Characterization of ADAM10 in Cervical and Oesophageal Cancers

Cleo Julie-Jean Williams

Dissertation presented in fulfilment of the requirements for the degree of Master of Science (M.Sc.) (Med) in Medical Biochemistry in the

Faculty of Health Sciences
University of Cape Town

Supervisor: A/Prof. Virna Leaner

Co-supervisor: A/Prof. Denver Hendricks

August 2013

Dedication

In loving memory of my grandfather Ernest David Williams who passed away from lung cancer. He will forever be missed, and never forgotten.

6 December 1932 - 16 June 2007

Plagiarism Declaration

I, Cleo Williams, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is submitted for another degree in this or any other university.

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Date October 30th 2013

Acknowledgements

I would like to thank God, for providing me with the strength and wisdom throughout this project. Opening my mind and guiding my thoughts leading me in the right direction when I needed it most.

I would sincerely like to thank my supervisor, A/Prof. Virna Leaner for affording me the opportunity to join her research group. I am grateful for her patience, invaluable guidance, support and advice during the project.

I would also like to thank the cancer lab for willingness to provide advice and alternative ideas when I was in need of it. Also being a lab family that any student would be loved and supported as blood relatives.

I would like to thank my family and friend for their support, patience and understanding throughout the duration of this project. A special thanks to my parents, Roderick Williams and Julie-Jean Williams, who always believed in my abilities and Zalda Lagardien for her motivation and consistent support during my postgraduate career.

I would also like to thank Prof. J Blackburn for nomination and funding in the form of a NRF Grant Holder-linked Bursary, iThembi Labs (NECSA) for funding the project and providing a bursary.

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Abbreviations

ADAM	A Disintegrin and Metalloproteinase
APP	Amyloid Precursor Protein
APS	Ammonium persulfate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CNS	Central Nervous System
DEPC	diethylpyrocarbonate
DHT	Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
dsDNA	double stranded DNA
DMEM	Dulbecco's modified Eagle's medium
EZH2	enchancer of zeste homolog 2
EGF	Epidermal Growth Factor
EMT	Epithelial-mesenchymal transition
E.coli	*Escherichia coli*
FCS	Foetal Calf Serum
HSIL	High Squamous Intraepithelial Lesions
HPV	*Human papillomavirus*
IGF1	insulin-like growth factor 1
KSFM	Keratinocyte Serum Free Medium
L1 –CAM	L1 Cell Adhesion Molecule
LSIL	Low Squamous Intraepithelial Lesions
MET	mesenchymal-epithelial transition

MTT	3-(4,5-Dimethylhtiazol-z-yl)-2,5-diphenyltetrazolium bromide
NSCLC	non-small cell lung cancer
PDACs	pancreatic ductal adenocarcinomas
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real-time PCR
RIPA	radioimmunoprecipitation assay
shRNA	short hairpin RNA
siRNA	short interference RNA
TEMED	N,N,N´,N´tetramethylethylenediamine
TNFα	tumour necrosis factor α
WHO	World Health Organisation

Abstract

A Disintegrin And Metalloproteinase 10 (ADAM10) is a cell surface molecule that activates proteins such as; adhesion molecules, cytokines, and growth factors. ADAM10 has been associated with the proliferation and metastasis of cancers including, breast, lung, and colon. While ADAM10 has been investigated in these cancers, very little is known of its role in cervical and oesophageal cancer. The aim of this study was to investigate the endogenous expression of ADAM10 in cervical and oesophageal patient material and representative cell lines. Our data shows that ADAM10 expression is elevated in both cervical and oesophageal cancers. To investigate the biological relevance of elevated ADAM10 expression in these cancers we inhibited either its expression or activity using an ADAM10 short-hairpin RNA plasmid, a small interference RNA, and a small molecule inhibitor, GI254023X in HeLa (cervical cancer cell line) and WHCO5 (oesophageal cancer cell line). The effects on cancer cell biology was analysed in the presence of ADAM10 inhibition. Our results showed no effect on cervical and oesophageal cancer cell proliferation when ADAM10 expression or activity was reduced. However, a clear change in cell morphology and Actin organisation was observed. Inhibition of ADAM10 also caused a significant increase in cell adhesion accompanied by decreased migration and invasion. The change in Actin organisation, cell adhesion, motility and invasion in ADAM10 inhibited cells was indicative of mesenchymal-epithelial transition (MET). In conclusion, the data presented in this study suggest that ADAM10 has a role in the metastasis and invasion of cervical and oesophageal cancer.

Chapter 1: Introduction

1.1 Preamble

ADAM10 is a cell surface protein that activates various cell surface molecules. It is a protein that has been associated with the proliferation and development of cancers such as; colon, lung, and gastric, amongst others. In studies conducted on cancer, metastasis and epithelial to mesenchymal transition (EMT) has been identified as one of the six major characteristics of cancer tumour progression. ADAM10 activates a number of proteins, adhesion molecules such as E-cadherin and L1-CAM, as well as growth factor molecules such as EGF, and alters their expression to increase cancer cell motility and proliferation. In cervical and oesophageal cancer the functionality of ADAM10 has not been investigated. As there are high incidence and mortality rate associated with cervical and oesophageal cancer in South Africa, the role of ADAM10 in these cancers formed the basis of this investigation.

1.2 Hallmarks of cancer

Cancer is a disease characterised by the deregulation of normal cell homeostasis and metabolism. This deregulation is identified in six key factors including; sustaining proliferative signalling, resisting cell death, inducing angiogenesis, enabling replicative immortality, activating invasion and metastasis, and evading growth suppressors (fig 1.1) (Cavallo et al. 2011, Hanahan & Weinberg 2011).

