **Tumor necrosis factor-alpha converting enzyme/ADAM 17 mediates MUC1 shedding.** *Journal of Biological Chemistry* 2003, **278:**3386-3394.
25. Thathiah A, Carson DD: **MT1-MMP mediates MUC1 shedding independent of TACE/ADAM17.** *Biochemical Journal* 2004, **382:**363-373.
26. Lillehoj EP, Han F, Kim KC: **Mutagenesis of a Gly-Ser cleavage site in MUC1 inhibits ectodomain shedding.** *Biochemical and Biophysical Research Communications* 2003, **307:**743-749.
27. Norum LF, Erikstein B, Nustad K: **Elevated CA125 in breast cancer--A sign of advanced disease.** *Tumour biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine* 2001, **22:**223-228.
28. Al-Azawi D, Kelly G, Myers E, McDermott EW, Hill ADK, Duffy MJ, O Higgins N: **CA 15-3 is predictive of response and disease recurrence following treatment in locally advanced breast cancer.** *Bmc Cancer* 2006, **6**.
29. O'Malley FP, Pinder SE: *Breast Pathology.* Philidelphia, PA; 2006.
30. Hovey RC, Trott JF, Vonderhaar BK: **Establishing a framework for the functional mammary gland: From endocrinology to morphology.** *Journal of Mammary Gland Biology and Neoplasia* 2002, **7:**17-38.
31. Hennighausen L, Robinson GW: **Signaling pathways in mammary gland development.** *Developmental Cell* 2001, **1:**467-475.
32. Li P, Barraclough R, Fernig DG, Smith JA, Rudland PS: **Stem cells in breast epithelia.** *International Journal of Experimental Pathology* 1998, **79:**193-206.
33. Neville MC, McFadden TB, Forsyth I: **Hormonal regulation of mammary differentiation and milk secretion.** *Journal of Mammary Gland Biology and Neoplasia* 2002, **7:**49-66.
34. Leblond CP, Inoue S: **Structure, composition, and assembly of the basement membrane.** *American Journal of Anatomy* 1989, **185:**367-390.
35. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB: **Myofibroblasts. I. Paracrine cells important in health and disease.** *American Journal of Physiology-Cell Physiology* 1999, **277:**C1-C19.
36. Cummings MC, Chambers R, Simpson PT, Lakhani SR: **Molecular classification of breast cancer: is it time to pack up our microscopes?** *Pathology* 2011, **43:**1-8.

37. Kruger S, Fahrenkrog T, Muller H: **Proliferative and apoptotic activity in lobular breast carcinoma.** *International Journal of Molecular Medicine* 1999, **4:**171-174.
38. Korkola JE, DeVries S, Fridlyand J, Hwang ES, Estep ALH, Chen YY, Chew KL, Dairkee SH, Jensen RM, Waldman FM: **Differentiation of lobular versus ductal breast carcinomas by expression microarray analysis.** *Cancer Research* 2003, **63:**7167-7175.
39. Thomas JS, Kerr GR, Jack WJL, Campbell F, McKay L, Pedersen HC, Kunkler IH, Cameron DA, Chetty U, Bartlett JMS: **Histological grading of invasive breast carcinoma - a simplification of existing methods in a large conservation series with long-term follow-up.** *Histopathology* 2009, **55:**724-731.
40. Edge S, Byrd D, Compton C, Fritz A, Greene F, Trotti A: *American Joint Cancer Committee Cancer staging manual.* 6th edn. New York: Springer; 2010.
41. Mohsin SK, Weiss H, Havighurst T, Clark GM, Berardo M, Roarth L, To TV, Zho Q, Love RR, Allred DC: **Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study.** *Modern Pathology* 2004, **17:**1545-1554.
42. Nguyen PL, Taghian AG, Katz MS, Niemierko A, Raad RFA, Boon WL, Bellon JR, Wong JS, Smith BL, Harris JR: **Breast cancer subtype approximated by estrogen receptor, progesterone receptor, and HER-2 is associated with local and distant recurrence after breast-conserving therapy.** *Journal of Clinical Oncology* 2008, **26:**2373-2378.
43. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: **Human breast cancer - correlation of relapse and survival with amplification of the HER-2/neu oncogene.** *Science* 1987, **235:**177-182.
44. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, et al: **Molecular portraits of human breast tumours.** *Nature* 2000, **406:**747-752.
45. Hennessy BT, Gonzalez-Angulo AM, Stemke-Hale K, Gilcrease MZ, Krishnamurthy S, Lee JS, Fridlyand J, Sahin A, Agarwal R, Joy C, et al: **Characterization of a Naturally Occurring Breast Cancer Subset Enriched in Epithelial-to-Mesenchymal Transition and Stem Cell Characteristics.** *Cancer Research* 2009, **69:**4116-4124.
46. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, He XP, Perou CM: **Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer.** *Breast Cancer Research* 2010, **12**.
47. Millikan RC, Newman B, Tse CK, Moorman PG, Conway K, Smith LV, Labbok MH, Geradts J, Bensen JT, Jackson S, et al: **Epidemiology of basal-like breast cancer.** *Breast Cancer Research and Treatment* 2008, **109:**123-139.
48. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, Karaca G, Troester MA, Tse CK, Edmiston S, et al: **Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study.** *Jama-Journal of the American Medical Association* 2006, **295:**2492-2502.
49. Kennecke H, Yerushalmi R, Woods R, Cheang MCU, Voduc D, Speers CH, Nielsen TO, Gelmon K: **Metastatic Behavior of Breast Cancer Subtypes.** *Journal of Clinical Oncology* 2010, **28:**3271-3277.
50. Huober J, von Minckwitz G, Denkert C, Tesch H, Weiss E, Zahm DM, Belau A, Khandan F, Hauschild M, Thomssen C, et al: **Effect of neoadjuvant anthracycline-taxane-based chemotherapy in different biological breast**

cancer phenotypes: overall results from the GeparTrio study. *Breast Cancer Research and Treatment* 2010, **124:**133-140.

51. Symmans WF, Hatzis C, Sotiriou C, Andre F, Peintinger F, Regitnig P, Daxenbichler G, Desmedt C, Domont J, Marth C, et al: **Genomic Index of Sensitivity to Endocrine Therapy for Breast Cancer.** *Journal of Clinical Oncology* 2010, **28:**4111-4119.
52. Chang HR, Glaspy J, Allison MA, Kass FC, Elashoff R, Chung DU, Gornbein J: **Differential Response of Triple-Negative Breast Cancer to a Docetaxel and Carboplatin-Based Neoadjuvant Treatment.** *Cancer* 2010, **116:**4227-4237.
53. Hugh J, Hanson J, Cheang MCU, Nielsen TO, Perou CM, Dumontet C, Reed J, Krajewska M, Treilleux I, Rupin M, et al: **Breast Cancer Subtypes and Response to Docetaxel in Node-Positive Breast Cancer: Use of an Immunohistochemical Definition in the BCIRG 001 Trial.** *Journal of Clinical Oncology* 2009, **27:**1168-1176.
54. Crowley L: *An introduction to human disease : Pathology and pathophysiology correlations.* 6th edn: Jones and Bartlett; 2004.
55. Tse GM, Tan PH, Pang ALM, Tang APY, Cheung HS: **Calcification in breast lesions: pathologists' perspective.** *Journal of Clinical Pathology* 2008, **61:**145-151.
56. Kalluri R: **EMT: When epithelial cells decide to become mesenchymal-like cells.** *Journal of Clinical Investigation* 2009, **119:**1417-1419.
57. Kiemer AK, Takeuchi K, Quinlan MP: **Identification of genes involved in epithelial-mesenchymal transition and tumor progression.** *Oncogene* 2001, **20:**6679-6688.
58. Moreno-Bueno G, Portillo F, Cano A: **Transcriptional regulation of cell polarity in EMT and cancer.** *Oncogene* 2008, **27:**6958-6969.
59. Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, Palacios J: **Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype.** *Cancer Research* 2008, **68:**989-997.
60. Gilles C, Polette M, Seiki M, Birembaut P, Thompson EW: **Implication of collagen type 1-induced membrane-type 1 matrix metalloproteinase expression and matrix metalloproteinase-2 activation in the metastatic progression of breast carcinoma.** *Laboratory Investigation* 1997, **76:**651-660.
61. Gilles C, Polette M, Coraux C, Tournier JM, Meneguzzi G, Munaut C, Volders L, Rousselle P, Birembaut P, Foidart JM: **Contribution of MT1-MMP and of human laminin-5 gamma 2 chain degradation to mammary epithelial cell migration.** *Journal of Cell Science* 2001, **114:**2967-2976.
62. Aokage K, Ishii G, Ohtaki Y, Yamaguchi Y, Hishida T, Yoshida J, Nishimura M, Nagai K, Ochiai A: **Dynamic molecular changes associated with epithelial-mesenchymal transition and subsequent mesenchymal-epithelial transition in the early phase of metastatic tumor formation.** *International Journal of Cancer* 2011, **128:**1585-1595.
63. Vrana JA, Stang MT, Grande JP, Getz MJ: **Expression of tissue factor in tumor stroma correlates with progression to invasive human breast cancer: Paracrine regulation by carcinoma cell-derived members of the transforming growth factor beta family.** *Cancer Research* 1996, **56:**5063-5070.
64. Nielsen BS, Rank F, Lopez JM, Balbin M, Vizoso F, Lund LR, Dano K, Lopez-Otin C: **Collagenase-3 expression in breast myofibroblasts as a molecular**

marker of transition of ductal carcinoma in situ lesions to invasive ductal carcinomas. *Cancer Research* 2001, **61:**7091-7100.

65. Bisson C, Blacher S, Polette M, Blanc JF, Kebers F, Desreux J, Tetu B, Rosenbaum J, Foidart JM, Birembaut P, Noel A: **Restricted expression of membrane type 1-matrix metalloproteinase by myofibroblasts adjacent to human breast cancer cells.** *International Journal of Cancer* 2003, **105:**7-13.
66. Guidi AJ, Schnitt SJ, Fischer L, Tognazzi K, Harris JR, Dvorak HF, Brown LF: **Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in patients with ductal carcinoma in situ of the breast.** *Cancer* 1997, **80:**1945-1953.
67. Polyak K, Kalluri R: **The Role of the Microenvironment in Mammary Gland Development and Cancer.** *Cold Spring Harbor Perspectives in Biology* 2010, **2:**14.
68. Elder EE, Kennedy CW, Gluch L, Carmalt HL, Janu NC, Joseph MG, Donellan MJ, Molland JG, Gillett DJ: **Patterns of breast cancer relapse.** *Ejso* 2006, **32:**922-927.
69. Klein A, Olendrowitz C, Schmutzler R, Hampl J, Schlag PM, Maass N, Arnold N, Wessel R, Ramser J, Meindl A, et al: **Identification of brain- and bone-specific breast cancer metastasis genes.** *Cancer Letters* 2009, **276:**212-220.
70. Koo JS, Jung W, Jeong J: **Metastatic breast cancer shows different immunohistochemical phenotype according to metastatic site.** *Tumori* 2010, **96:**424-432.
71. Mendoza M, Khanna C: **Revisiting the seed and soil in cancer metastasis.** *International Journal of Biochemistry & Cell Biology* 2009, **41:**1452-1462.
72. Muller A, Homey B, Soto H, Ge NF, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, et al: **Involvement of chemokine receptors in breast cancer metastasis.** *Nature* 2001, **410:**50-56.
73. Miles FL, Pruitt FL, van Golen KL, Cooper CR: **Stepping out of the flow: capillary extravasation in cancer metastasis.** *Clinical & Experimental Metastasis* 2008, **25:**305-324.
74. Wyble CW, Hynes KL, Kuchibhotla J, Marcus BC, Hallahan D, Gewertz BL: **TNF-alpha and IL-1 upregulate membrane-bound and soluble E-selectin through a common pathway.** *Journal of Surgical Research* 1997, **73:**107-112.
75. van Wely CA, Beverley PCL, Brett SJ, Britten CJ, Tite JP: **Expression of L-selectin on Th1 cells is regulated by IL-12.** *Journal of Immunology* 1999, **163:**1214-1221.
76. Galdiero M, DeLero GC, Marcatili A: **Cytokine and adhesion molecule expression in human monocytes and endothelial cells stimulated with bacterial heat shock proteins.** *Infection and Immunity* 1997, **65:**699-707.
77. Somers WS, Tang J, Shaw GD, Camphausen RT: **Insights into the molecular basis of leukocyte tethering and rolling revealed by structures of P- and E-selectin bound to SLe(X) and PSGL-1.** *Cell* 2000, **103:**467-479.
78. Kobayashi H, Boelte KC, Lin PC: **Endothelial cell adhesion molecules and cancer progression.** *Current Medicinal Chemistry* 2007, **14:**377-386.
79. Oppenheimermarks N, Davis LS, Bogue DT, Ramberg J, Lipsky PE: **Differential utilization of ICAM-1 and VCAM-1 during the adhesion and transendothelial migration of human lymphocytes.** *Journal of Immunology* 1991, **147:**2913-2921.
80. Wong D, Prameya R, Dorovini-Zis K: **In vitro adhesion and migration of T lymphocytes across monolayers of human brain microvessel endothelial**

cells: Regulation by ICAM-1,VCAM-1, E-selectin and PECAM-1. *Journal of Neuropathology and Experimental Neurology* 1999, **58:**138-152.

81. Bianchi E, Bender JR, Blasi F, Pardi R: **Through and beyond the wall: late steps in leukocyte transendothelial migration.** *Immunology Today* 1997, **18:**586-591.
82. Barthel SR, Gavino JD, Descheny L, Dimitroff CJ: **Targeting selectins and selectin ligands in inflammation and cancer.** *Expert Opinion on Therapeutic Targets* 2007, **11:**1473-1491.
83. Auguste P, Fallavollita L, Wang N, Burnier J, Bikfalvi A, Brodt P: **The host inflammatory response promotes liver metastasis by increasing tumor cell arrest and extravasation.** *American Journal of Pathology* 2007, **170:**1781-1792.
84. Kannagi R, Izawa M, Koike T, Miyazaki K, Kimura N: **Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis.** *Cancer Science* 2004, **95:**377-384.
85. Kim YJ, Varki A: **Perspectives on the significance of altered glycosylation of glycoproteins in cancer.** *Glycoconjugate Journal* 1997, **14:**569-576.
86. Kannagi R: **Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer.** *Glycoconjugate Journal* 1997, **14:**577-584.
87. Hayashi T, Takahashi T, Motoya S, Ishida T, Itoh F, Adachi M, Hinoda Y, Imai K: **MUC1 mucin core protein binds to the domain 1 of ICAM-1.** *Digestion* 2001, **63:**87-92.
88. Ando H, Aihara R, Ohno T, Ogata K, Mochiki E, Kuwano H: **Prognostic significance of the expression of MUC1 and collagen type IV in advanced gastric carcinoma.** *British Journal of Surgery* 2009, **96:**901-909.
89. de Roos MAJ, van der Vegt B, Peterse JL, Patriarca C, de Vries J, de Bock GH, Wesseling J: **The expression pattern of MUC1 (EMA) is related to tumour characteristics and clinical outcome in 'pure' ductal carcinoma in situ of the breast.** *Histopathology* 2007, **51:**227-238.
90. Lin YS, Jung SM, Yeh CN, Chen YC, Tsai FC, Shiu TF, Wu HH, Lin PJ, Chu PH: **MUC1, MUC2 and MUC5AC expression in hepatocellular carcinoma with cardiac metastasis.** *Molecular Medicine Reports* 2009, **2:**291-294.
91. van der Vegt B, de Roos MAJ, Peterse JL, Patriarca C, Hilkens J, de Bock GH, Wesseling J: **The expression pattern of MUC1 (EMA) is related to tumour characteristics and clinical outcome of invasive ductal breast carcinoma.** *Histopathology* 2007, **51:**322-335.
92. Mazzocca A, Carloni V: **The Metastatic Process: Methodological Advances and Pharmacological Challenges.** *Current Medicinal Chemistry* 2009, **16:**1704-1717.
93. Jiang P, Enomoto A, Takahashi M: **Cell biology of the movement of breast cancer cells: Intracellular signalling and the actin cytoskeleton.** *Cancer Letters* 2009, **284:**122-130.
94. Pani G, Galeotti T, Chiarugi P: **Metastasis: cancer cell's escape from oxidative stress.** *Cancer and Metastasis Reviews* 2010, **29:**351-378.
95. Ansieau S, de Fromentel CC, Bastid J, Morel AP, Puisieux A: **Role of the epithelial-mesenchymal transition during tumor progression.** *Bulletin Du Cancer* 2010, **97:**7-15.
96. Wells A: *Cell motility in cancer invasion and metastasis.* Eighth edn. Dordrecht, The Netherlands: Springer; 2006.

97. Ananthakrishnan R, Ehrlicher A: **The forces behind cell movement.** *International Journal of Biological Sciences* 2007, **3:**303-317.
98. Heasman SJ, Ridley AJ: **Mammalian Rho GTPases: new insights into their functions from in vivo studies.** *Nature Reviews Molecular Cell Biology* 2008, **9:**690-701.
99. Kurisu S, Takenawa T: **The WASP and WAVE family proteins.** *Genome Biology* 2009, **10:**9.
100. Fritz G, Just I, Kaina B: **Rho GTPases are over-expressed in human tumors.** *International Journal of Cancer* 1999, **81:**682-687.
101. Bellizzi A, Mangia A, Chiriatti A, Petroni S, Quaranta M, Schittull F, Malfettone A, Cardone RA, Paradiso A, Reshkin SJ: **RhoA protein expression in primary breast cancers and matched lymphocytes is associated with progression of the disease.** *International Journal of Molecular Medicine* 2008, **22:**25-31.
102. Pollard TD, Borisy GG: **Cellular motility driven by assembly and disassembly of actin filaments.** *Cell* 2003, **112:**453-465.
103. Holly SP, Larson MK, Parise LV: **Multiple roles of integrins in cell motility.** *Experimental Cell Research* 2000, **261:**69-74.
104. Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF: **Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness.** *Nature* 1997, **385:**537-540.
105. Kornberg L, Earp HS, Parsons JT, Schaller M, Juliano RL: **Cell adhesion or integrin clustering increases phosphorylation of focal adhesion associated tyrosine kinase.** *Journal of Biological Chemistry* 1992, **267:**23439-23442.
106. Hanks SK, Ryzhova L, Shin NY, Brabek J: **Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility.** *Frontiers in Bioscience* 2003, **8:**D982-D996.
107. Geiger B, Bershadsky A, Pankov R, Yamada KM: **Transmembrane extracellular matrix-cytoskeleton crosstalk.** *Nature Reviews Molecular Cell Biology* 2001, **2:**793-805.
108. de Heer P, Koudijs MM, van de Velde CJH, Aalbers R, Tollenaar R, Putter H, Morreau J, van de Water B, Kuppen PJK: **Combined expression of the non-receptor protein tyrosine kinases FAK and Src in primary colorectal cancer is associated with tumor recurrence and metastasis formation.** *Ejso* 2008, **34:**1253-1261.
109. Planas-Silva MD, Bruggeman RD, Grenko RT, Smith JS: **Role of c-Src and focal adhesion kinase in progression and metastasis of estrogen receptor-positive breast cancer.** *Biochemical and Biophysical Research Communications* 2006, **341:**73-81.
110. Franzen CA, Amargo E, Todorovic V, Desai BV, Huda S, Mirzoeva S, Chiu K, Grzybowski BA, Chew TL, Green KJ, Pelling JC: **The Chemopreventive Bioflavonoid Apigenin Inhibits Prostate Cancer Cell Motility through the Focal Adhesion Kinase/Src Signaling Mechanism.** *Cancer Prevention Research* 2009, **2:**830-841.
111. Hsu YL, Wu LY, Hou MF, Tsai EM, Lee JN, Liang HL, Jong YJ, Hung CH, Kuo PL: **Glabridin, an isoflavan from licorice root, inhibits migration, invasion and angiogenesis of MDA-MB-231 human breast adenocarcinoma cells by inhibiting focal adhesion kinase/Rho signaling pathway.** *Molecular Nutrition & Food Research* 2011, **55:**318-327.

112. Sawyer TK: **Cancer metastasis therapeutic targets and drug discovery: emerging small-molecule protein kinase inhibitors.** *Expert Opinion on Investigational Drugs* 2004, **13:**1-19.
113. Yang JC, Bai LF, Yap S, Gao AC, Kung HJ, Evans CP: **Effect of the Specific Src Family Kinase Inhibitor Saracatinib on Osteolytic Lesions Using the PC-3 Bone Model.** *Molecular Cancer Therapeutics* 2010, **9:**1629-1637.
114. Wadhawan A, Smith C, Nicholson RI, Barrett-Lee P, Hiscox S: **Src-mediated regulation of homotypic cell adhesion: implications for cancer progression and opportunities for therapeutic intervention.** *Cancer Treatment Reviews* 2011, **37:**234-241.
115. Irby RB, Yeatman TJ: **Increased Src activity disrupts cadherin/catenin-mediated homotypic adhesion in human colon cancer and transformed rodent cells.** *Cancer Research* 2002, **62:**2669-2674.
116. Alteraifi AM, Zhelev DV: **Transient increase of free cytosolic calcium during neutrophil motility responses.** *Journal of Cell Science* 1997, **110:**1967-1977.
117. Publicover SJ, Giojalas LC, Teves ME, de Oliveira G, Garcia AAM, Barratt CLR, Harper CV: **Ca2+ signalling in the control of motility and guidance in mammalian sperm.** *Frontiers in Bioscience* 2008, **13:**5623-5637.
118. Komuro H, Kumada T: **Ca2+ transients control CNS neuronal migration.** *Cell Calcium* 2005, **37:**387-393.
119. Kumada T, Komuro H: **Completion of neuronal migration regulated by loss of Ca2+ transients.** *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101:**8479-8484.
120. Li HY, Cook JD, Terry M, Spitzer NC, Ferrari MB: **Calcium transients regulate patterned actin assembly during myofibrillogenesis.** *Developmental Dynamics* 2004, **229:**231-242.
121. Parekh AB: **Decoding cytosolic Ca2+ oscillations.** *Trends in Biochemical Sciences* 2011, **36:**78-87.
122. Jimenez C, Hernandez C, Pimental B, Carrera AC: **The p85 regulatory subunit controls sequential activation of phosphoinositide 3-kinase by Tyr kinases and Ras.** *Journal of Biological Chemistry* 2002, **277:**41556-41562.
123. Cuevas BD, Lu YL, Mao ML, Zhang JY, LaPushin R, Siminovitch K, Mills GB: **Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase.** *Journal of Biological Chemistry* 2001, **276:**27455-27461.
124. Rameh LE, Rhee SG, Spokes K, Kazlauskas A, Cantley LC, Cantley LG: **Phosphoinositide 3-kinase regulates phospholipase C gamma-mediated calcium signaling.** *Journal of Biological Chemistry* 1998, **273:**23750-23757.
125. Harootunian AT, Kao JPY, Paranjape S, Tsien RY: **Generation of calcium oscillations in fibroblasts by positive feedback between calcium and IP3.** *Science* 1991, **251:**75-78.
126. Berridge MJ, Lipp P, Bootman MD: **The versatility and universality of calcium signalling.** *Nature Reviews Molecular Cell Biology* 2000, **1:**11-21.
127. Myoui A, Nishimura R, Williams PJ, Hiraga T, Tamura D, Michigami T, Mundy GR, Yoneda T: **C-src tyrosine kinase activity is associated with tumor colonization in bone and lung in an animal model of human breast cancer metastasis.** *Cancer Research* 2003, **63:**5028-5033.
128. Hiscox S, Morgan L, Green TP, Barrow D, Gee J, Nicholson RI: **Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells.** *Breast cancer research and treatment* 2006, **97:**263-274.

129. Elsberger B, Stewart B, Tatarov O, Edwards J: **Is Src a Viable Target for Treating Solid Tumours?** *Current Cancer Drug Targets* 2010, **10:**683-694.
130. Bafna S, Kaur S, Batra SK: **Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells.** *Oncogene* 2010, **29:**2893-2904.
131. Hollingsworth MA, Swanson BJ: **Mucins in cancer: Protection and control of the cell surface.** *Nature Reviews Cancer* 2004, **4:**45-60.
132. Levitin F, Stern O, Weiss M, Gil-Henn C, Ziv R, Prokocimer Z, Smorodinsky NI, Rubinstein DB, Wreschner DH: **The MUC1 SEA module is a self-cleaving domain.** *Journal of Biological Chemistry* 2005, **280:**33374-33386.
133. Palmai-Pallag T, Khodabukus N, Kinarsky L, Leir SH, Sherman S, Hollingsworth MA, Harris A: **The role of the SEA (sea urchin sperm protein, enterokinase and agrin) module in cleavage of membrane-tethered mucins.** *FEBS Journal* 2005, **272:**2901-2911.
134. Wreschner DH, McGuckin MA, Williams SJ, Baruch A, Yoeli M, Ziv R, Okun L, Zaretsky J, Smorodinsky N, Keydar I, et al: **Generation of ligand-receptor alliances by "SEA" module-mediated cleavage of membrane-associated mucin proteins.** *Protein Science* 2002, **11:**698-706.
135. Hattrup CL, Gendler SJ: **Structure and function of the cell surface (tethered) mucins.** *Annual Review of Physiology* 2008, **70:**431-457.
136. Yonezawa S, Goto M, Yamada N, Higashi M, Nomoto M: **Expression profiles of MUC1, MUC2, and MUC4 mucins in human neoplasms and their relationship with biological behavior.** *Proteomics* 2008, **8:**3329-3341.
137. Rakha EA, Boyce RW, Abd El-Rehim D, Kurien T, Green AR, Paish EC, Robertson JF, Ellis IO: **Expression of mucins (MUC1, MUC2, MUC3, MUC4, MUC5AC and MUC6) and their prognostic significance in human breast cancer.** *Modern Pathology* 2005, **18:**1295-1304.
138. Hamada T, Matsukita S, Goto M, Kitajima S, Batra SK, Irimura T, Sueyoshi K, Sugihara K, Yonezawa S: **Mucin expression in pleomorphic adenoma of salivary gland: a potential role for MUC1 as a marker to predict recurrence.** *Journal of Clinical Pathology* 2004, **57:**813-821.
139. Saitou M, Goto M, Horinouchi M, Tamada S, Nagata K, Hamada T, Osako M, Takao S, Batra SK, Aikou T, et al: **MUC4 expression is a novel prognostic factor in patients with invasive ductal carcinoma of the pancreas.** *Journal of Clinical Pathology* 2005, **58:**845-852.
140. Tamada S, Goto M, Nomoto M, Nagata K, Shimizu T, Tanaka S, Sakoda K, Imai K, Yonezawa S: **Expression of MUC1 and MUC2 mucins in extrahepatic bile duct carcinomas: Its relationship with tumor progression and prognosis.** *Pathology International* 2002, **52:**713-723.
141. Togami S, Nomoto M, Higashi M, Goto M, Yonezawa S, Tsuji T, Batra SK, Douchi T: **Expression of mucin antigens (MUC1 and MUC16) as a prognostic factor for mucinous adenocarcinoma of the uterine cervix.** *Journal of Obstetrics and Gynaecology Research* 2010, **36:**588-597.
142. Matsuyama T, Ishikawa T, Mogushi K, Yoshida T, Iida S, Uetake H, Mizushima H, Tanaka H, Sugihara K: **MUC12 mRNA expression is an independent marker of prognosis in stage II and stage III colorectal cancer.** *International Journal of Cancer* 2010, **127:**2292-2299.
143. Gendler SJ, Spicer AP, Lalani EN, Duhig T, Peat N, Burchell J, Pemberton L, Boshell M, Taylor-Papadimitriou J: **Structure and biology of a carcinoma-**

associated mucin, MUC1. *The American Review of Respiratory Disease* 1991, **144:**S42-47.
144. Li Y, Liu D, Chen D, Kharbanda S, Kufe D: **Human DF3/MUC1 carcinoma-associated protein functions as an oncogene.** *Oncogene* 2003, **22:**6107-6110.
145. van de Wiel-van Kemenade E, Ligtenberg MJ, de Boer AJ, Buijs F, Vos HL, Melief CJ, Hilkens J, Figdor CG: **Episialin (MUC1) inhibits cytotoxic lymphocyte-target cell interaction.** *Journal of immunology (Baltimore, Md: 1950)* 1993, **151:**767-776.
146. Duffy MJ, Shering S, Sherry F, McDermott E, O'Higgins N: **CA 15-3: a prognostic marker in breast cancer.** *The International journal of biological markers* 2000, **15:**330-333.
147. Chan AK, Lockhart DC, von Bernstorff W, Spanjaard RA, Joo HG, Eberlein TJ, Goedegebuure PS: **Soluble MUC1 secreted by human epithelial cancer cells mediates immune suppression by blocking T-cell activation.** *International Journal of Cancer* 1999, **82:**721-726.
148. Tsutsumida H, Swanson BJ, Singh PK, Caffrey TC, Kitajima S, Goto M, Yonezawa S, Hollingsworth MA: **RNA interference suppression of MUC1 reduces the growth rate and metastatic phenotype of human pancreatic cancer cells.** *Clinical Cancer Research* 2006, **12:**2976-2987.
149. Gendler SJ, Burchell JM, Duhig T, Lamport D, White R, Parker M, Taylor-Papadimitriou J: **Cloning of partial cDNA encoding differentiation and tumor-associated mucin glycoproteins expressed by human mammary epithelium.** *Proceedings of the National Academy of Sciences of the United States of America* 1987, **84:**6060-6064.
150. Gendler SJ, Burchell J, Duhig T, Girling A, Millis R, Taylorpapadimitriou J: **Cloning the cDNA for the core protein of a breast cancer associated mucin recognized by antibodies to the milk-fat globule.** *British Journal of Cancer* 1987, **56:**511-511.
151. Ligtenberg MJL, Vos HL, Gennissen AMC, Hilkens J: **Episialin, a carcinoma-associated mucin, is generated by a polymorphic gene encoding splice variants with alternative amino termini.** *Journal of Biological Chemistry* 1990, **265:**5573-5578.
152. Gendler SJ, Lancaster CA, Taylor-Papadimitriou J, Duhig T, Peat N, Burchell J, Pemberton L, Lalani EN, Wilson D: **Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin.** *The Journal of biological chemistry* 1990, **265:**15286-15293.
153. Lan MS, Batra SK, Qi WN, Metzgar RS, Hollingsworth MA: **Cloning and sequencing of a human pancreatic tumor mucin cDNA.** *The Journal of biological chemistry* 1990, **265:**15294-15299.
154. Jentoft N: **Why are proteins O-glycosylated?** *Trends in Biochemical Sciences* 1990, **15:**291-294.
155. Fukuda M: **Possible roles of tumor-associated carbohydrate antigens.** *Cancer Research* 1996, **56:**2237-2244.
156. Rottger S, White J, Wandall HH, Olivo JC, Stark A, Bennett EP, Whitehouse C, Berger EG, Clausen H, Nilsson T: **Localization of three human polypeptide GalNAc-transferases in HeLa cells suggests initiation of O-linked glycosylation throughout the Golgi apparatus.** *Journal of Cell Science* 1998, **111:**45-60.