Figure 1.1: The Hallmarks of cancer. There are 6 characteristics that aid in the development and sustainability of cancer progression. These characteristics enable the proliferation, resistance to cell death, evading growth suppressors, inducing angiogenesis, enabling replicative immortality, and activating invasion and metastasis of cancer (Hanahan & Weinberg 2011)

Cancer cells can sustain proliferative signalling by producing growth factor ligands themselves; alternatively they can stimulate surrounding normal cells to supply these growth factors (Pietras & Ostman 2010). High-throughput DNA sequencing analysis of cancer cells has shown that cancer cells often contain somatic mutations that lead to activated growth factor receptors (Glaire et al. 2012). The proliferation of cancer cells is also enabled by their ability to disrupt the negative-feedback system of cells. In normal cells the negative-feedback system regulates the production and reduction of molecules in the cell, and the disruption of this cycle induces the uncontrolled proliferation of cancer cells (Hanahan & Weinberg 2011).

Cancer cells also have the ability to resist cell death and evade growth suppressors. They can circumvent powerful programs that negatively regulate cell proliferation by deactivating the functionality of tumour suppressors. Proteins such as retinoblastoma (RB) and p53 operate

the central control nodes that govern the regulation and decisions of cell proliferation (Sherr & McCormick 2002). Tumour cells evade apoptosis by commonly deregulating these proteins. Inhibitors of apoptosis, such as Bcl-2, Bcl-x_L, A1, etc. are often up-regulated to prevent cancer cell death and promote cancer pathogenesis (Hanahan & Weinberg 2011).

Other hallmarks of cancer include the ability of cancer cells to induce angiogenesis (Carmeliet & Jain 2000) (fig 1.1) (Ma et al. 2007) and achieve immortality. By inducing angiogenesis the tumorigenic cells are able to produce vasculature that enables the constant supply of nutrients and oxygen to and through the tumour (Carmeliet & Jain 2000). Cancer cells can achieve replicative immortality by maintaining telomeric DNA at sufficient lengths, thereby avoiding triggers for senescence and apoptosis (Reed 1999). These immortalised populations of cancer cells are frequently found after treatment in culture. They are a small population of cells that are resistant to treatment and proliferate uncontrollably.

Cancers are known for their exploitation of epithelial cells into proliferating tumorigenic cells, and adhesion molecules such as E-cadherin are up-regulated in these tumour bodies for their maintenance and establishment (Nijkamp et al. 2011). When the tumour is established and developed, cancer cells can communicate with the stroma cells to induce invasion and metastasis. Matastesis is caused by Epithelial-mesenchymal transition (EMT). EMT is a mechanism by which proliferative epithelial cells produce limipodia protrusions that transform the cells into migratory mesenchymal cells. These cells are able to migrate

through the stroma and establish a new tumour body by the reversion of mesenchymal cells to epithelial cells known as MET (Tsuji et al. 2009; Nijkamp et al. 2011).

These identified hallmarks are summarized as the ability of cancer cells to manipulate the established mechanisms of normal cells, disrupting the homeostasis, and causing uncontrolled and unmanaged proliferation, cell death evasion, invasion and metastasis. In addition to the six classic hallmarks, additional characteristics of cancer cells have been identified. An increasing focus area in research highlights two emerging hallmarks necessary for cancer development. One involves the capability to modify cellular metabolism to effectively support tumour progression and proliferation. The second allows cancer cells to evade immunological destruction by T and B lymphocytes, macrophages, and natural killer cells (Hanahan & Weinberg 2011).

Another area of cancer research that has received much attention are those focused on secondary treatments involved in reducing cancer cell metastasis. EMT is a process by which epithelial cells become mesenchyme-like, enabling them to move through an extracellular matrix during the process of metastasis (fig 1.2). It is characterised be the loss of cell adhesion, the repression of E-cadherin expression, and increased cell motility. The metastatic process of cancer cells throughout the body to seed secondary tumours at distant sites requires cancer cells to leave the primary tumour and to acquire migratory and invasive capabilities. In a process of EMT, cancer cells use developmental processes to increase migratory and invasive properties that involve the significant reorganization of the

actin cytoskeleton and the associated formation of membrane protrusions required for invasive growth (Yilmaz & Christofori 2009).

Epithelial cells are cells that establish the primary tumour body, which are compacted into clusters of growing cells. These cells have high cell-cell connectivity and inter-cell communication networks. High expression of communication and adhesion molecules such as E-cadherin (Larue & Bellacosa 2005) are generally notably present, with decreased expression of N-cadherin and vimentin (Nijkamp et al. 2011). At tumour developmental saturation, the epithelial cells at the surface transition into mesenchymal cells. Mesenchymal cells are cells that have decreased adhesion and connectivity to adjacent cells to allow for mobility and migration of tumour cells to other areas, having increased expression of N-cadherin (Yang & Weinberg 2008) and vimentin, and decreased expression of E-cadherin. These cells defuse into blood vessels, and invade areas to establish new tumour bodies. At this point the metastatic mesenchymal cells revert back to epithelial tumour forming cells, in a process known as MET, or mesenchymal-epithelial transition (fig 1.2) (Thompson & Newgreen 2005). Cells may at any point be in transition from EMT or MET, depending on the stage of tumour development (Zavadil et al. 2008).