157. Burchell JM, Mungul A, Taylor-Papadimitriou J: **O-linked glycosylation in the mammary gland: Changes that occur during malignancy.** *Journal of Mammary Gland Biology and Neoplasia* 2001, **6:**355-364.
158. Taylor-Papadimitriou J, Burchell J, Miles DW, Dalziel M: **MUC1 and cancer.** *Biochimica Et Biophysica Acta-Molecular Basis of Disease* 1999, **1455:**301-313.
159. Lloyd KO, Burchell J, Kudryashov V, Yin BWT, TaylorPapadimitriou J: **Comparison of O-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines - Demonstration of simpler and fewer glycan chains in tumor cells.** *Journal of Biological Chemistry* 1996, **271:**33325-33334.
160. Muller S, Alving K, Peter-Katalinic J, Zachara N, Gooley AA, Hanisch FG: **High density O-glycosylation on tandem repeat peptide from secretory MUC1 of T47D breast cancer cells.** *Journal of Biological Chemistry* 1999, **274:**18165-18172.
161. Muller S, Goletz S, Packer N, Gooley A, Lawson AM, Hanisch FG: **Localization of O-glycosylation sites on glycopeptide fragments from lactation-associated MUC1 - All putative sites within the tandem repeat are glycosylation targets in vivo.** *Journal of Biological Chemistry* 1997, **272:**24780-24793.
162. Picco G, Julien S, Brockhausen I, Beatson R, Antonopoulos A, Haslam S, Mandel U, Dell A, Pinder S, Taylor-Papadimitriou J, Burchell J: **Over-expression of ST3Gal-I promotes mammary tumorigenesis.** *Glycobiology* 2010, **20:**1241-1250.
163. Rughetti A, Pellicciotta I, Biffoni M, Backstrom M, Link T, Bennet EP, Clausen H, Noll T, Hansson GC, Burchelll JM, et al: **Recombinant tumor-associated MUC1 glycoprotein impairs the differentiation and function of dendritic cells.** *Journal of Immunology* 2005, **174:**7764-7772.
164. Baldus SE, Hanisch FG, Kotlarek GM, Zirbes TK, Thiele J, Isenberg J, Karsten UR, Devine PL, Dienes HP: **Coexpression of MUC1 mucin peptide core and the Thomsen-Friedenreich antigen in colorectal neoplasms.** *Cancer* 1998, **82:**1019-1027.
165. Baldus SE, Hanisch FG, Monaca E, Karsten UR, Zirbes TK, Thiele J, Dienes HP: **Immunoreactivity of Thomsen-Friedenreich (TF) antigen in human neoplasms: The importance of carrier-specific glycotope expression on MUC1.** *Histology and Histopathology* 1999, **14:**1153-1158.
166. Yu LG, Andrews N, Zhao Q, McKean D, Williams JF, Connor LJ, Gerasimenko OV, Hilkens J, Hirabayashi J, Kasai K, Rhodes JM: **Galectin-3 interaction with Thomsen-Friedenreich disaccharide on cancer-associated MUC1 causes increased cancer cell endothelial adhesion.** *The Journal of biological chemistry* 2007, **282:**773-781.
167. Zhao Q, Guo X, Nash GB, Stone PC, Hilkens J, Rhodes JM, Yu LG: **Circulating galectin-3 promotes metastasis by modifying MUC1 localization on cancer cell surface.** *Cancer research* 2009, **69:**6799-6806.
168. Zhao Q, Barclay M, Hilkens J, Guo X, Barrow H, Rhodes JM, Yu LG: **Interaction between circulating galectin-3 and cancer-associated MUC1 enhances tumour cell homotypic aggregation and prevents anoikis.** *Molecular cancer* 2010, **9:**154.
169. Burchell J, Gendler S, Taylorpapadimitriou J, Girling A, Lewis A, Millis R, Lamport D: **Development and characterization of breast-cancer reactive**

monoclonal-antibodies directed to the core protein of the human-milk mucin. *Cancer Research* 1987, **47:**5476-5482.
170. Ryan SO, Turner MS, Gariepy J, Finn OJ: **Tumor Antigen Epitopes Interpreted by the Immune System as Self or Abnormal-Self Differentially Affect Cancer Vaccine Responses.** *Cancer Research* 2010, **70:**5788-5796.
171. Singh R, Bandyopadhyay D: **MUC1 - A target molecule for cancer therapy.** *Cancer Biology & Therapy* 2007, **6:**481-486.
172. Apostolopoulos V, Pietersz GA, Tsibanis A, Tsikkinis A, Drakaki H, Loveland BE, Piddlesden SJ, Plebanski M, Pouniotis DS, Alexis MN, et al: **Pilot phase III immunotherapy study in early-stage breast cancer patients using oxidized mannan-MUC1 ISRCTN71711835.** *Breast Cancer Research* 2006, **8**.
173. Jin D, Rong Y, Lou W, Qin X, Wu W, Nin X, Wang D, Kuang T, Qin Y: **Therapeutic vaccination against advanced pancreatic cancer by autologous dendritic cells pulsed with a MUC1 peptide: Preclinical results of a clinical phase I trial.** *Journal of Clinical Oncology* 2009, **27:**e15652.
174. Ivanov PK, Chmutin EF, Yakubovskaya RI, Manzjuk LV, Hanson KP, Moiseenko VM, Artamonova EV, Bolotina LV, Nemtsova ER, Karmakova TA, Baryshnikov AY: **Therapeutic monoclonal anti-MUC1 antibodies (IMUTERAN): Interim analysis of the results of the phase II clinical trial.** *Annals of Oncology* 2005, **16:**47-47.
175. Pecher G, Haring A, Kaiser L, Thiel E: **Mucin gene (MUC1) transfected dendritic cells as vaccine: results of a phase I/II clinical trial.** *Cancer Immunology Immunotherapy* 2002, **51:**669-673.
176. Casadio R, Fariselli P, Taroni C, Compiani M: **A predictor of transmembrane alpha-helix domains of proteins based on neural networks.** *European Biophysics Journal with Biophysics Letters* 1996, **24:**165-178.
177. Lichtenthaler SF, Beher D, Grimm HS, Wang R, Shearman MS, Masters CL, Beyreuther K: **The intramembrane cleavage site of the amyloid precursor protein depends on the length of its transmembrane domain.** *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99:**1365-1370.
178. Julian J, Dharmaraj N, Carson DD: **MUC1 Is a Substrate for gamma-Secretase.** *Journal of Cellular Biochemistry* 2009, **108:**802-815.
179. Pemberton LF, Rughetti A, TaylorPapadimitriou J, Gendler SJ: **The epithelial mucin MUC1 contains at least two discrete signals specifying membrane localization in cells.** *Journal of Biological Chemistry* 1996, **271:**2332-2340.
180. Kinlough CL, McMahan RJ, Poland PA, Bruns JB, Harkleroad KL, Stremple RJ, Kashlan OB, Weixel KM, Weisz OA, Hughey RP: **Recycling of MUC1 is dependent on its palmitoylation.** *Journal of Biological Chemistry* 2006, **281:**12112-12122.
181. Hanzal-Bayer MF, Hancock JF: **Lipid rafts and membrane traffic.** *Febs Letters* 2007, **581:**2098-2104.
182. Lindner R, Naim HY: **Domains in biological membranes.** *Experimental Cell Research* 2009, **315:**2871-2878.
183. Yin L, Ahmad R, Kosugi M, Kawano T, Avigan D, Stone R, Kharbanda S, Kufe D: **Terminal differentiation of chronic myelogenous leukemia cells is induced by targeting of the MUC1-C oncoprotein.** *Cancer biology & therapy* 2010, **10**.
184. Raina D, Ahmad R, Joshi MD, Yin L, Wu Z, Kawano T, Vasir B, Avigan D, Kharbanda S, Kufe D: **Direct targeting of the mucin 1 oncoprotein blocks**

survival and tumorigenicity of human breast carcinoma cells. *Cancer research* 2009, **69:**5133-5141.

185. Zrihanlicht S, Baruch A, Elroystein O, Keydar I, Wreschner DH: **Tyrosine phosphorylation of the MUC1 breast-cancer membrane proteins - cytokine receptor-like molecules.** *Febs Letters* 1994, **356:**130-136.
186. Li Y, Kuwahara H, Ren J, Wen G, Kufe D: **The c-Src tyrosine kinase regulates signaling of the human DF3/MUC1 carcinoma-associated antigen with GSK3 beta and beta-catenin.** *Journal of Biological Chemistry* 2001, **276:**6061-6064.
187. Li Q, Ren J, Kufe D: **Interaction of human MUC1 and beta-catenin is regulated by Lck and ZAP-70 in activated Jurkat T cells.** *Biochemical & Biophysical Research Communications* 2004, **315:**471-476.
188. Schroeder JA, Thompson MC, Gardner MM, Gendler SJ: **Transgenic MUC1 interacts with epidermal growth factor receptor and correlates with mitogen-activated protein kinase activation ill the mouse mammary gland.** *Journal of Biological Chemistry* 2001, **276:**13057-13064.
189. Li Y, Chen W, Ren J, Yu WH, Li Q, Yoshida K, Kufe D: **DF3/MUC1 signaling in multiple myeloma cells is regulated by intarleukin-7.** *Cancer Biology & Therapy* 2003, **2:**187-193.
190. Kato K, Lu W, Kai H, Kim C: **Phosphoinositide 3-kinase is activated by MUC1 but not responsible for MUC1-induced suppression of Toll-like receptor 5 signaling.** *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2007, **293:**L686-L692.
191. Pandey P, Kharbanda S, Kufe D: **Association of the DF3/MUC1 breast cancer antigen with Grb2 and the Sos/Ras exchange protein.** *Cancer research* 1995, **55:**4000-4003.
192. Shen Q, Rahn JJ, Zhang J, Gunasekera N, Sun X, Shaw AR, Hendzel MJ, Hoffman P, Bernier A, Hugh JC: **MUC1 initiates Src-CrkL-Rac1/Cdc42-mediated actin cytoskeletal protrusive motility after ligating intercellular adhesion molecule-1.** *Molecular cancer research : MCR* 2008, **6:**555-567.
193. Raina D, Ahmad R, Kumar S, Ren J, Yoshida K, Kharbanda S, Kufe D: **MUC1 oncoprotein blocks nuclear targeting of c-Abl in the apoptotic response to DNA damage.** *Embo Journal* 2006, **25:**3774-3783.
194. Wei XL, Xu H, Kufe D: **MUC1 oncoprotein stabilizes and activates estrogen receptor alpha.** *Molecular Cell* 2006, **21:**295-305.
195. Yamamoto M, Bharti A, Li Y, Kufe D: **Interaction of the DF3/MUC1 breast carcinoma-associated antigen and beta-catenin in cell adhesion.** *The Journal of biological chemistry* 1997, **272:**12492-12494.
196. Wen Y, Caffrey TC, Wheelock MJ, Johnson KR, Hollingsworth MA: **Nuclear association of the cytoplasmic tail of MUC1 and beta-catenin.** *Journal of Biological Chemistry* 2003, **278:**38029-38039.
197. Li Y, Yu WH, Ren J, Chen W, Huang L, Kharbanda S, Loda M, Kufe D: **Heregulin targets gamma-catenin to the nucleolus by a mechanism dependent on the DF3/MUC1 oncoprotein.** *Molecular Cancer Research: MCR* 2003, **1:**765-775.
198. Bennett R, Jr., Jarvela T, Engelhardt P, Kostamovaara L, Sparks P, Carpen O, Turunen O, Vaheri A: **Mucin MUC1 is seen in cell surface protrusions together with ezrin in immunoelectron tomography and is concentrated at tips of filopodial protrusions in MCF-7 breast carcinoma cells.** *The journal of*

histochemistry and cytochemistry : official journal of the Histochemistry Society 2001, **49:**67-77.

199. Julian J, Carson DD: **Formation of MUC1 metabolic complex is conserved in tumor-derived and normal epithelial cells.** *Biochemical & Biophysical Research Communications* 2002, **293:**1183-1190.
200. Johansson DGA, Macao B, Sandberg A, Hard T: **SEA domain autoproteolysis accelerated by conformational strain: Mechanistic aspects.** *Journal of Molecular Biology* 2008, **377:**1130-1143.
201. Macao B, Johansson DG, Hansson GC, Hard T: **Autoproteolysis coupled to protein folding in the SEA domain of the membrane-bound MUC1 mucin.** *Nature Structural & Molecular Biology* 2006, **13:**71-76.
202. Brou C: **Intracellular trafficking of Notch receptors and ligands.** *Experimental Cell Research* 2009, **315:**1549-1555.
203. Blaumueller CM, Qi HL, Zagouras P, ArtavanisTsakonas S: **Intracellular cleavage of notch leads to a heterodimeric receptor on the plasma membrane.** *Cell* 1997, **90:**281-291.
204. Gordon WR, Vardar-Ulu D, L'Heureux S, Ashworth T, Malecki MJ, Sanchez-Irizarry C, McArthur DG, Histen G, Mitchell JL, Aster JC, Blacklow SC: **Effects of S1 Cleavage on the Structure, Surface Export, and Signaling Activity of Human Notch1 and Notch2.** *Plos One* 2009, **4:**12.
205. Bush G, diSibio G, Miyamoto A, Denault JB, Leduc R, Weinmaster G: **Ligand-induced signaling in the absence of furin processing of Notch1.** *Developmental Biology* 2001, **229:**494-502.
206. Kidd S, Lieber T: **Furin cleavage is not a requirement for Drosophila Notch function.** *Mechanisms of Development* 2002, **115:**41-51.
207. Nakamura H, Hinoda Y, Nakagawa N, Makiguchi Y, Itoh F, Endo T, Imai K: **Detection of circulating anti-MUC1 mucin core protein antibodies in patients with colorectal cancer.** *Journal of gastroenterology* 1998, **33:**354-361.
208. Martin A, Corte MD, Alvarez AM, Rodriguez JC, Andicoechea A, Bongera M, Junquera S, Pidal D, Allende MT, Muniz JLG, Vizoso F: **Prognostic value of pre-operative serum CA 15.3 levels in breast cancer.** *Anticancer Research* 2006, **26:**3965-3971.
209. Xu Y, Zhang L, Hu GX: **Potential application of alternatively glycosylated serum MUC1 and MUC5AC in gastric cancer diagnosis.** *Biologicals* 2009, **37:**18-25.
210. Thathiah A, Brayman M, Dharmaraj N, Julian JJ, Lagow EL, Carson DD: **Tumor necrosis factor alpha stimulates MUC1 synthesis and ectodomain release in a human uterine epithelial cell line.** *Endocrinology* 2004, **145:**4192-4203.
211. Black RA, Doedens JR, Mahimkar R, Johnson R, Guo L, Wallace A, Virca D, Eisenman J, Slack J, Castner B, et al: **Substrate specificity and inducibility of TACE (tumour necrosis factor alpha-converting enzyme) revisited: the Ala-Val preference, and induced intrinsic activity.** *Biochemical Society symposium* 2003, **(70):**39-52.
212. Wen YF, Caffrey TC, Wheelock MJ, Johnson KR, Hollingsworth MA: **Nuclear association of the cytoplasmic tail of MUC1 and beta-catenin.** *Journal of Biological Chemistry* 2003, **278:**38029-38039.
213. Brou C, Logeat F, Gupta N, Bessia C, LeBail O, Doedens JR, Cumano A, Roux P, Black RA, Israel A: **A novel proteolytic cleavage involved in Notch**

signaling: the role of the disintegrin-metalloprotease TACE. *Molecular cell* 2000, **5:**207-216.

214. van Tetering G, van Diest P, Verlaan I, van der Wall E, Kopan R, Vooijs M: **Metalloprotease ADAM10 Is Required for Notch1 Site 2 Cleavage.** *Journal of Biological Chemistry* 2009, **284:**31018-31027.
215. Six E, Ndiaye D, Laabi Y, Brou C, Gupta-Rossi N, Israel A, Logeat F: **The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and gamma-secretase.** *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100:**7638-7643.
216. Nichols JT, Miyamoto A, Olsen SL, D'Souza B, Yao C, Weinmaster G: **DSL ligand endocytosis physically dissociates Notch 1 heterodimers before activating proteolysis can occur.** *Journal of Cell Biology* 2007, **176:**445-458.
217. Bozkulak EC, Weinmaster G: **Selective Use of ADAM10 and ADAM17 in Activation of Notch1 Signaling.** *Molecular and Cellular Biology* 2009, **29:**5679-5695.
218. Mumm JS, Schroeter EH, Saxena MT, Griesemer A, Tian X, Pan DJ, Ray WJ, Kopan R: **A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1.** *Molecular cell* 2000, **5:**197-206.
219. Sorensen EB, Conner SD: **gamma-Secretase-Dependent Cleavage Initiates Notch Signaling from the Plasma Membrane.** *Traffic* 2010, **11:**1234-1245.
220. Gao Y, Pimplikar SW: **The gamma -secretase-cleaved C-terminal fragment of amyloid precursor protein mediates signaling to the nucleus.** *Proceedings of the National Academy of Sciences of the United States of America* 2001, **98:**14979-14984.
221. Das I, Craig C, Funahashi Y, Jung KM, Kim TW, Byers R, Weng AP, Kutok JL, Aster JC, Kitajewski J: **Notch oncoproteins depend on gamma-secretase/presenilin activity for processing and function.** *Journal of Biological Chemistry* 2004, **279:**30771-30780.
222. Merchant NB, Voskresensky I, Rogers CM, LaFleur B, Dempsey PJ, Graves-Deal R, Revetta F, Foutch AC, Rothenberg ML, Washington MK, Coffey RJ: **TACE/ADAM-17: A component of the epidermal growth factor receptor axis and a promising therapeutic target in colorectal cancer.** *Clinical Cancer Research* 2008, **14:**1182-1191.
223. Arribas J, Esselens C: **ADAM17 as a Therapeutic Target in Multiple Diseases.** *Current Pharmaceutical Design* 2009, **15:**2319-2335.
224. Kornfeld JW, Meder S, Wohlberg M, Friedrich RE, Rau T, Riethdorf L, Loning T, Pantel K, Riethdorf S: **Overexpression of TACE and TIMP3 mRNA in head and neck cancer: association with tumour development and progression.** *British Journal of Cancer* 2011, **104:**138-145.
225. Liu PC, Liu X, Li Y, Covington M, Wynn R, Huber R, Hillman M, Yang G, Ellis D, Marando C, et al: **Identification of ADAM10 as a major source of HER2 ectodomain sheddase activity in HER2 overexpressing breast cancer cells.** *Cancer biology & therapy* 2006, **5:**657-664.
226. Friedman S, Levy R, Garrett W, Doval DC, Bondarde S, Sahoo TP, Lokanatha D, Julka PK, Shenoy K, Nagarkar R, et al: **Clinical Benefit of INCB7839, a Potent and Selective Inhibitor of ADAM10 and ADAM17, in Combination with Trastuzumab in Metastatic HER2 Positive Breast Cancer Patients.** *Cancer Research* 2009, **69:**783S-784S.

227. Soundararajan R, Sayat R, Robertson GS, Marignani PA: **Triptolide An inhibitor of a disintegrin and metalloproteinase 10 (ADAM10) in cancer cells.** *Cancer Biology & Therapy* 2009, **8:**2054-2062.
228. Wei P, Walls M, Qiu M, Ding R, Denlinger RH, Wong A, Tsaparikos K, Jani JP, Hosea N, Sands M, et al: **Evaluation of Selective gamma-Secretase Inhibitor PF-03084014 for Its Antitumor Efficacy and Gastrointestinal Safety to Guide Optimal Clinical Trial Design.** *Molecular Cancer Therapeutics* 2010, **9:**1618-1628.
229. Al-Hussaini H, Subramanyam D, Reedijk M, Sridhar SS: **Notch Signaling Pathway as a Therapeutic Target in Breast Cancer.** *Molecular Cancer Therapeutics* 2011, **10:**9-15.
230. Zrihan-Licht S, Vos HL, Baruch A, Elroy-Stein O, Sagiv D, Keydar I, Hilkens J, Wreschner DH: **Characterization and molecular cloning of a novel MUC1 protein, devoid of tandem repeats, expressed in human breast cancer tissue.** *European journal of biochemistry / FEBS* 1994, **224:**787-795.
231. Baruch A, Hartmann ML, Yoeli M, Adereth Y, Greenstein S, Stadler Y, Skornik Y, Zaretsky J, Smorodinsky NI, Keydar I, Wreschner DH: **The breast cancer-associated MUC1 gene generates both a receptor and its cognate binding protein.** *Cancer Research* 1999, **59:**1552-1561.
232. Schut IC, Waterfall PM, Ross M, O'Sullivan C, Miller WR, Habib FK, Bayne CW: **MUC1 expression, splice variant and short form transcription (MUC1/Z, MUC1/Y) in prostate cell lines and tissue.** *Bju International* 2003, **91:**278-283.
233. Baruch A, Hartmann M, Zrihan-Licht S, Greenstein S, Burstein M, Keydar I, Weiss M, Smorodinsky N, Wreschner DH: **Preferential expression of novel MUC1 tumor antigen isoforms in human epithelial tumors and their tumor-potentiating function.** *International Journal of Cancer* 1997, **71:**741-749.
234. Obermair A, Schmid BC, Packer LM, Leodolter S, Birner P, Ward BG, Crandon AJ, McGuckin MA, Zeillinger R: **Expression of MUC1 splice variants in benign and malignant ovarian tumours.** *International Journal of Cancer* 2002, **100:**166-171.
235. Oosterkamp HM, Scheiner L, Stefanova MC, Lloyd KO, Finstad CL: **Comparison of MUC-1 mucin expression in epithelial and non-epithelial cancer cell lines and demonstration of a new short variant form (MUC-1/Z).** *International Journal of Cancer* 1997, **72:**87-94.
236. Lancaster CA, Peat N, Duhig T, Wilson D, Taylorpapadimitriou J, Gendler SJ: **Structure and expression of the human polymorphic epithelial mucin gene - an expressed VNTR unit.** *Biochemical and Biophysical Research Communications* 1990, **173:**1019-1029.
237. Gendler SJ, Cohen EP, Craston A, Duhig T, Johnstone G, Barnes D: **The locus of the polymorphic epithelial mucin (PEM) tumor-antigen on chromosome 1q21 shows a high frequency of alteration in primary human breast tumors.** *International Journal of Cancer* 1990, **45:**431-435.
238. Gaemers IC, Vos HL, Volders HH, van der Valk SW, Hilkens J: **A stat-responsive element in the promoter of the episialin/MUC1 gene is involved in its overexpression in carcinoma cells.** *The Journal of biological chemistry* 2001, **276:**6191-6199.
239. Li Y, Chen W, Ren J, Yu WH, Li Q, Yoshida K, Kufe D: **DF3/MUC1 signaling in multiple myeloma cells is regulated by interleukin-7.** *Cancer biology & therapy* 2003, **2:**187-193.

240. Verma M, Davidson EA: **MUC1 upregulation by ethanol.** *Cancer biochemistry biophysics* 1999, **17:**1-11.
241. Hong DH, Petrovics G, Anderson WB, Forstner J, Forstner G: **Induction of mucin gene expression in human colonic cell lines by PMA is dependent on PKC-epsilon.** *American Journal of Physiology-Gastrointestinal and Liver Physiology* 1999, **277:**G1041-G1047.
242. Zotter S, Hageman P, A L, WJ M, Hilgers J: **Tissue and tumor distribution of human polymorphic epithelial mucin.** *Cancer Reviews* 1988, **11/12:**101.
243. Kovarik A, Peat N, Wilson D, Gendler SJ, Taylor-Papadimitriou J: **Analysis of the tissue-specific promoter of the MUC1 gene.** *Journal of Biological Chemistry* 1993, **268:**9917-9926.
244. Correa I, Plunkett T, Vlad A, Mungul A, Candelora-Kettel J, Burchell JM, Taylor-Papadimitriou J, Finn OJ: **Form and pattern of MUC1 expression on T cells activated in vivo or in vitro suggests a function in T-cell migration.** *Immunology* 2003, **108:**32-41.
245. Agrawal B, Krantz MJ, Parker J, Longenecker BM: **Expression of MUC1 mucin on activated human T cells: Implications for a role of MUC1 in normal immune regulation.** *Cancer Research* 1998, **58:**4079-4081.
246. Agrawal B, Krantz MJ, Reddish MA, Longenecker BM: **Cancer-associated MUC1 mucin inhibits human T-cell proliferation, which is reversible by IL-2.** *Nature Medicine* 1998, **4:**43-49.
247. Agrawal B, Longenecker BM: **MUC1 mucin-mediated regulation of human T cells.** *International Immunology* 2005, **17:**391-399.
248. Agrawal B, Gendler SJ, Longenecker BM: **The biological role of mucins in cellular interactions and immune regulation: prospects for cancer immunotherapy.** *Molecular Medicine Today* 1998, **4:**397-403.
249. Mukherjee P, Ginardi AR, Madsen CS, Tinder TL, Jacobs F, Parker J, Agrawal B, Longenecker BM, Gendler SJ: **MUC1-specific CTLs are non-functional within a pancreatic tumor microenvironment.** *Glycoconjugate Journal* 2001, **18:**931-942.
250. Paul S, Bizouarne N, Paul A, Price MR, Hansson GC, Kieny MP, Acres RB: **Lack of evidence for an immunosuppressive role for MUC1.** *Cancer Immunology Immunotherapy* 1999, **48:**22-28.
251. Lacunza E, Baudis M, Colussi AG, Segal-Eiras A, Croce MV, Abba MC: **MUC1 oncogene amplification correlates with protein overexpression in invasive breast carcinoma cells.** *Cancer genetics and cytogenetics* 2010, **201:**102-110.
252. Zrihanlicht S, Weiss M, Keydar I, Wreschner DH: **DNA methylation status of the MUC1 gene coding for a breast cancer associated protein.** *International Journal of Cancer* 1995, **62:**245-251.
253. Yamada N, Nishida Y, Tsutsumida H, Hamada T, Goto M, Higashi M, Nomoto M, Yonezawa S: **MUC1 expression is regulated by DNA methylation and histone H3 lysine 9 modification in cancer cells.** *Cancer Research* 2008, **68:**2708-2716.
254. Gilles F, Goy A, Remache Y, Shue P, Zelenetz AD: **MUC1 dysregulation as the consequence of a t(1;14)(q21;q32) translocation in an extranodal lymphoma.** *Blood* 2000, **95:**2930-2936.
255. Dyomin VG, Palanisamy N, Lloyd KO, Dyomina K, Jhanwar SC, Houldsworth J, Chaganti RSK: **MUC1 is activated in a B-cell lymphoma by the t(1;14)(q21;q32) translocation and is rearranged and amplified in B-cell lymphoma subsets.** *Blood* 2000, **95:**2666-2671.

256. Kondo K, Kohno N, Yokoyama A, Hiwada K: **Decreased MUC1 expression induces E-cadherin-mediated cell adhesion of breast cancer cell lines.** *Cancer research* 1998, **58:**2014-2019.
257. Wesseling J, van der Valk SW, Vos HL, Sonnenberg A, Hilkens J: **Episialin (MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components.** *The Journal of cell biology* 1995, **129:**255-265.
258. Lillehoj EP, Hyun SW, Kim BT, Zhang XG, Lee DI, Rowland S, Kim KC: **Muc1 mucins on the cell surface are adhesion sites for Pseudomonas aeruginosa.** *American journal of physiologyLung cellular and molecular physiology* 2001, **280:**L181-187.
259. Lillehoj EP, Kim BT, Kim KC: **Identification of Pseudomonas aeruginosa flagellin as an adhesin for Muc1 mucin.** *American journal of physiologyLung cellular and molecular physiology* 2002, **282:**L751-756.
260. Lillehoj EP, Kim H, Chun EY, Kim KC: **Pseudomonas aeruginosa stimulates phosphorylation of the airway epithelial membrane glycoprotein Muc1 and activates MAP kinase.** *American journal of physiologyLung cellular and molecular physiology* 2004, **287:**L809-815.
261. Lu WJ, Hisatsune A, Koga T, Kato K, Kuwahara I, Lillehoj EP, Chen W, Cross AS, Gendler SJ, Gewirtz AT, Kim KC: **Cutting edge: Enhanced pulmonary clearance of Pseudomonas aeruginosa by Muc1 knockout mice.** *Journal of Immunology* 2006, **176:**3890-3894.
262. Albelda SM: **Endothelial and epithelial cell adhesion molecules.** *American journal of respiratory cell and molecular biology* 1991, **4:**195-203.
263. Vereecken P, Boudjeltia KZ, Debray C, Awada A, Legssyer I, Sales F, Petein M, Vanhaeverbeek M, Ghanem G, Heenen M: **High serum galectin-3 in advanced melanoma: preliminary results.** *Clinical and Experimental Dermatology* 2006, **31:**105-109.
264. Kim SJ, Lee SJ, Sung HJ, Choi IK, Choi CW, Kim BS, Kim JS, Yu W, Hwang HS, Kim IS: **Increased Serum 90K and Galectin-3 Expression Are Associated with Advanced Stage and a Worse Prognosis in Diffuse Large B-Cell Lymphomas.** *Acta Haematologica* 2008, **120:**211-216.
265. Zhang K, Baeckstrom D, Brevinge H, Hansson GC: **Secreted MUC1 mucins lacking their cytoplasmic part and carrying sialyl-Lewis a and x epitopes from a tumor cell line and sera of colon carcinoma patients can inhibit HL-60 leukocyte adhesion to E-selectin-expressing endothelial cells.** *Journal of cellular biochemistry* 1996, **60:**538-549.
266. Zhang K, Baeckstrom D, Brevinge H, Hansson GC: **Comparison of sialyl-Lewis a carrying CD43 and MUC1 mucins secreted from a colon carcinoma cell line for E-selectin binding and inhibition of leukocyte adhesion.** *Tumor Biology* 1997, **18:**175-187.
267. Li YQ, Kuwahara H, Ren J, Wen GY, Kufe D: **The c-Src tyrosine kinase regulates signaling of the human DF3/MUC1 carcinoma-associated antigen with GSK3 beta and beta-catenin.** *Journal of Biological Chemistry* 2001, **276:**6061-6064.
268. Al Masri A, Gendler SJ: **Muc1 affects c-Src signaling in PyV MT-induced mammary tumorigenesis.** *Oncogene* 2005, **24:**5799-5808.
269. Ren J, Bharti A, Raina D, Chen W, Ahmad R, Kufe D: **MUC1 oncoprotein is targeted to mitochondria by heregulin-induced activation of c-Src and the molecular chaperone HSP90.** *Oncogene* 2006, **25:**20-31.

270. Mukherjee P, Tinder TL, Basu GD, Gendler SJ: **MUC1 (CD227) interacts with lck tyrosine kinase in Jurkat lymphoma cells and normal T cells.** *Journal of Leukocyte Biology* 2005, **77:**90-99.
271. Li Y, Bharti A, Chen D, Gong J, Kufe D: **Interaction of glycogen synthase kinase 3beta with the DF3/MUC1 carcinoma-associated antigen and beta-catenin.** *Molecular and cellular biology* 1998, **18:**7216-7224.
272. Huang L, Chen D, Liu D, Yin L, Kharbanda S, Kufe D: **MUC1 oncoprotein blocks glycogen synthase kinase 3beta-mediated phosphorylation and degradation of beta-catenin.** *Cancer research* 2005, **65:**10413-10422.
273. Schroeder JA, Adriance MC, Thompson MC, Camenisch TD, Gendler SJ: **MUC1 alters beta-catenin-dependent tumor formation and promotes cellular invasion.** *Oncogene* 2003, **22:**1324-1332.
274. Baldus SE, Monig SP, Huxel S, Landsberg S, Hanisch FG, Engelmann K, Schneider PM, Thiele J, Holscher AH, Dienes HP: **MUC1 and nuclear beta-catenin are coexpressed at the invasion front of colorectal carcinomas and are both correlated with tumor prognosis.** *Clinical Cancer Research* 2004, **10:**2790-2796.
275. Lillehoj EP, Lu WJ, Kiser T, Goldblum SE, Kim KC: **MUC1 inhibits cell proliferation by beta-catenin-dependent mechanism.** *Biochimica Et Biophysica Acta-Molecular Cell Research* 2007, **1773:**1028-1038.
276. Magkou C, Nakopoulou L, Zoubouli C, Karali K, Theohari I, Bakarakos P, Giannopoulou I: **Expression of the epidermal growth factor receptor (EGFR) and the phosphorylated EGFR in invasive breast carcinomas.** *Breast Cancer Research* 2008, **10:**8.
277. Meche A, Cimpean AM, Raica M: **Immunohistochemical expression and significance of epidermal growth factor receptor (EGFR) in breast cancer.** *Romanian Journal of Morphology and Embryology* 2009, **50:**217-221.
278. Li Y, Ren J, Yu W, Li Q, Kuwahara H, Yin L, Carraway KL, 3rd, Kufe D: **The epidermal growth factor receptor regulates interaction of the human DF3/MUC1 carcinoma antigen with c-Src and beta-catenin.** *Journal of Biological Chemistry* 2001, **276:**35239-35242.
279. Li XJ, Wang L, Nunes DP, Troxler RF, Offner GD: **Suppression of MUC1 synthesis downregulates expression of the epidermal growth factor receptor.** *Cancer Biology & Therapy* 2005, **4:**968-973.
280. Bitler BG, Menzl I, Huerta CL, Sands B, Knowlton W, Chang A, Schroeder JA: **Intracellular MUC1 Peptides Inhibit Cancer Progression.** *Clinical Cancer Research* 2009, **15:**100-109.
281. Bitler BG, Goverdhan A, Schroeder JA: **MUC1 regulates nuclear localization and function of the epidermal growth factor receptor.** *Journal of cell science* 2010, **123:**1716-1723.
282. Hisatsune A, Nakayama H, Kawasaki M, Horie I, Miyata T, Isohama Y, Kim KC, Katsuki H: **Anti-MUC1 antibody inhibits EGF receptor signaling in cancer cells.** *Biochemical and Biophysical Research Communications* 2011, **405:**377-381.
283. Ren J, Raina D, Chen W, Li GL, Huang L, Kufe D: **MUC1 oncoprotein functions in activation of fibroblast growth factor receptor signaling.** *Molecular Cancer Research* 2006, **4:**873-883.
284. Wang HG, Lillehoj EP, Kim KC: **Identification of four sites of stimulated tyrosine phosphorylation in the MUC1 cytoplasmic tail.** *Biochemical and Biophysical Research Communications* 2003, **310:**341-346.