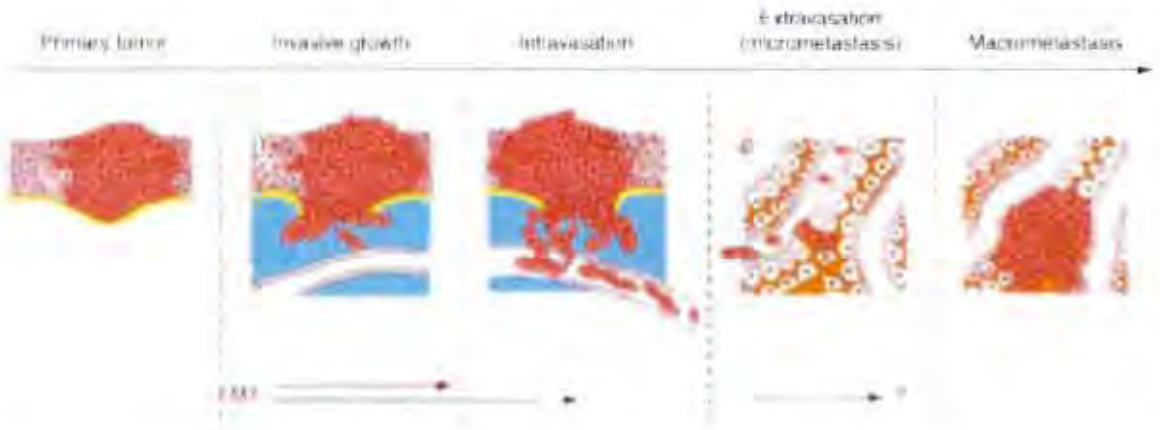


Figure 1.2 The epithelial–mesenchymal transition (EMT) and cancer progression. Tumour metastasis entails a series of distinct, sequential steps, involving local tumour growth, invasion by transmigration through basement membranes and non-tumour host tissue, intravasation into blood vessels, dissemination and survival in the bloodstream, extravasation, and re-establishment at distant sites (Huber et al. 2005)

The conversion of epithelial cells into mesenchymal cells requires alterations in morphology, cellular architecture, adhesion, and migration capacity. Commonly used molecular markers for EMT include increased expression of N-cadherin and vimentin, nuclear localization of β-catenin, and increased production of the transcription factors such as Snail1 (Snail), Snail2 (Slug), Twist, EF1/ZEB1, SIP1/ZEB2, and/or E47 that inhibit E-cadherin production. Phenotypic markers for an EMT include an increased capacity for migration and three-dimensional invasion, as well as resistance to apoptosis (Lee et al. 2006). During EMT, non-motile, polarized epithelial cells, embedded via cell-cell junctions in a cell collective, dissolve their cell-cell junctions and convert into individual, non-polarized, motile and invasive mesenchymal cells (Yilmaz & Christofori 2009). In colon cancer, the overexpression of ADAM10 causes enhanced L1-CAM cleavage and induced liver metastasis (Gavert et al. 2007), suggesting a role for ADAM10 in EMT.

1.3 Cervical and oesophageal cancer are major cancers in the South African context

1.3.1 Cancer of the *cervix uteri*

Cancer of the *cervix uteri* is the second most common cancer among women, and worldwide statistics estimated 529,800 new cases of women were diagnosed with cervical cancer, with more than 275, 100 deaths in 2008 (Jemal et al. 2011). In developed countries 76,500 women were diagnosed, with all of the lesions being detected early and no incidences of death recorded according to the statistics of 2008. However in developing countries of 453,300 women diagnosed with cervical cancer, 242,000 of these patients died from the disease (Jemal et al. 2011).

In South Africa the incidence of cervical cancer in all ages of women in relation to other cancers is ranked second, after breast cancer. According to statistical analysis performed by the World Health Organisation (WHO), their results suggested that cervical cancer is most prevalent in women aged 15-45, with the South African statistics showing an incidence and mortality rate of 26.8 and 14.8 (fig 1.3). The World Health Organization global estimations, demonstrate that the incidence of cervical cancer in the Southern African population to be the third highest, following Western and Eastern Africa. Collectively Africa has the highest incidence recorded for 2008, suggesting that cervical cancer is a significant threat to African and South African women (Aguado et al. 2010).