285. Huang L, Ren J, Chen D, Li Y, Kharbanda S, Kufe D: **MUC1 cytoplasmic domain coactivates Wnt target gene transcription and confers transformation.** *Cancer Biology & Therapy* 2003, **2:**702-706.
286. Thompson EJ, Shanmugam K, Hattrup CL, Kotlarczyk KL, Gutierrez A, Bradley JM, Mukherjee P, Gendler SJ: **Tyrosines in the MUC1 cytoplasmic tail modulate transcription via the extracellular signal-regulated kinase 1/2 and nuclear factor-kappa B pathways.** *Molecular Cancer Research* 2006, **4:**489-497.
287. Khodarev NN, Pitroda SP, Beckett MA, MacDermed DM, Huang L, Kufe DW, Weichselbaum RR: **MUC1-induced transcriptional programs associated with tumorigenesis predict outcome in breast and lung cancer.** *Cancer research* 2009, **69:**2833-2837.
288. Kufe D: **Oncogenic function of the MUC1 receptor subunit in gene regulation.** *Oncogene* 2010, **29:**5663-5666.
289. Behrens ME, Grandgenett PM, Bailey JM, Singh PK, Yi CH, Yu F, Hollingsworth MA: **The reactive tumor microenvironment: MUC1 signaling directly reprograms transcription of CTGF.** *Oncogene* 2010.
290. Lillehoj EP, Lu W, Kiser T, Goldblum SE, Kim KC: **MUC1 inhibits cell proliferation by a beta-catenin-dependent mechanism.** *Biochimica et biophysica acta* 2007, **1773:**1028-1038.
291. Hattrup CL, Gendler SJ: **MUC1 alters oncogenic events and transcription in human breast cancer cells.** *Breast Cancer Research* 2006, **8**.
292. Ahmad R, Rajabi H, Kosugi M, Joshi MD, Alam M, Vasir B, Kawano T, Kharbanda S, Kufe D: **MUC1-C Oncoprotein Promotes STAT3 Activation in an Autoinductive Regulatory Loop.** *Science Signaling* 2011, **4:**13.
293. Schroeder JA, Al Masri A, Adriance MC, Tessier JC, Kotlarczyk KL, Thompson MC, Gendler SJ: **MUC1 overexpression results in mammary gland tumorigenesis and prolonged alveolar differentiation.** *Oncogene* 2004, **23:**5739-5747.
294. Yin L, Li Y, Ren J, Kuwahara H, Kufe D: **Human MUC1 carcinoma antigen regulates intracellular oxidant levels and the apoptotic response to oxidative stress.** *The Journal of biological chemistry* 2003, **278:**35458-35464.
295. Yin L, Huang L, Kufe D: **MUC1 oncoprotein activates the FOXO3a transcription factor in a survival response to oxidative stress.** *Journal of Biological Chemistry* 2004, **279:**45721-45727.
296. Sachdeva M, Mo YY: **MicroRNA-145 suppresses cell invasion and metastasis by directly targeting mucin 1.** *Cancer research* 2010, **70:**378-387.
297. van de Stolpe A, van der Saag PT: **Intercellular adhesion molecule-1.** *Journal of Molecular Medicine (Berlin, Germany)* 1996, **74:**13-33.
298. Jun CD, Carman CV, Redick SD, Shimaoka M, Erickson HP, Springer TA: **Ultrastructure and function of dimeric, soluble intercellular adhesion molecule-1 (ICAM-1).** *The Journal of biological chemistry* 2001, **276:**29019-29027.
299. Oh HM, Kwon MS, Kim HJ, Jeon BH, Kim HR, Choi HO, Na BR, Eom SH, Song NW, Jun CD: **Intermediate monomer-dimer equilibrium structure of native ICAM-1: Implication for enhanced cell adhesion.** *Experimental Cell Research* 2011, **317:**163-172.
300. Casasnovas JM, Stehle T, Liu JH, Wang JH, Springer TA: **A dimeric crystal structure for the N-terminal two domains of intercellular adhesion molecule-**

1. *Proceedings of the National Academy of Sciences of the United States of America* 1998, **95:**4134-4139.
301. Jun CD, Shimaoka M, Carman CV, Takagi J, Springer TA: **Dimerization and the effectiveness of ICAM-1 in mediating LFA-1-dependent adhesion.** *Proceedings of the National Academy of Sciences of the United States of America* 2001, **98:**6830-6835.
302. Yang YT, Jun CD, Liu JH, Zhang RG, Joachimiak A, Springer TA, Wang JH: **Structural basis for dimerization of ICAM-1 on the cell surface.** *Molecular Cell* 2004, **14:**269-276.
303. Oh HM, Lee S, Na BR, Wee H, Kim SH, Choi SC, Lee KM, Jun CD: **RKIKK motif in the intracellular domain is critical for spatial and dynamic organization of ICAM-1: Functional implication for the leukocyte adhesion and transmigration.** *Molecular Biology of the Cell* 2007, **18:**2322-2335.
304. Barreiro O, Yanez-Mo M, Serrador JM, Montoya MC, Vicente-Manzanares M, Tejedor R, Furthmayr H, Sanchez-Madrid F: **Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes.** *The Journal of cell biology* 2002, **157:**1233-1245.
305. Tilghman RW, Hoover RL: **E-selectin and ICAM-1 are incorporated into detergent-insoluble membrane domains following clustering in endothelial cells.** *Febs Letters* 2002, **525:**83-87.
306. Kuk JE, Nakane M, Lane B, Luly J: **ICAM-1 induction in human cultured cells by inflammatory cytokines.** *Faseb Journal* 1996, **10:**2788-2788.
307. Basoglu M, Yildirgan MI, Taysi S, Yilmaz I, Kiziltunc A, Balik AA, Celebi F, Atamanalp SS: **Levels of soluble intercellular adhesion molecule-1 and total sialic acid in serum of patients with colorectal cancer.** *Journal of Surgical Oncology* 2003, **83:**180-184.
308. Alexiou D, Karayiannakis AJ, Syrigos KN, Zbar A, Sekara E, Michail P, Rosenberg T, Diamantis T: **Clinical significance of serum levels of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in gastric cancer patients.** *American Journal of Gastroenterology* 2003, **98:**478-485.
309. Kamiyoshihara M, Kawashima O, Otani Y, Morishita Y: **Clinical significance of the preoperative serum-soluble intercellular adhesion molecule-1 in non-small cell lung cancer.** *Journal of Cardiovascular Surgery* 2002, **43:**729-734.
310. Kostler WJ, Tomek S, Brodowicz T, Budinsky AC, Flamm M, Hejna M, Krainer M, Wiltschke C, Zielinski CC: **Soluble ICAM-1 in breast cancer: clinical significance and biological implications.** *Cancer Immunology Immunotherapy* 2001, **50:**483-490.
311. Staunton DE, Dustin ML, Erickson HP, Springer TA: **The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus.** *Cell* 1990, **61:**243-254.
312. Diamond MS, Staunton DE, Marlin SD, Springer TA: **Binding of the integrin Mac-1 (CD11B/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation.** *Cell* 1991, **65:**961-971.
313. Stanley P, Hogg N: **The I domain of integrin LFA-1 interacts with ICAM-1 domain 1 at residue Glu-34 but not Gln-73.** *Journal of Biological Chemistry* 1998, **273:**3358-3362.
314. Sanders VM, Vitetta ES: **B cell-associated LFA-1 and T cell-associated ICAM-1 transiently cluster in the area of contact between interacting cells.** *Cellular immunology* 1991, **132:**45-55.

315. Etienne-Manneville S, Manneville JB, Adamson P, Wilbourn B, Greenwood J, Couraud PO: **ICAM-1-coupled cytoskeletal rearrangements and transendothelial lymphocyte migration involve intracellular calcium signaling in brain endothelial cell lines.** *Journal of Immunology* 2000, **165:**3375-3383.
316. Etienne S, Adamson P, Greenwood J, Strosberg AD, Cazaubon S, Couraud PO: **ICAM-1 signaling pathways associated with Rho activation in microvascular brain endothelial cells.** *Journal of Immunology* 1998, **161:**5755-5761.
317. Sumagin R, Sarelius IH: **Intercellular Adhesion Molecule-1 Enrichment near Tricellular Endothelial Junctions Is Preferentially Associated with Leukocyte Transmigration and Signals for Reorganization of These Junctions To Accommodate Leukocyte Passage.** *Journal of Immunology* 2010, **184:**5242-5252.
318. Cook-Mills JM, Deem TL: **Active participation of endothelial cells in inflammation.** *Journal of Leukocyte Biology* 2005, **77:**487-495.
319. Lewalle JM, Bajou K, Desreux J, Mareel M, Dejana E, Noel A, Foidart JM: **Alteration of interendothelial adherens junctions following tumor cell-endothelial cell interaction in vitro.** *Experimental Cell Research* 1997, **237:**347-356.
320. Lewalle JM, Cataldo D, Bajou K, Lambert CA, Foidart JM: **Endothelial cell intracellular Ca2+ concentration is increased upon breast tumor cell contact and mediates tumor cell transendothelial migration.** *Clinical & Experimental Metastasis* 1998, **16:**21-29.
321. Roskoski R: **Src protein-tyrosine kinase structure and regulation.** *Biochemical and Biophysical Research Communications* 2004, **324:**1155-1164.
322. Patwardhan P, Resh MD: **Myristoylation and Membrane Binding Regulate c-Src Stability and Kinase Activity.** *Molecular and Cellular Biology* 2010, **30:**4094-4107.
323. Boggon TJ, Eck MJ: **Structure and regulation of Src family kinases.** *Oncogene* 2004, **23:**7918-7927.
324. Li SSC: **Specificity and versatility of SH3 and other proline-recognition domains: structural basis and implications for cellular signal transduction.** *Biochemical Journal* 2005, **390:**641-653.
325. Irby RB, Mao WG, Coppola D, Kang J, Loubeau JM, Trudeau W, Karl R, Fujita DJ, Jove R, Yeatman TJ: **Activating SRC mutation in a subset of advanced human colon cancers.** *Nature Genetics* 1999, **21:**187-190.
326. Thomas SM, Brugge JS: **Cellular functions regulated by Src family kinases.** *Annual Review of Cell and Developmental Biology* 1997, **13:**513-609.
327. Summy JM, Gallick GE: **Src family kinases in tumor progression and metastasis.** *Cancer and Metastasis Reviews* 2003, **22:**337-358.
328. Liang FB, Liang J, Wang WQ, Sun AP, Udho E, Zhang ZY: **PRL3 promotes cell invasion and proliferation by down-regulation of Csk leading to Src activation.** *Journal of Biological Chemistry* 2007, **282:**5413-5419.
329. Cam WR, Masaki T, Shiratori Y, Kato N, Ikenoue T, Okamoto M, Igarashi K, Sano T, Omata M: **Reduced C-terminal Src kinase activity is correlated inversely with pp60(c-src) activity in colorectal carcinoma.** *Cancer* 2001, **92:**61-70.
330. Porter M, Schindler T, Kuriyan J, Miller WT: **Reciprocal regulation of Hck activity by phosphorylation of Tyr(527) and Tyr(416). Effect of introducing**

a high affinity intramolecular SH2 ligand. *The Journal of Biological Chemistry* 2000, **275:**2721-2726.

331. Yadav SS, Miller WT: **Cooperative activation of Src family kinases by SH3 and SH2 ligands.** *Cancer Letters* 2007, **257:**116-123.
332. Alexandropoulos K, Baltimore D: **Coordinate activation of c-Src by SH3- and SH2-binding sites on a novel, p130(Cas)-related protein, Sin.** *Genes & Development* 1996, **10:**1341-1355.
333. Thomas JW, Ellis B, Boerner RJ, Knight WB, White GC, Schaller MD: **SH2- and SH3-mediated interactions between focal adhesion kinase and Src.** *Journal of Biological Chemistry* 1998, **273:**577-583.
334. Boonyaratanakornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, Maller JL, Miller WT, Edwards DP: **Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases.** *Molecular Cell* 2001, **8:**269-280.
335. Liu XQ, Brodeur SR, Gish G, Songyang Z, Cantley LC, Laudano AP, Pawson T: **Regulation of c-Src tyrosine kinase activity by the Src SH2 domain.** *Oncogene* 1993, **8:**1119-1126.
336. Mendieta J, Gago F: **In silico activation of Src tyrosine kinase reveals the molecular basis for intramolecular autophosphorylation.** *Journal of Molecular Graphics & Modelling* 2004, **23:**189-198.
337. Frame MC: **Src in cancer: deregulation and consequences for cell behaviour.** *Biochimica Et Biophysica Acta-Reviews on Cancer* 2002, **1602:**114-130.
338. Zhu S, Bjorge JD, Fujita DJ: **PTP1B contributes to the oncogenic properties of colon cancer cells through src activation.** *Cancer Research* 2007, **67:**10129-10137.
339. Carlucci A, Porpora M, Garbi C, Galgani M, Santoriello M, Mascolo M, di Lorenzo D, Altieri V, Quarto M, Terracciano L, et al: **PTPD1 Supports Receptor Stability and Mitogenic Signaling in Bladder Cancer Cells.** *Journal of Biological Chemistry* 2010, **285:**39260-39270.
340. Verbeek BS, Vroom TM, Adriaansen-Slot SS, Ottenhoff-Kalff AE, Geertzema JG, Hennipman A, Rijksen G: **c-Src protein expression is increased in human breast cancer. An immunohistochemical and biochemical analysis.** *The Journal of Pathology* 1996, **180:**383-388.
341. Mandal M, Myers JN, Lippman SM, Johnson FM, Williams MD, Rayala S, Ohshiro K, Rosenthal DI, Weber RS, Gallick GE, El-Naggar AK: **Epithelial to mesenchymal transition in head and neck squamous carcinoma - Association of src activation with E-cadherin down-regulation, vimentin expression, and aggressive tumor features.** *Cancer* 2008, **112:**2088-2100.
342. Liu J, Bailey K: **Caveolin-1 up-regulation during epithelial-mesenchymal transition is mediated by Src and FAK.** *Clinical & Experimental Metastasis* 2009, **26:**898-898.
343. Liu XA, Feng RQ: **Inhibition of epithelial to mesenchymal transition in metastatic breast carcinoma cells by c-Src suppression.** *Acta Biochimica Et Biophysica Sinica* 2010, **42:**496-501.
344. Nagaharu K, Zhang XH, Yoshida T, Katoh D, Hanamura N, Kozuka Y, Ogawa T, Shiraishi T, Imanaka-Yoshida K: **Tenascin C Induces Epithelial-Mesenchymal Transition-Like Change Accompanied by SRC Activation and Focal Adhesion Kinase Phosphorylation in Human Breast Cancer Cells.** *American Journal of Pathology* 2011, **178:**754-763.

345. Guarino M: **Src Signaling in Cancer Invasion.** *Journal of Cellular Physiology* 2010, **223:**14-26.
346. Caccia D, Micciche F, Cassinelli G, Mondellini P, Casalini P, Bongarzone I: **Dasatinib reduces FAK phosphorylation increasing the effects of RPI-1 inhibition in a RET/PTC1-expressing cell line.** *Molecular Cancer* 2010, **9:**14.
347. Dong MY, Rice L, Lepler S, Pampo C, Siemann DW: **Impact of the Src Inhibitor Saracatinib on the Metastatic Phenotype of a Fibrosarcoma (KHT) Tumor Model.** *Anticancer Research* 2010, **30:**4405-4413.
348. Schweppe RE, Kerege AA, French JD, Sharma V, Grzywa RL, Haugen BR: **Inhibition of Src with AZD0530 Reveals the Src-Focal Adhesion Kinase Complex as a Novel Therapeutic Target in Papillary and Anaplastic Thyroid Cancer.** *Journal of Clinical Endocrinology & Metabolism* 2009, **94:**2199-2203.
349. Kong L, Deng ZH, Shen HY, Zhang YX: **Src family kinase inhibitor PP2 efficiently inhibits cervical cancer cell proliferation through down-regulating phospho-Src-Y416 and phospho-EGFR-Y1173.** *Molecular and Cellular Biochemistry* 2011, **348:**11-19.
350. Xu Y, Benlimame N, Su J, He Q, Alaoui-Jamali MA: **Regulation of focal adhesion turnover by ErbB signalling in invasive breast cancer cells.** *British Journal of Cancer* 2009, **100:**633-643.
351. Nakaigawa N, Weirich G, Schmidt L, Zbar B: **Tumorigenesis mediated by MET mutant M1268T is inhibited by dominant-negative Src.** *Oncogene* 2000, **19:**2996-3002.
352. Emaduddin M, Bicknell DC, Bodmer WF, Feller SM: **Cell growth, global phosphotyrosine elevation, and c-Met phosphorylation through Src family kinases in colorectal cancer cells.** *Proceedings of the National Academy of Sciences of the United States of America* 2008, **105:**2358-2362.
353. Sen B, Peng SH, Saigal B, Williams MD, Johnson FM: **Distinct Interactions Between c-Src and c-Met in Mediating Resistance to c-Src Inhibition in Head and Neck Cancer.** *Clinical Cancer Research* 2011, **17:**514-524.
354. Araujo J, Logothetis C: **Dasatinib: A potent SRC inhibitor in clinical development for the treatment of solid tumors.** *Cancer Treatment Reviews* 2010, **36:**492-500.
355. Nagaraj NS, Revetta F, Washington MK, Merchant NB: **Combined Inhibition of Src and EGFR Abrogates Tumor-Stromal Interaction and Angiogenesis in Pancreatic Cancer In Vivo.** *Pancreas* 2010, **39:**1335-1336.
356. Nagaraj NS, Washington MK, Merchant NB: **Combined Blockade of Src Kinase and Epidermal Growth Factor Receptor with Gemcitabine Overcomes STAT3-Mediated Resistance of Inhibition of Pancreatic Tumor Growth.** *Clinical Cancer Research* 2011, **17:**483-493.
357. Seoane S, Montero JC, Ocana A, Pandiella A: **Effect of Multikinase Inhibitors on Caspase-Independent Cell Death and DNA Damage in HER2-Overexpressing Breast Cancer Cells.** *Journal of the National Cancer Institute* 2010, **102:**1432-1446.
358. Mao W, Irby R, Coppola D, Fu L, Wloch M, Turner J, Yu H, Garcia R, Jove R, Yeatman TJ: **Activation of c-Src by receptor tyrosine kinases in human colon cancer cells with high metastatic potential.** *Oncogene* 1997, **15:**3083-3090.
359. Zhao Y, Planas-Silva MD: **Mislocalization of cell-cell adhesion complexes in tamoxifen-resistant breast cancer cells with elevated c-Src tyrosine kinase activity.** *Cancer Letters* 2009, **275:**204-212.

360. Nam JS, Ino Y, Sakamoto M, Hirohashi S: **Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis.** *Clinical Cancer Research* 2002, **8:**2430-2436.
361. Li YQ, Ren J, Yu WH, Li Q, Kuwahara H, Yin L, Carraway KL, Kufe D: **The epidermal growth factor receptor regulates interaction of the human DF3/MUC1 carcinoma antigen with c-Src and beta-catenin.** *Journal of Biological Chemistry* 2001, **276:**35239-35242.
362. Thirkill TL, Cao T, Stout M, Blankenship TN, Barakat A, Douglas GC: **MUC1 is involved in trophoblast transendothelial migration.** *Biochimica et biophysica acta* 2007, **1773:**1007-1014.
363. McDermott KM, Crocker PR, Harris A, Burdick MD, Hinoda Y, Hayashi T, Imai K, Hollingsworth MA: **Overexpression of MUC1 reconfigures the binding properties of tumor cells.** *International Journal of Cancer* 2001, **94:**783-791.
364. Kam JL, Regimbald LH, Hilgers JH, Hoffman P, Krantz MJ, Longenecker BM, Hugh JC: **MUC1 synthetic peptide inhibition of intercellular adhesion molecule-1 and MUC1 binding requires six tandem repeats.** *Cancer Research* 1998, **58:**5577-5581.
365. Shen Q: **The role of MUC1/ICAM-1 interaction in breast cancer cell motility.** University of Alberta, Laboratory medicine and pathology; 2006.
366. Mader CC, Oser M, Magalhaes MAO, Bravo-Cordero JJ, Condeelis J, Koleske AJ, Gil-Henn H: **An EGFR-Src-Arg-Cortactin Pathway Mediates Functional Maturation of Invadopodia and Breast Cancer Cell Invasion.** *Cancer Research* 2011, **71:**1730-1741.
367. Mellado M, Vila-Coro AJ, Martinez C, Rodriguez-Frade JM: **Receptor dimerization: A key step in chemokine signaling.** *Cellular and Molecular Biology* 2001, **47:**575-582.
368. Grotzinger J: **Molecular mechanisms of cytokine receptor activation.** *Biochimica Et Biophysica Acta-Molecular Cell Research* 2002, **1592:**215-223.
369. Brown RJ, Adams JJ, Pelekanos RA, Wan Y, McKinstry WJ, Palethorpe K, Seeber RM, Monks TA, Eidne KA, Parker MW, Waters MJ: **Model for growth hormone receptor activation based on subunit rotation within a receptor dimer.** *Nature Structural & Molecular Biology* 2005, **12:**814-821.
370. Livnah O, Stura EA, Middleton SA, Johnson DL, Jolliffe LK, Wilson LA: **Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation.** *Science* 1999, **283:**987-990.
371. Mendrola JM, Berger MB, King MC, Lemmon MA: **The single transmembrane domains of ErbB receptors self-associate in cell membranes.** *Journal of Biological Chemistry* 2002, **277:**4704-4712.
372. Constantinescu SN, Keren T, Socolovsky M, Nam HS, Henis YI, Lodish HF: **Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain.** *Proceedings of the National Academy of Sciences of the United States of America* 2001, **98:**4379-4384.
373. Zhang Y, Jiang J, Kopchick JJ, Frank SJ: **Disulfide linkage of growth hormone (GH) receptors (GHR) reflects GH-induced GHR dimerization - Association of JAK2 with the GHR is enhanced by receptor dimerization.** *Journal of Biological Chemistry* 1999, **274:**33072-33084.
374. Goldsmith JF, Lee SJ, Jiang J, Frank SJ: **Growth hormone induces detergent insolubility of GR receptors in IM-9 cells.** *American Journal of Physiology-Endocrinology and Metabolism* 1997, **273:**E932-E941.

375. Zhang Y, Guan R, Jiang J, Kopchick JJ, Black RA, Baumann G, Frank SJ: **Growth hormone (GH)-induced dimerization inhibits phorbol ester-stimulated GH receptor proteolysis.** *Journal of Biological Chemistry* 2001, **276:**24565-24573.
376. Yang RY, Yang KS, Pike LJ, Marshall GR: **Targeting the dimerization of epidermal growth factor receptors with small-molecule inhibitors.** *Chemical Biology & Drug Design* 2010, **76:**1-9.
377. Johnson BE, Janne PA: **Rationale for a phase II trial of pertuzumab, a HER-2 dimerization inhibitor, in patients with non-small cell lung cancer.** *Clinical Cancer Research* 2006, **12:**4436S-4440S.
378. Baselga J, Swain SM: **CLEOPATRA: A Phase III Evaluation of Pertuzumab and Trastuzumab for HER2-Positive Metastatic Breast Cancer.** *Clinical Breast Cancer* 2010, **10:**489-491.
379. Llovera M, Pichard C, Bernichtein S, Jeay S, Touraine P, Kelly PA, Goffin V: **Human prolactin (hPRL) antagonists inhibit hPRL-activated signaling pathways involved in breast cancer cell proliferation.** *Oncogene* 2000, **19:**4695-4705.
380. Meerzaman D, Xing PX, Kim KC: **Construction and characterization of a chimeric receptor containing the cytoplasmic domain of MUC1 mucin.** *American Journal of Physiology - Lung Cellular & Molecular Physiology* 2000, **278:**625-629.
381. Wang HH, Lillehoj EP, Kim KC: **MUC1 tyrosine phosphorylation activates the extracellular signal-regulated kinase.** *Biochemical and Biophysical Research Communications* 2004, **321:**448-454.
382. Blanchetot C, den Hertog J: **Antibody-induced dimerization of HARPTP alpha-EGFR chimera suggests a ligand dependent mechanism of regulation for RPTP alpha.** *Febs Letters* 2000, **484:**235-240.
383. Liu Y, Ross J, Komm BS, Bodine PVN, Billiard J: **Antibody-induced dimerization activates the Ror2 tyrosine kinase receptor and stimulates 14-3-3 phosphorylation and osteogenesis in human mesenchymal stem cells.** *Journal of Bone and Mineral Research* 2006, **21:**S98-S98.
384. Erickson-Miller CL, Taylor AH, Hopson CB, Lehr R, Trinh HN, Fischer EI, Doyle ML, Brigham-Burke MR, Young PR: **Dimerization of Epo receptor by a specific monoclonal antibody is sufficient for JAK2 activation, but not for proliferation or differentiation.** *Experimental Hematology* 1999, **27:**76.
385. Satoh S, Hinoda Y, Hayashi T, Burdick MD, Imai K, Hollingsworth MA: **Enhancement of metastatic properties of pancreatic cancer cells by MUC1 gene encoding an anti-adhesion molecule.** *International Journal of Cancer* 2000, **88:**507-518.
386. Croce MV, Isla-Larrain M, Remes-Lenicov F, Colussi AG, Lacunza E, Kim KC, Gendler SJ, Segal-Eiras A: **MUC1 cytoplasmic tail detection using CT33 polyclonal and CT2 monoclonal antibodies in breast and colorectal tissue.** *Histology & Histopathology* 2006, **21:**849-855.
387. Clackson T, Yang W, Rozamus LW, Hatada M, Amara JF, Rollins CT, Stevenson LF, Magari SR, Wood SA, Courage NL, et al: **Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity.** *Proceedings of the National Academy of Sciences of the United States of America* 1998, **95:**10437-10442.
388. ARIAD Pharmaceuticals I: **ARGENT™ Regulated homodimerization kit.** 2.0 edition. Cambridge, MA; 2002.

389. ARIAD Pharmaceuticals I: **RPD™ Regulated secretion/aggregation kit.** 2.0 edition. Cambridge, MA; 2002.
390. Zhou Y, Rajabi H, Kufe D: **MUC1-C oncoprotein is a target for small molecule inhibitors.** *Molecular Pharmacology Fast Forward* 2011, **79:**886-893.
391. Yin L, Wu Z, Avigan D, Rosenblatt J, Stone R, Kharbanda S, D. K: **MUC1-C oncoprotein suppresses reactive oxygen species-induced terminal differentiation of acute myelogenous leukemia cells.** *Blood* 2011.
392. Whitney ML, Otto KG, Blau CA, Reinecke H, Murry CE: **Control of myoblast proliferation with a synthetic ligand.** *Journal of Biological Chemistry* 2001, **276:**41191-41196.
393. Song GJ, Hinkle PM: **Regulated dimerization of the thyrotropin releasing hormone receptor affects receptor trafficking but not signaling.** *Molecular Endocrinology* 2005, **19:**2859-2870.
394. Parnes JR: **Molecular biology and function of CD4 and CD8.** *Advances in Immunology* 1989, **44:**265-311.
395. Boursier JP, Alcover A, Herve F, Laisney I, Acuto O: **Evidence for an extended structure of the T-cell coreceptor CD8-alpha as deduced from the hydrodynamic properties of soluble forms of the extracellular region.** *Journal of Biological Chemistry* 1993, **268:**2013-2020.
396. Meseguer M, Aplin JD, Caballero-Campo P, O'Connor JE, Martin JC, Remohi J, Pellicer A, Simon C: **Human endometrial mucin MUC1 is up-regulated by progesterone and down-regulated in vitro by the human blastocyst.** *Biology of Reproduction* 2001, **64:**590-601.
397. Maretzky T, Reiss K, Ludwig A, Buchholz J, Scholz F, Proksch E, de Strooper B, Hartmann D, Saftig P: **ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and, beta-catenin translocation.** *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102:**9182-9187.
398. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok K, Connelly PA: **Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor - Study of Lck- and FynT-dependent T cell activation.** *Journal of Biological Chemistry* 1996, **271:**695-701.
399. Chen JR, Capdevila J, Harris RC: **Overexpression of C-terminal Src kinase blocks 14,15-epoxyeicosatrienoic acid-induced tyrosine phosphorylation and mitogenesis.** *Journal of Biological Chemistry* 2000, **275:**13789-13792.
400. Carlomagno F, Vitagliano D, Guida T, Basolo F, Castellone MD, Melillo RM, Fusco A, Santoro M: **Efficient inhibition of RET/papillary thyroid carcinoma oncogenic kinases by 4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo 3,4-d pyrimidine (PP2).** *Journal of Clinical Endocrinology & Metabolism* 2003, **88:**1897-1902.
401. Gonzalez L, Agullo-Ortuno MT, Garcia-Martinez JM, Calcabrini A, Gamallo C, Palacios J, Aranda A, Martin-Perez J: **Role of c-Src in human MCF7 breast cancer cell tumorigenesis.** *Journal of Biological Chemistry* 2006, **281:**20851-20864.
402. Hiscox S, Jordan NJ, Smith C, James M, Morgan L, Taylor KM, Green TP, Nicholson RI: **Dual targeting of Src and ER prevents acquired antihormone resistance in breast cancer cells.** *Breast Cancer Research and Treatment* 2009, **115:**57-67.
403. Hanson JM, Browell DA, Cunliffe WJ, Varma J, Allen A, Hemming D, Shenton BK, Young JR, Higgs MJ, Brotherick I, Pearson JP: **MUC1 expression in**