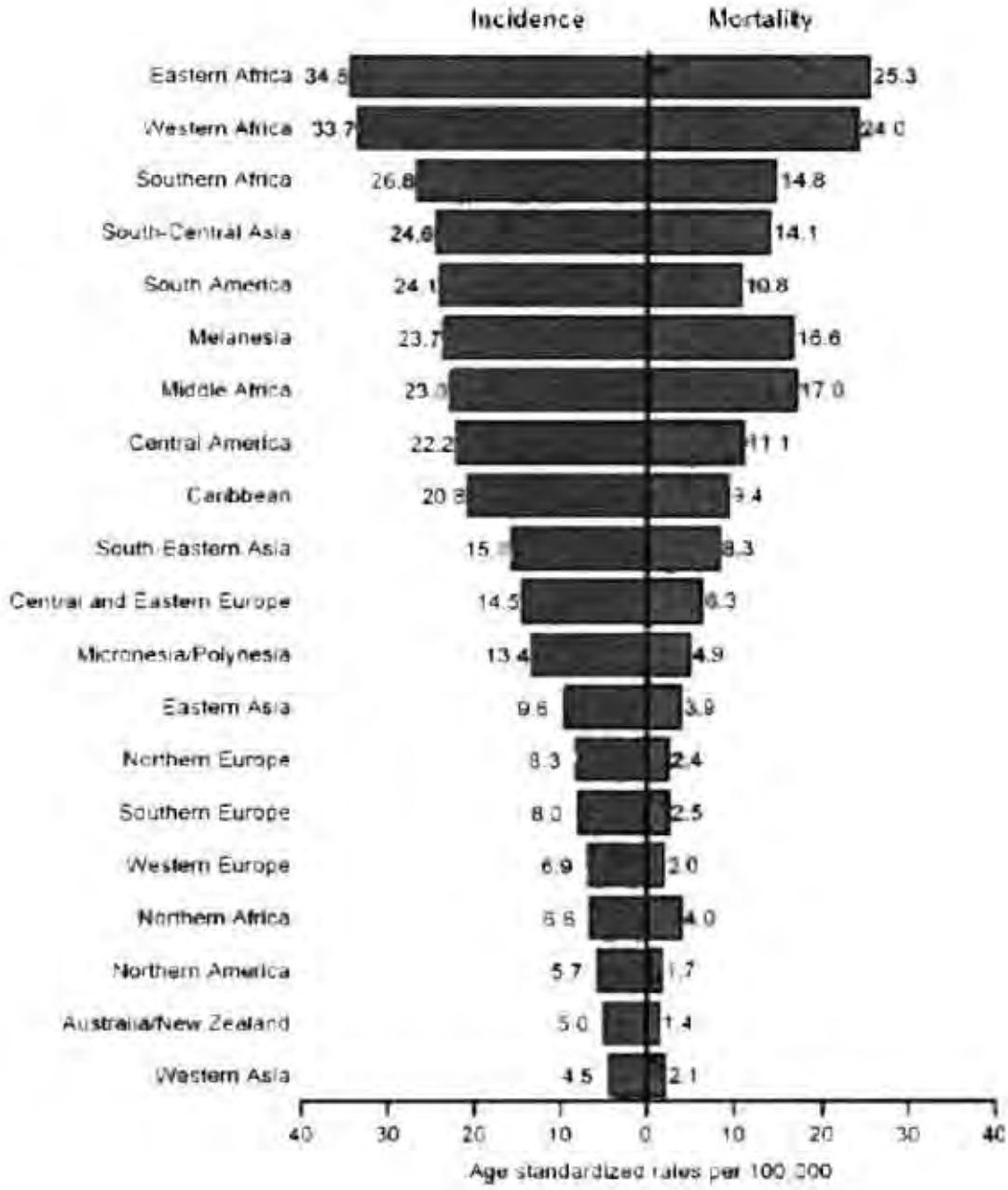


Figure 1.3 World Health Organisation statistical analysis of incidence and mortality rate of cervical cancer worldwide. This data depicts the incidence and mortality rates of women worldwide. Results listed in descending order, with Eastern, Western, and Southern Africa demonstrating the highest incidence and mortality rates. Statistics calculated rates per 100 000 women per year (Jemal et al. 2011).

The prevalence of cervical cancer in the developing world is largely due to limited access to good screening programs. The cancer develops in the cervix, which forms the lower third of the uterus. Cervical cancer typically develops slowly over a period of many years and can eventually spread to surrounding areas if left undetected. Disease progression is characterized in 3 stages; low squamous intraepithelial lesions (LSIL), high squamous intraepithelial lesions (HSIL), and invasive carcinoma. Lesions are detectable early by for example the papsmear, and can be surgically removed (Andersson et al. 2005). If the lesions are not treated they can progress into invasive carcinomas, where the cancer metastasizes and invades the deep stroma, causing the cancer to localize and infect other organs in the body. The only treatments available at this stage are hysterectomy, radiotherapy and/or chemotherapy (Im et al. 2003). Approximately 70% of cervical tumours develop from infections caused by the *Human papillomavirus* (HPV) 16 and 18 (Muñoz et al. 2006). It is anticipated that the occurrence of cervical will be significantly reduced as HPV vaccines such as Gardasil and Cervarix are rolled out in developing countries. These vaccines target the high risk HPV16 and HPV18 (Cervarix) and HPV16, HPV18, HPV6 and HPV11 (Gardasil).

1.3.2 Cancer of the oesophagus

The World Health Organization statistics of 2008 show an estimated 326,600 new cases of males and 482,300 females diagnosed with oesophageal cancer worldwide, with 276,100 deaths in males and 130,700 deaths of females recorded (Jemal et al. 2011). Although there were no statistical incidences recorded for the developed countries, an estimated 53,100 deaths were recorded in males with no fatalities recorded for females. In developing

countries however records have shown that there were 262,600 new cases in males and 137,900 in females, of which 223,000 of diagnosed males and 115,900 diagnosed female were estimated to have died from this disease (Jemal et al. 2011).

Globally, the incidence rates vary by nearly 16-fold, with the highest rates found in Southern and Eastern Africa and Eastern Asia and lowest rates observed in Western and Middle Africa and Central America in both males and females. Oesophageal cancer is three to four times more common among males than females. Southern Africa results indicated an incidence rate of 22.3 and 11.7 in males and females, respectively (fig 1.4).

Oesophageal cancer is classified into 2 subtypes; squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma arises from the squamous epithelial cells and forms in the upper part of the oesophagus, whereas adenocarcinoma arises from the glandular cells of the oesophagus at the junction of the oesophagus and the stomach. There are various reasons for the incidence of this poorly understood cancer, and compelling evidence suggests that chemicals such as nitrosomines, mycotoxins, cigarette smoke, excess alcohol intake, and opium abuse, are associated with the development of this cancer. Certain vitamin deficiencies have also been associated with the development of oesophageal cancer (Syrjänen 2002). HPV has been implicated in the aetiology of squamous cell carcinoma of the oesophagus, although not all oesophageal tumours contain the HPV DNA (Schiffman & Castle 2003). Symptoms of oesophageal cancer include dysplasia, pain and discomfort when eating. The treatments available include; surgery, chemotherapy and/or radiation therapy (Enzinger & Mayer 2003).

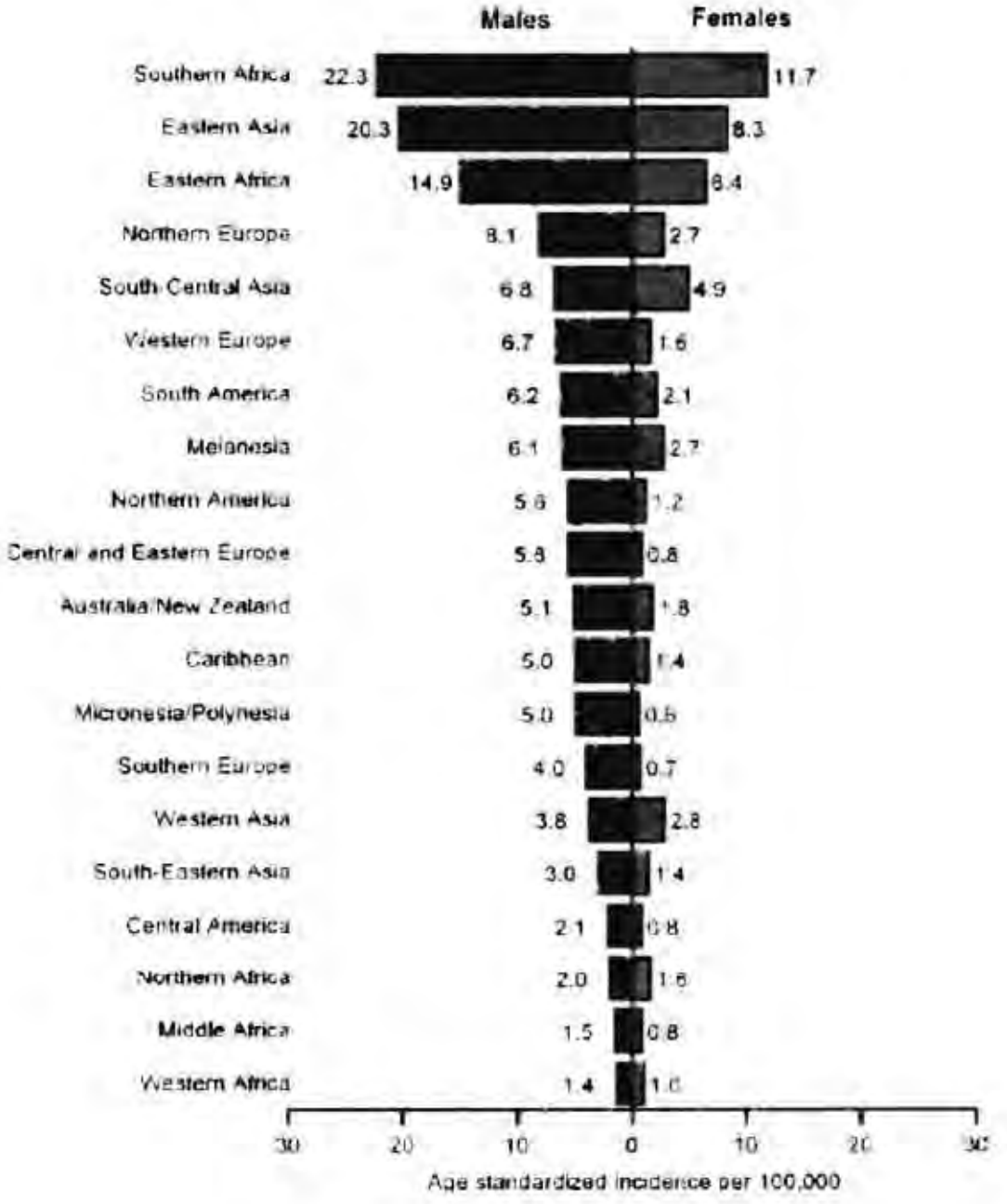


Figure 1.4 Incidence rate of oesophageal cancer in countries worldwide. This data depicts the incidence rates of males and females worldwide. Results listed in descending order, with Southern Africa, Eastern Asia, and Eastern Africa, demonstrating the highest incidence rates. Statistics calculated rates per 100 000 women per year (Jemal et al. 2011).

1.4 Biomarker discovery and targeted therapy

On-going cancer research has yielded many promising avenues for prevention, detection and treatment, however, newer and improved methods of treatment and diagnostics are still required. A more recent approach to cancer therapy is that of targeted therapy, where drugs are designed to specifically target proteins involved in the progression and maintenance of the tumour. With the designed selectively of the targeted drug, it is anticipated that these agents will potentially be less damaging to normal cells compared to treatments such as current chemotherapies and radiation that kill fast-dividing normal cells and thus cause unwanted side-effects.

Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules involved in tumour growth and progression (Srinivas et al. 2002). By focusing on molecular and cellular changes that are specific to cancer, targeted cancer therapies have the potential to be more effective than other types of treatment, including chemotherapy and radiotherapy, and less harmful to normal cells (Bang et al. 2010; Schiess et al. 2009). Biomarker discovery involves the identification of specific molecules in the cancer cell that may be targeted for treatment. Biomarkers are useful for the applications in, diagnoses, therapeutic target design, and provide predictions for patient response in the manipulation of the targeted biomarker (Adam et al. 2001).

Targeted cancer therapies interfere with cancer proliferation and spread in different ways. Many of these therapies focus on molecules involved in cell signalling pathways, which form complex communication systems that operate basic cellular functions and activities, such as cell division, cell movement, cell responses to specific external stimuli, and cell death (Srinivas et al. 2002). By blocking cell signaling pathways, targeted cancer therapies can help inhibit cancer progression and may induce cancer cell death through apoptosis, necrosis, or autophagy. Furthermore, cell surface proteins have been identified as key targets as both biomarkers and cancer therapeutics, as integration would not add to the complexity of the targeted drug designed (Brand et al. 2011).

A family of cell surface proteins that have received interest of late as potential targets for cancer therapy are the ADAM family of proteins (Duffy et al. 2011). Proteases belonging to the ADAMs family have been identified as targets for cancer therapeutic design, and have been associated with the migration and invasion of cancers.