primary breast cancer: the effect of tamoxifen treatment. *Breast Cancer Research and Treatment* 2001, **67:**215-222.
404. Paszkiewicz-Gadek A, Porowska H, Anchim T, Wotczynski S, Gindzienski A: **Biosynthesis of MUC1 mucin in human endometrial adenocarcinoma is modulated by estradiol and tamoxifen.** *Gynecological Endocrinology* 2003, **17:**37-44.
405. Jackson AL, Burchard J, Leake D, Reynolds A, Schelter J, Guo J, Johnson JM, Lim L, Karpilow J, Nichols K, et al: **Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing.** *RNA* 2006, **12:**1197-1205.
406. Meerzaman D, Shapiro PS, Kim KC: **Involvement of the MAP kinase ERK2 in MUC1 mucin signaling.** *American journal of physiologyLung cellular and molecular physiology* 2001, **281:**L86-91.
407. Leichert LI, Jakob U: **Protein thiol modifications visualized in vivo.** *Plos Biology* 2004, **2:**1723-1737.
408. Linke K, Jakob U: **Not every disulfide lasts forever: Disulfide bond formation as a redox switch.** *Antioxidants & Redox Signaling* 2003, **5:**425-434.
409. Jin CN, Rajabi H, Kufe D: **miR-1226 targets expression of the mucin 1 oncoprotein and induces cell death.** *International Journal of Oncology* 2010, **37:**61-69.
410. Fanayan S, Shehata M, Agterof AP, McGuckin MA, Alonso MA, Byrne JA: **Mucin 1 (MUC1) is a novel partner for MAL2 in breast carcinoma cells.** *Bmc Cell Biology* 2009, **10:**13.
411. Staubach S, Razawi H, Hanisch FG: **Proteomics of MUC1-containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF-7.** *Proteomics* 2009, **9:**2820-2835.
412. Lindfors HE, Venkata BS, Drijfhout JW, Ubbink M: **Linker length dependent binding of a focal adhesion kinase derived peptide to the Src SH3-SH2 domains.** *Febs Letters* 2011, **585:**601-605.
413. Melkonian KA, Ostermeyer AG, Chen JZ, Roth MG, Brown DA: **Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts - Many raft proteins are acylated, while few are prenylated.** *Journal of Biological Chemistry* 1999, **274:**3910-3917.
414. Michel V, Bakovic M: **Lipid rafts in health and disease.** *Biology of the Cell* 2007, **99:**129-140.
415. Mukai H: **Treatment strategy for HER2-positive breast cancer.** *International Journal of Clinical Oncology* 2010, **15:**335-340.
416. Ghosh R, Narasanna A, Wang SE, Liu SY, Chakrabarty A, Balko JM, Gonzalez-Angulo AM, Mills GB, Penuel E, Winslow J, et al: **Trastuzumab Has Preferential Activity against Breast Cancers Driven by HER2 Homodimers.** *Cancer Research* 2011, **71:**1871-1882.
417. Reilly PL, Woska JR, Jeanfavre DD, McNally E, Rothlein R, Bormann BJ: **The native structure of intercellular-adhesion molecule-1 (ICAM-1) is a dimer - correlation with binding to LFA-1.** *Journal of Immunology* 1995, **155:**529-532.
418. Tsakadze NL, Sithu SD, Sen U, English WR, Murphy G, D'Souza SE: **Tumor necrosis factor-alpha-converting enzyme (TACE/ADAM-17) mediates the ectodomain cleavage of intercellular adhesion molecule-1 (ICAM-1).** *Journal of Biological Chemistry* 2006, **281:**3157-3164.
419. Riedle S, Kiefel H, Gast D, Bondong S, Wolterink S, Gutwein P, Altevogt P: **Nuclear translocation and signalling of L1-CAM in human carcinoma cells**

requires ADAM10 and presenilin/gamma-secretase activity. *Biochemical Journal* 2009, **420:**391-402.
420. Li YP, Galileo DS: **Soluble L1CAM promotes breast cancer cell adhesion and migration in vitro, but not invasion.** *Cancer Cell International* 2010, **10:**12.
421. Zheng X, Jiang F, Katakowski M, Zhang ZG, Lu QE, Chopp M: **ADAM17 promotes breast cancer cell malignant phenotype through EGFR-PI3K-AKT activation.** *Cancer Biology & Therapy* 2009, **8:**1045-1054.
422. Gavert N, Sheffer M, Raveh S, Spaderna S, Shtutman M, Brabletz T, Barany F, Paty P, Notterman D, Domany E, Ben-Ze'ev A: **Expression of L1-CAM and ADAM10 in human colon cancer cells induces metastasis.** *Cancer Research* 2007, **67:**7703-7712.
423. Rosso O, Piazza T, Bongarzone I, Rossello A, Mezzanzanica D, Canevari S, Orengo AM, Puppo A, Ferrini S, Fabbi M: **The ALCAM shedding by the metalloprotease ADAM17/TACE is involved in motility of ovarian carcinoma cells.** *Molecular Cancer Research* 2007, **5:**1246-1253.
424. Chalaris A, Gewiese J, Paliga K, Fleig L, Schneede A, Krieger K, Rose-John S, Scheller J: **ADAM17-mediated shedding of the IL6R induces cleavage of the membrane stub by gamma-secretase.** *Biochimica Et Biophysica Acta-Molecular Cell Research* 2010, **1803:**234-245.
425. Micciche F, Da Riva L, Fabbi M, Pilotti S, Mondellini P, Ferrini S, Canevari S, Pierotti MA, Bongarzone I: **Activated Leukocyte Cell Adhesion Molecule Expression and Shedding in Thyroid Tumors.** *Plos One* 2011, **6**.
426. Liu PCC, Liu XD, Li YL, Covington M, Wynn R, Huber R, Hillman M, Yang G, Ellis D, Marando C, et al: **Identification of ADAM10 as a major source of HER2 ectodomain sheddase activity in HER2 overexpressing breast cancer cells.** *Cancer Biology & Therapy* 2006, **5:**657-664.
427. Esselens CW, Malapeira J, Colome N, Moss M, Canals F, Arribas J: **Metastasis-associated C4.4A, a GPI-anchored protein cleaved by ADAM10 and ADAM17.** *Biological Chemistry* 2008, **389:**1075-1084.
428. Sahin U, Blobel CP: **Ectodomain shedding of the EGF-receptor ligand epigen is mediated by ADAM17.** *Febs Letters* 2007, **581:**41-44.
429. Nagano O, Murakami D, Hartmann D, de Strooper B, Saftig P, Iwatsubo T, Nakajima M, Shinohara M, Saya H: **Cell-matrix interaction via CD44 is independently regulated by different metal loproteinases activated in response to extracellular Ca2+ influx and PKC activation.** *Journal of Cell Biology* 2004, **165:**893-902.
430. Horiuchi K, Le Gall S, Schulte M, Yamaguchi T, Reiss K, Murphy G, Toyama Y, Hartmann D, Saftig P, Blobel CP: **Substrate selectivity of epidermal growth factor-receptor ligand sheddases and their regulation by phorbol esters and calcium influx.** *Molecular Biology of the Cell* 2007, **18:**176-188.
431. Sanderson MP, Erickson SN, Gough PJ, Garton KJ, Wille PT, Raines EW, Dunbar AJ, Dempsey PJ: **ADAM10 mediates ectodomain shedding of the betacellulin precursor activated by p-aminophenylmercuric acetate and extracellular calcium influx.** *Journal of Biological Chemistry* 2005, **280:**1826-1837.
432. Hundhausen C, Schulte A, Schulz B, Andrzejewski MG, Schwarz N, von Hundelshausen P, Winter U, Paliga K, Reiss K, Saftig P, et al: **Regulated shedding of transmembrane chemokines by the disintegrin and metalloproteinase 10 facilitates detachment of adherent leukocytes.** *Journal of Immunology* 2007, **178:**8064-8072.

433. Maretzky T, Zhou W, Huang XY, Blobel CP: **A transforming Src mutant increases the bioavailability of EGFR ligands via stimulation of the cell-surface metalloproteinase ADAM17.** *Oncogene* 2011, **30:**611-618.
434. Pitroda SP, Khodarev NN, Beckett MA, Kufe DW, Weichselbaum RR: **MUC1-induced alterations in a lipid metabolic gene network predict response of human breast cancers to tamoxifen treatment.** *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106:**5837-5841.
435. Mohan MJ, Seaton T, Mitchell J, Howe A, Blackburn K, Burkhart W, Moyer M, Patel I, Waitt GM, Becherer JD, et al: **The tumor necrosis factor-alpha converting enzyme (TACE): a unique metalloproteinase with highly defined substrate selectivity.** *Biochemistry* 2002, **41:**9462-9469.
436. Black RA, Doedens JR, Mahimkar R, Johnson R, Guo L, Wallace A, Virca D, Eisenman J, Slack J, Castner B, et al: **Substrate specificity and inducibility of TACE (tumour necrosis factor alpha-converting enzyme) revisited: the Ala-Val preference, and induced intrinsic activity.** In *Proteases and the Regulation of Biological Processes. Volume* 70. Edited by Saklatvala J, Nagase H, Salvesen G; 2003: 39-52: *Biochemical Society Symposium*].
437. Caescu CI, Jeschke GR, Turk BE: **Active-site determinants of substrate recognition by the metalloproteinases TACE and ADAM10.** *Biochemical Journal* 2009, **424:**79-88.
438. Ring A, Dowsett M: **Mechanisms of tamoxifen resistance.** *Endocrine-Related Cancer* 2004, **11:**643-658.
439. Khodarev N, Ahmad R, Rajabi H, Pitroda S, Kufe T, McClary C, Joshi MD, MacDermed D, Weichselbaum R, Kufe D: **Cooperativity of the MUC1 oncoprotein and STAT1 pathway in poor prognosis human breast cancer.** *Oncogene* 2010, **29:**920-929.
440. Xu RD, Povlsen GK, Soroka V, Bock E, Berezin V: **A peptide antagonist of the ErbB1 receptor inhibits receptor activation, tumor cell growth and migration in vitro and xenograft tumor growth in vivo.** *Cellular Oncology* 2010, **32:**259-274.
441. Guarneri V, Barbieri E, Dieci MV, Piacentini F, Conte P: **Anti-HER2 neoadjuvant and adjuvant therapies in HER2 positive breast cancer.** *Cancer Treatment Reviews* 2010, **36:**S62-S66.
442. Yang RYC, Yang KS, Pike LJ, Marshall GR: **Targeting the Dimerization of Epidermal Growth Factor Receptors with Small-Molecule Inhibitors.** *Chemical Biology & Drug Design* 2010, **76:**1-9.
443. Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN: **The HER-2 Receptor and Breast Cancer: Ten Years of Targeted Anti-HER-2 Therapy and Personalized Medicine.** *Oncologist* 2009, **14:**320-368.
444. Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J: **Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells.** *Cancer research* 2001, **61:**4744-4749.
445. Baselga J, Albanell J, Molina MA, Arribas J: **Mechanism of action of trastuzumab and scientific update.** *Seminars in oncology* 2001, **28:**4-11.
446. Witters L, Scherle P, Friedman S, Fridman J, Caulder E, Newton R, Lipton A: **Synergistic inhibition with a dual epidermal growth factor receptor/HER-2/neu tyrosine kinase inhibitor and a disintegrin and metalloprotease inhibitor.** *Cancer Research* 2008, **68:**7083-7089.

447. Escrevente C, Morais VA, Keller S, Soares CM, Altevogt P, Costa J: **Functional role of N-glycosylation from ADAM10 in processing, localization and activity of the enzyme.** *Biochimica Et Biophysica Acta-General Subjects* 2008, **1780:**905-913.
448. Mohler KM, Sleath PR, Fitzner JN, Cerretti DP, Alderson M, Kerwar SS, Torrance DS, Ottenevans C, Greenstreet T, Weerawarna K, et al: **Protection against a lethal dose of endotoxin by an inhibitor of tumor-necrosis-factor processing.** *Nature* 1994, **370:**218-220.
449. Garton KJ, Gough PJ, Philalay J, Wille PT, Blobel CP, Whitehead RH, Dempsey PJ, Raines EW: **Stimulated shedding of vascular cell adhesion molecule 1 (VCAM-1) is mediated by tumor necrosis factor-alpha-converting enzyme (ADAM 17).** *Journal of Biological Chemistry* 2003, **278:**37459-37464.
450. Razawi H, Hughey R, Hanisch FG: **Lipid-raft-associated Re-Endocytosis and Recycling of MUC1 Play a Role in the Modulation of O-Glycan Profiles.** *Glycobiology* 2008, **18:**38.
451. Tellier E, Canault M, Rebsomen L, Bonardo B, Juhan-Vague I, Nalbone G, Peiretti F: **The shedding activity of ADAM17 is sequestered in lipid rafts.** *Experimental Cell Research* 2006, **312:**3969-3980.
452. Parr-Sturgess CA, Rushton DJ, Parkin ET: **Ectodomain shedding of the Notch ligand Jagged1 is mediated by ADAM17, but is not a lipid-raft-associated event.** *Biochemical Journal* 2010, **432:**283-294.
453. Berezovska O, Jack C, McLean P, Aster JC, Hicks C, Xia W, Wolfe MS, Kimberly WT, Weinmaster G, Selkoe DJ, Hyman BT: **Aspartate mutations in presenilin and gamma-secretase inhibitors both impair notch1 proteolysis and nuclear translocation with relative preservation of notch1 signaling.** *Journal of neurochemistry* 2000, **75:**583-593.
454. Pocobelli G, Newcomb PA, Trentham-Dietz A, Titus-Ernstoff L, Hampton JM, Egan KM: **Statin use and risk of breast cancer.** *Cancer* 2008, **112:**27-33.
455. Klawitter J, Shokati T, Moll V, Christians U: **Effects of lovastatin on breast cancer cells: a proteo-metabonomic study.** *Breast Cancer Research* 2010, **12:**20.

Appendix 1: Determining the effect of inhibition of MUC1-ECD cleavage on MUC1 DRM residency

A.1.1. Introduction and objectives

In recent years, the fluid mosaic model of cell membrane composition has been challenged by the discovery that biological membranes contain multiple distinct domains. These domains are defined by their protein, lipid, and cholesterol composition, and allow for spatial compartmentalization of cellular signalling. One such domain, loosely termed detergent-resistant membrane (DRM), is enriched in cholesterol, sphingolipids, and GPI-anchored or acylated membrane proteins and is resistant to extraction in Triton X-100 detergent at 4°C [182]. MUC1 has been reported to reside in DRMs, with evidence suggesting that recycling and glycosylation of MUC1 [407, 436] and partnership with other transmembrane proteins [406] are controlled by DRM localized processes. In addition, disruption of DRMs *in vivo* by cholesterol sequesterization with methyl-betacyclodextrin (MβCD) has been shown to disrupt ICAM-1 binding induced CaOs, indicating that DRMs are important in transmission of the MUC1/ICAM-1 signal [13]. The reported MUC1 protease, ADAM17, has been shown to exert both DRM-dependent and independent activity [437, 438], and the relationship between MUC1 cleavage and DRM residency has not been investigated. As we demonstrated in Chapter 6 that MUC1 cleavage is required for cell migration, elucidation of the relationship between MUC1-ECD cleavage and DRMs would provide information on the potential of synergistic combinations of metalloprotease inhibitors and DRM-disrupting compounds, such as statins, in a clinical setting. Also, as we demonstrated in Chapter 4 that MUC1 dimerization is required for Src recruitment and ICAM-1 binding induced signalling, we investigated the relationship between MUC1 dimerization and DRMs. Although these experiments are preliminary, they provide insight on the relationship between MUC1 function and DRM residency and direct future research.

A.1.2. Materials and Methods

A.1.2.1. Cell lines and reagents

Armenian Hamster CT2 monoclonal antibody (mAb), directed against the last 17 C-terminal amino acids of MUC1-CD (hereafter referred to as anti-MUC1-CD), was generously provided by Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ) [381]. Anti-Armenian hamster horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). Mouse anti-tubulin mAb were from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified eagle medium (DMEM), Opti-MEM media, Geneticidin (G418), trypsin, fetal bovine serum (FBS), and Lipofectamine 2000 were from Invitrogen, Inc. (Carlsbad, CA). Triton X-100, ammonium persulfate (AP) was from Fisher Scientific (Nepean, ON). Glycine, 40% (w/v) acrylamide solution, bromophenol blue, and Bradford protein assay reagent were from Bio-Rad Laboratories Ltd. (Hercules, CA). TEMED was from EMD Chemicals Inc. (Gibbstown, NJ).

Human breast cancer cell line T47D was from the American Type Culture Collection (ATCC) and were maintained in DMEM with 10% FBS and 6ug/ml insulin. 293T human embryonic kidney epithelial cells (HEK 293T) were from ATCC and maintained in DMEM with 10% FBS. Generation of MUC1-CFP-Fv HEK 293T transfectants was performed using Lipofectamine 2000 and Opti-MEM according to manufacturer's instructions, and maintained in DMEM with 10% FBS and 200ug/ml G418. All cell lines were cultured at 37°C in a humidified incubator containing 5% CO_2 (Water-Jacketed Incubator, Forma Scientific, Marietta, OH).