1.5 The ADAMs family of proteases

The ADAMs family of proteases (a disintergrin and metalloprotease) are cell surface proteins belonging to the zinc protease subfamily and are key proteins in the ectodomain shedding of various membrane substrates, including growth factors, cytokines, growth factor receptors, cytokines receptors, and several different types of adhesion molecules (Duffy et al. 2011). ADAMs are composed of 6 domains each performing specific functions (Seals & Courtneidge 2003a). The domain structures of ADAMs consist of a prodomain (blocks

protease activity), a metalloprotease domain (protease activity), a disintegrin domain (adhesion activity), a cysteine-rich domain (adhesion activity), an EGF-like domain (stimulates membrane fusion), and a cytoplasmic tail domain (binds protein kinaseC-δ phosphorylated) (fig 1.6).

ADAM proteins are expressed as zymogens, or proenzymes, and removal of their prodomain results in their activation. The prodomain is typically removed by a proprotein convertase or by autocatalysis, depending on the specific ADAM (Duffy et al., 2011). In addition, certain ADAM family members can activate other family members. As an example, it has been suggested that the prodomain of ADAM10 can be cleaved by ADAM9 and ADAM15 (Tousseyn et al., 2009). Once activated, ADAM proteins cleave their substrates, and while different family members are reported to have different substrates, under certain circumstances the proteins in the ADAMs family can functionally compensate for each other (Tousseyn, *et al.*, 2009).

A.

B.

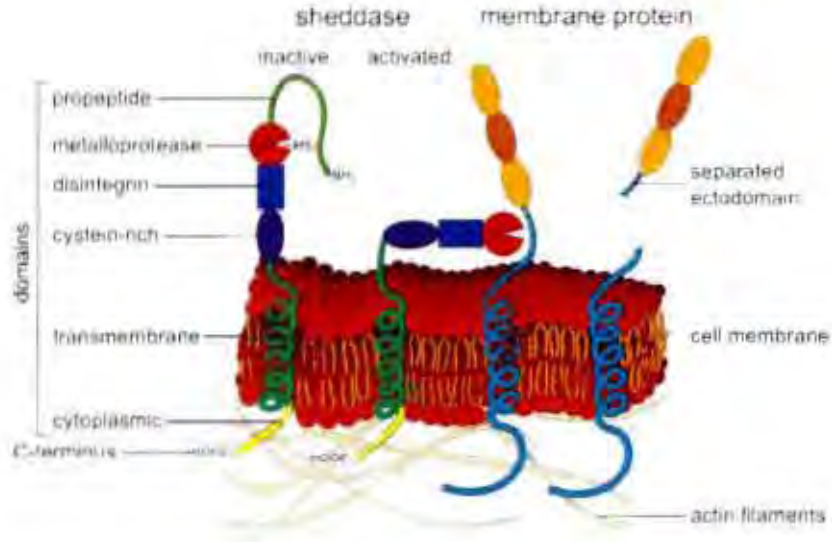


Figure 1.6: Schematic representation of ADAMs family domains and mechanism of action. **(A)** ADAMs are comprised of 6 domains; a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, and a cytoplasmic tail domain (Seals & Courtneidge 2003b). **(B)** A functional model for the localization of ADAMs in the cell membrane. The model diagram demonstrates the activation of an ADAM protease, causing the releasing the prodomain, cleaving a nearby molecule, causing its activation (Kuebi 2010).

Table 1.1 Human ADAMs proteins. Collective total of 19 ADAM proteases found in the human body. Each of these ADAMs are found to be expressed in various parts of the body.

ADAM	Expression	Potential function
2	testis	Sperm/egg binding/fusion
7	Epididymis	Undefined
8	Granulocytes/monocytes	Undefined
9	somatic	Sheddase/cell migration
10	somatic	Sheddase/cell fate determination
11	brain	Putative tumour suppressor
12	somatic	Sheddase/myoblast fusion
15	somatic	Cell-cell binding
17	somatic	Sheddase
18	testis	Undefined
19	somatic	Sheddase/dendritic cell division
20	testis	Undefined
21	testis	Undefined
22	brain	Undefined
23	brain	Cell adhesion/neural dev.
28	Epididymis/lung/lymphocytes	Immune surveillance
29	Testis	Undefined
30	Testis	Undefined
33	Somatic	Genetically linked to asthma

Information consolidated; (Seals & Courtneidge 2003a)

Proteins of the ADAMs family are expressed in; vertebrates, *Caenorhabditis elegans*, *Drosophila*, and *Xenopus* species, however they have not been found in *Escherichia coli*, *Saccharomyces cerevisiae*, or plants (Seals & Courtneidge 2003a). Table 1.1 Provides a list of some of the ADAMs expressed in humans as well as their localization and function. Even though the function of some of the ADAMs identified in humans that remain undefined, others have been associated with cell-cell communication, adhesion, and cell migration (Seals & Courtneidge 2003a).

1.6 ADAMs role in cancer progression

The ADAMs protein family has been associated with many cancers including breast, colon, lung, stomach, ovarian, and prostate cancer amongst others. In these cancers the ADAMs assists in various aspects of cancer cell proliferation, adhesion, migration and invasion.

ADAM8 has been associated with lung, kidney and brain cancers (Mochizuki & Okada 2007). ADAM8 was found to be highly expressed in lung cancer tissue compared to normal, and its high expression was significantly more common in advanced stage IIIB/IV adenocarcinomas compared to adenocarcinomas at stage I-IIIA, suggesting that ADAM8 could potentially be useful as a diagnostics target for lung cancer (Ishikawa et al. 2004). ADAM8 has also been reported to be overexpressed in renal cell carcinomas (Mochizuki and Okada, 2007). In brain cancer, ADAM8 has been shown to be overexpressed in the endothelia of blood vessels of tumour specimens compared to normal, and its activation has been associated with the invasive activity of glioma cells, suggesting that ADAM8 plays a significant role in brain tumour invasion (Wildeboer et al. 2006).