The pC1-Neo-hMUC1-TR+ FLAG plasmid carrying the MUC1 gene was kindly provided by Dr. Sandra Gendler and was used to generate MUC1-CFP-Fv (work by J. Rahn, Q.Shen, J. Zhang, and A. Bernier). The plasmid pC4-Fv1E encoding the FKBP

F36V variant followed by a c-terminal hemagglutinin (HA) epitope was generously provided by ARIAD Pharmaceuticals Inc and is described in [382]. To generate the MUC1-CFP-Fv, the FvHA domain of pC4-Fv1E was amplified by polymerase chain reaction (PCR) with a 5' primer (ATTTGTACAT GGCTTCTAGAGGAGTGC**)** and a 3' primer (CTCTTGTACACTGAAGTTCTCAGG ATCC**)** which introduced 3' and 5' BsrG1 restriction sites (underlined). The PCR product and the MUC1-CFP plasmid were digested with BsrG1, ligated, and sequenced to confirm insertion and orientation.

A.1.2.2. SDS-PAGE and Western blot analysis

For "DRM exclusion" experiments, cells were lysed in ice-cold Triton X-100 buffer (20mM Tris-HCl pH7.4, 150mM NaCl, 2mM EDTA, 1% glycerol, 1% Triton X-100, with fresh 0.5% (w/v) protease inhibitor cocktail and phosphatase inhibitor cocktail), homogenized with a 26 gauge needle and insoluble components pelleted by centrifugation at 14000 x g for 3 minutes. For "DRM inclusion" experiments, cells were lysed in RIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, with fresh 0.5% (w/v) protease inhibitor cocktail and phosphatase inhibitor cocktail), sonicated for 30 seconds, and centrifuged at 14000 x g for 3 minutes. Supernatants were removed and assayed for protein concentration using Bradford assay (Bio-Rad), followed by boiling for 10 minutes in 4x Laemmli sample buffer (LSB) (0.5M Tris-HCl pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol. Samples were either stored at -20°C or subjected to 4-20% SDS-PAGE. Electrophoresis was performed using the Bio-Rad Mini-PROTEAN II system at 60mA per gel, 80 V (constant voltage) for 15 minutes followed by 120 V for the remainder in running buffer (25mM Tris-Base pH 8.3, 192mM glycine, 0.1% SDS). The proteins were then transferred to nitrocellulose membrane (Bio-Rad) using the Bio-Rad Mini Trans-blot system on ice for 60 minutes at 100 V and 350 mA in transfer buffer (25mM Tris-Base

pH 8.3, 192mM glycine, 20% MeOH). Nitrocellulose membranes were then blocked in 5% (w/v) skim milk in Tris-buffered saline (TBS) (50mM Tris-Base pH 7.5, 150mM NaCl) with 0.05% (v/v) Tween-20 (TBS-T) for one hour at room temperature with gentle agitation. The membranes were then incubated with primary antibody overnight at 4°C at the concentration recommended by the manufacturer, followed by two 10 minute washes in TBS and one 10 minute wash in TBS-T. The membranes were then incubated with HRP-conjugated seconday antibody for one hour at RT at the concentration recommended by the manufacturer, following by another washing step identical to the first. Membranes were incubated with ECL plus western blotting reagent according to manufacturer's instructions, developed, scanned using a Canon Canoscan 8600F, imported into Image J (National Institutes of Health), analyzed for densitometry and/or adjusted and cropped for presentation.

If membranes were to be probed by additional antibodies to proteins such as tubulin, used as a protein loading control, they were first stripped of all original antibody by incubation in stripping buffer (62.5mM Tris-HCl pH 6.7, 2% SDS, 100mM β-mercaptoethanol) for 30 minutes at 50°C with gentle agitation. Following a wash step and re-blocking, membranes were probed as described above.

A.1.2.3. Densitometry

In order to quantitate changes in protein levels revealed by western blot, densitometric analysis of protein bands was performed. Using Image J software (NIH), each band in a series was assayed for pixel density. For assays in which changes in band ratio is of interest (ie. MUC1-CD dimer:total assays), only the ratio for each band set was calculated and comparisons between sets were made.

A.1.2.4. Statistics

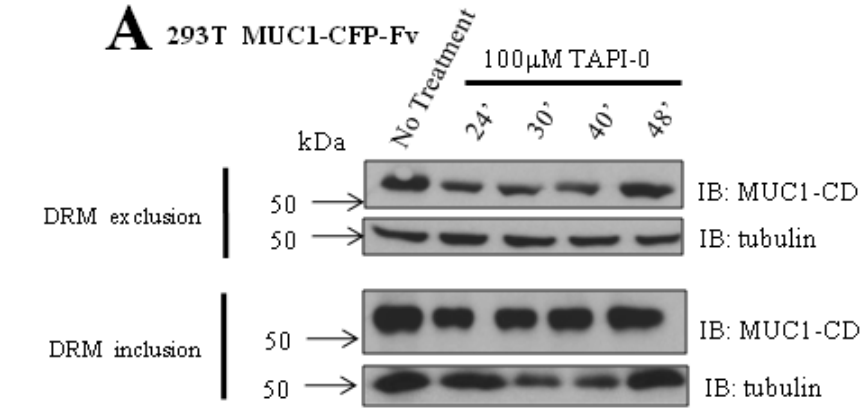
As the experiments presented in this chapter were only performed once, no statistical analysis was performed.

A.1.3. Results

A.1.3.1. Inhibition of MUC1-ECD cleavage results in increased residency of MUC1-on detergent resistant membranes

To determine the effect of cleavage inhibition on both MUC1 expression and residence on DRMs, we assayed MUC1-CD levels after treatment with TAPI-0 for 1, 6, 16, or 24 hours. Cells were lysed in either "DRM exclusion buffer" which would not recover proteins from DRMs, or "DRM inclusion buffer" which would solubilise DRMs and proteins from them would be recovered. Following densitometry of MUC1-CD bands and normalization to tubulin, the curves were compared to roughly determine MUC1-CD DRM residency (DRM inclusion – DRM exclusion = DRM). In other words, the difference between the two curves would represent the amount of MUC1-CD on the DRM.

In both MUC1-CFP-Fv transfected 293T cells (Fig A1.1A) and human breast cancer T47D cells (Fig A1.2A) treated with TAPI-0 for increasing periods, the levels of MUC1-CD in DRM exclusion experiments exhibited a downward trend, as illustrated by densitometry (Fig A1.1B; Fig A1.2B). When cells were lysed in DRM inclusion buffer, however, an upward trend was observed in both cell lines. The difference between these curves indicates increased residency of MUC1-CD on DRMs following TAPI-0 treatment for both MUC1-CFP-Fv and T47D cells. Interestingly, in both MUC1-CFP and T47D cells, treatment with TAPI-0 for 24 hours resulted in a return to near "No treatment"



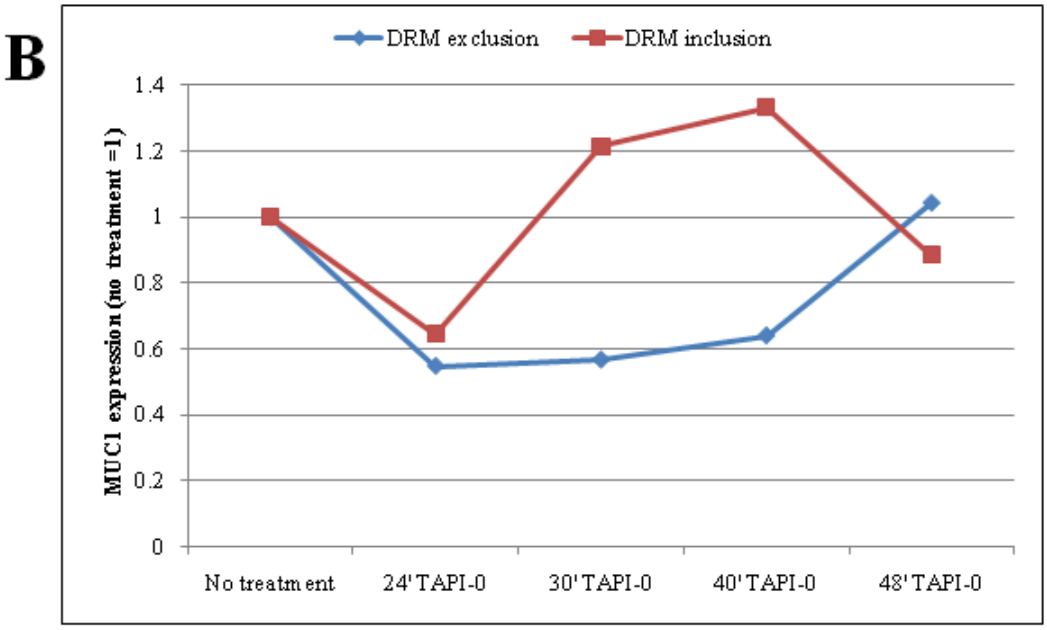


Figure A1.1. Effect of TAPI-0 treatment on MUC1-CD DRM residency in MUC1-CFP-Fv transfected HEK 293T cells. (A) Following treatment of MUC1-CFP-Fv cells with 100μM TAPI-0 for 24', 30', 40', or 48' hours, cells were lysed either in "DRM exclusion" (top panel) or "DRM inclusion" (bottom panel) buffer. Lysates were assayed for protein concentration and equal protein amounts were run on SDS-PAGE. Blots were probed with anti-MUC1-CD and anti-tubulin as a loading control. (**B**) Densitometry was performed on the MUC1-CD bands, with normalization to tubulin, and plotted with the "No treatment" conditions set to one and the remaining conditions expressed as a ratio.

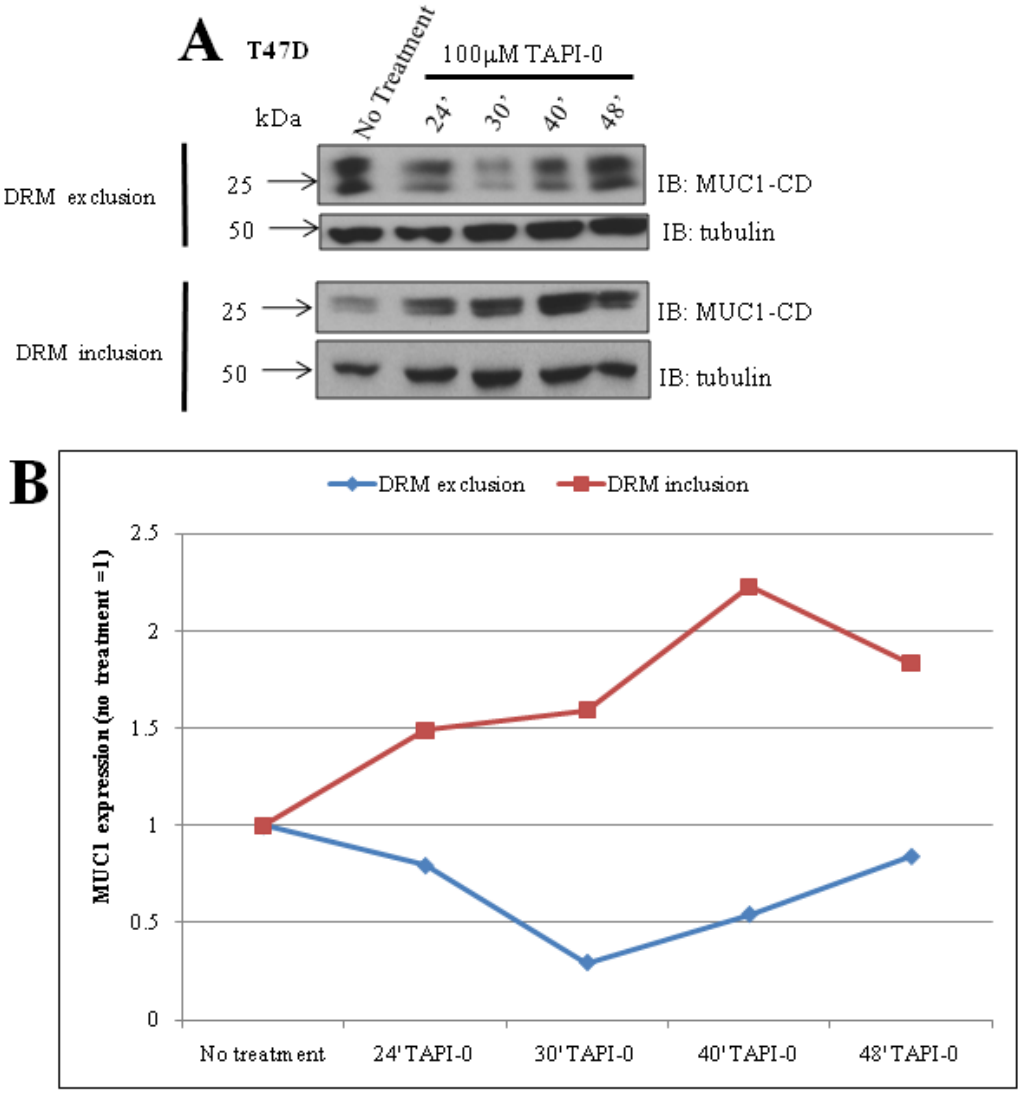


Figure A1.2. Effect of TAPI-0 treatment on MUC1-CD DRM residency in human breast cancer T47D cells. (A) Following treatment of T47D cells with 100μM TAPI-0 for 24', 30', 40', or 48' hours, cells were lysed either in "DRM exclusion" (top panel) or "DRM inclusion" (bottom panel) buffer. Lysates were assayed for protein concentration and equal protein amounts were run on SDS-PAGE. Blots were probed with anti-MUC1-CD and anti-tubulin as a loading control. (**B**) Densitometry was performed on the MUC1-CD bands, with normalization to tubulin, and plotted with the "No treatment" conditions set to one and the remaining conditions expressed as a ratio.

control values. As these experiments were only performed once, no statistical analysis was performed.

A.1.4. Discussion

For these sets of experiments, we assayed MUC1 levels in both DRM inclusive and exclusive lysis buffers following inhibition of MUC1-ECD cleavage to gain insight on the relationship between MUC1 cleavage and DRM residency. As these experiments were only performed once, we must limit our interpretation of these data to reflect the lack of statistical analysis.

We observed increased levels of MUC1-CD in DRM fractions in both transfected and T47D cells following treatment with TAPI-0. We conclude that inhibition of MUC1-ECD cleavage results in increased residence on MUC1 on DRMs. There are several possible mechanisms for this observation that could be investigated following completion of these studies. Inhibition of MUC1-ECD cleavage resulted in increased DRM residency, therefore it is possible that cleavage by ADAM17 on DRMs results in movement of MUC1 off DRMs. Non-DRM MUC1 could then be subject to interactions and further processing that could result in degradation. ADAM17 activity has been shown to occur on DRMs, supporting this hypothesis [437]. Contradictory to this, cleavage of the Notch ligand Jagged1 by ADAM17 was reported to be independent of DRMs, indicating that cell membrane localization of ADAM17 may be influenced by cell type and environment [437, 438]. Another possibility is that cleavage of MUC1 on DRMs results in DRM-dependent endocytosis and degradation, explaining the increased DRM residency following inhibition of cleavage. As MUC1 is a known substrate of γ-secretase, sequential cleavage by ADAM17 and γ-secretase could be followed by nuclear localization of MUC1 in a manner similar to that observed in NOTCH and amyloid

precursor protein [178, 219, 441]. As MUC1-induced transcription has been shown to upregulate genes involved in cell growth, motility, and angiogenesis, it is possible that nuclear translocation of MUC1 following DRM localized cleavage results in expression of this MUC1 associated "signature" [419]. Although this signature has been positively correlated with a poor prognosis, it is also possible that this signature functions in response to inflammation and insult in normal breast tissue, as cell growth, motility and angiogenesis are also components of the process of wound healing.

However, additional investigation of the relationship between MUC1 cleavage, DRM residency, and dimerization is needed to fully understand these phenomena, and their potential role in breast cancer, as the results presented here are preliminary.