ADAM9 has been associated with cancers of the breast, pancreas, skin, and lung (Mochizuki & Okada 2007). It was found to be overexpressed in breast cancer, and may contribute to the release of HB-EGF leading to tumour progression (Hea et al. 2003). In pancreatic cancer, ADAM9 expression distinguishes pancreatic ductal adenocarcinomas (PDACs) from other solid pancreatic tumours. In addition, cytoplasmic ADAM9 overexpression was associated with poor differentiation and shortened survival. The result suggested that ADAM9 overexpression may be a contributor to aggressive PDACs. In skin cancer, ADAM-9 was

found to be upregulated in melanomas at the border of the tumor cell nests, implicating ADAM-9 in melanoma progression (Zigrino et al. 2005). In lung cancer, results suggest that ADAM9 overexpression enhances cell adhesion and invasion of non-small cell lung cancer cells via modulation of other adhesion molecules and changes in sensitivity to growth factors, thereby promoting metastatic capacity to the brain (Shintani et al. 2004).

ADAM12 has been associated with cancers of the liver, prostate, skin, and breast (Mochizuki & Okada 2007). ADAM12 was upregulated in liver cancer compared to normal and was associated with tumor aggressiveness and progression (Pabic et al. 2003). ADAM15 has been associated with cancers of the breast, prostate, stomach, and lung (Mochizuki & Okada 2007). In breast and prostate cancers, ADAM15 has been shown to be upregulated in cancer compared to normal samples. It is shown to have high expression in adenocarcinomas and is associated with metastasis of breast and prostate cancers. (Kuefer et al. 2006).

ADAM17 is associated with cancers including; breast, ovary, kidney, colon, and prostate (Mochizuki & Okada 2007). ADAM17 has been reported to be responsible for the release of tumour necrosis factor α (TNFα) and amyloid precursor protein (APP) and it has been suggested that ADAM17 may be a potential target for drug development in diseases that cause deregulation of these proteins (Moss et al. 2001). These diseases include; arthritis, diabetes, HIV cachexia, sepsis and cancer. The ADAM17 gene sequence is ~35% identical to ADAM10 (Moss et al. 2001). ADAM17 is upstream of ADAM10 and has been shown to perform similar functions (Sahin et al. 2004). In colon cancer, ADAM17 has been identified

to be upregulated in cancer compared to normal. Its overexpression in human primary colon carcinoma, leading to co-expression with EGFR in both neoplastic and endothelial cells, suggests a role for ADAM17 in tumour growth and angiogenesis (Jarry et al. 2005).

A link between ADAM10 and cancer has also been established (see section 1.7). In a microarray analysis previously performed in our laboratory that compared the gene expression profiles of normal and cancer tissues of the cervix (Ward et al., 2011, unpublished data , van der Watt et al., 2009, unpublished data), ADAM10 was found to be one of the most significantly overexpressed genes in cervical tumours compared to normal. This highly significant overexpression of ADAM10 in cervical tumours has not been previously described and formed the basis for further investigation in this study.

1.7 ADAM10 and cancer

ADAM10 (also known as Kuzbanian) is a membrane bound sheddase protein with a size of ~85kDa in the inactive form and ~65kDa in its mature active form (Tousseyn et al. 2009). Although ADAM10 is similar to the other ADAM family proteins in that it contains the reprolysin and disintegrin domains, it lacks the cysteine rich and EGF-like domains commonly found in the ADAM family proteins (Reiss et al. 2005; Reiss et al. 2006).

The list of ADAM10 substrates is constantly growing, highlighting the central role of ADAM10 in cell biology. Its known functions include its α-secretase activity; its shedding of

substrates like TNF-α, EGF, betacellulin, HER2, Notch, and collagen IV; and its central role in cellular adhesion (Duffy et al., 2011), mediated by its activation of various adhesion molecules, like E-cadherin. The shedding of E-cadherin causes the increased expression of β-catenin, which through signalling activates cyclin D1 that enhances cell proliferation (Maretzky et al. 2005) and also enables invasion and metastasis (Ma et al. 2007). ADAM10 thus presents as a protein that associates with various aspects of cancer progression making it a promising target for cancer therapy.

ADAM10 performs its many functions in various locations in the human body, being found in the brain, testis and the body generally (table 1.1). ADAM10 has been associated with diseases relating to; defective heart and central nervous system (CNS) development, defective vasculogenesis and somitogenesis, as well as, defective Notch signalling, and Alzheimer's disease (Huovila et al. 2005). In cancers ADAM10 has been identified to be important for the proliferation, invasion, and metastasis of various cancer types including; gastric, oral cavity, stomach, ovary, uterine, colon, leukaemia, and prostate, etc. (Pan et al. 2012) In colon cancer ADAM10 is an activator of L1-CAM, which is a neuronal adhesion molecule expressed in many tumour types and has been shown to be involved in proliferation, invasion, and metastasis both *in vitro* and *in vivo*. In colorectal tumor tissue, L1-CAM was exclusively localized at the invasive front of the tumour tissue that expresses nuclear β-catenin together with ADAM10 (Gavert et al. 2005). The other ADAM10 substrates include EGFR family of growth factors that are cleaved and activated by ADAM10, with HB-EGF being identified as a biomarker for the treatment of ovarian cancer (Miyamoto et al.

2004). The surface molecule TNFα that is linked to mantle cell lymphoma is activated by the cleavage of ADAM10 (Armanious et al. 2011).

In non-small cell lung cancer (NSCLC), ADAM10 overexpression was found to be positively correlated with Notch1 expression in the NSCLC tissues. The down-regulation of ADAM10 expression using ADAM10 short hairpin RNA (shRNA) reduced the migration and invasion of NSCLC cells (Guo et al. 2012). In gastric cancer, protein levels of ADAM 10 were up-regulated in cancerous lesions compared with adjacent non-cancerous tissues. The positive expression of ADAM10 correlated with age, size of tumour, location of tumour, depth of invasion, vessel invasion, lymph node, and distant metastasis. In stages I, II, and III, the 5-year survival rate of patients with high ADAM 10 expression was significantly lower than in patients with low expression. This therefore suggested that ADAM10 may be a useful marker for predicting gastric tumour progression and prognosis (Wang et al. 2011).

While a role for ADAM10 has been shown in some cancers, there is little to no information regarding its potential as a biomarker and therapeutic target for cervical and oesophageal cancer. In our laboratory we identified elevated ADAM 10 expression in cervical cancer patient tissue. These findings formed the basis for further investigation of a role for ADAM 10 in cervical cancer. Moreover, no study has previously investigated the involvement of ADAM10 in oesophageal cancer. Since cervical and oesophageal cancers are of the predominant cancers in South Africa, and we have access for both patient material and cell

lines for these cancers, our study included a concurrent investigation of ADAM 10 expression and biological relevance in these cancer types.

1.8 Aim

The aim of this project is to investigate ADAM10 expression and functional relevance in cervical and oesophageal cancer.

1.9 Objectives

1. To determine ADAM10 expression in;
    a. cancer patient material
    b. cancer cell lines including;. cervical and oesophageal
2. Determining the effect of inhibiting ADAM10 expression on cancer cell biology using: Short hairpin RNA (shRNA), short interference RNA (siRNA) and a small molecule inhibitor, GI254023X. The biological endpoints to be investigated include; cell proliferation, adhesion, motility and invasion.

Chapter 2: Materials and Methods

2.1 Bacterial culture

2.1.1 Large-scale plasmid expression in bacterial culture

To isolate large quantities of the short-hairpin RNA (shRNA) (Addgene, Cambridge, MA, USA, 19140) for ADAM10 knock-down, *Escherichia coli* (E.coli) cells were transformed with ADAM10 shRNA plasmid. ADAM10 shRNA plasmid culture was streaked out on 100 µg/ml ampicillin containing Luria Broth (LB) agar plates and incubated inverted at 37 °C overnight. A single colony was isolated and inoculated into a subculture of 5 ml LB containing 100 µg/ml ampicillin for 6 hours in a 37 °C incubator shaking at 1500 rpm. After the 6 hour incubation 1 ml of the sub-culture was inoculated into a 1 L conical flask containing 250 ml Luria broth containing 100 µg/ml ampicillin and incubated with shaking overnight at 37 °C. The next day 500µl of the bacterial culture was added to 500 µl 100 % glycerol and stored at -80 °C as a stock. The remaining culture was transferred into centrifuge bottles and cells were collected by centrifugation at 6000 rpm for 30 minutes at 4 °C. The supernatant was discarded and the remaining pelleted bacterial culture was stored at -80 °C. The negative control shRNA plasmid (OriGene Technologies, Rockville, USA, TR30007) was previously purchased and was readily available in the laboratory.

2.1.2 Large-scale Plasmid DNA Purification

Plasmid DNA was harvested and purified from the pelleted bacterial culture using the Qiagen Plasmid Maxi Kit as per the manufacturer's instructions (Qiagen, Duesseldorf, Germany). Purified plasmid DNA was quantified by the Nanodrop 2000c Spectrophotometer (Thermo Scientific, Inqaba Biotechnology, South Africa) and resolved on a 1 % agarose gel to validate the integrity of the isolated plasmid. The remaining plasmid stock was stored at -20 ^{o}C.

2.2 Patient material

Twenty-six cervical unpaired specimens including sixteen cervical cancer biopsies and ten normal cervical specimens were collected from patients from Groote Schuur Hospital (South Africa). Fifty-four oesophageal paired patient specimens were donated from the International Centre for Genetic Engineering and Biotechnology (ICGEB). In this study five normal and fifteen cancer cervical specimens were utilized for analysis, and ten paired oesophageal patient specimens.

2.3 Mammalian cell culture

2.3.1 Cell lines

To investigate the expression of ADAM10 in normal and cancerous cultured cell lines, we used a panel of cell lines. Normal cell lines used were: skin fibroblasts FG, gifted by Dr A. D. Marais (Groote Schuur Hospital, Cape Town); lung fibroblasts Wi38, purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA); and the hTERT-immortalized human oesophageal keratinocyte EPC2-hTERT cell line, obtained from Prof. A.K. Rustgi

(University of Pennysylvania, Philadelphia, USA). Cervical cancer cell lines CaSki (HPV16 positive), HeLa (HPV18 positive), Me180 (HPV68 positive), Ms751 (HPV18 and HPV45 positive), SiHa (HPV16 positive), and C33A (HPV negative, mutant p53) were acquired from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Oesophageal cancer cell lines WHCO1, 5, and 6, were obtained as a generous gift from Professor Robin Veale (Brown et al. 2011), and KYSE70, 410, and 520 were obtained from the German Resource Centre for Biological Material (Berlin, Germany).

2.3.2 Cell culture conditions

All cell lines, with the exception of EPC2, were cultured and maintained at a confluency of 60 % - 80 % in full medium comprised of; Dulbecco's modified Eagle's medium (DMEM) supplemented with; 10 % heat-inactivated foetal calf serum (FCS), 100 units/ml penicillin and 100 µg/ml streptomycin (P/S), and incubated at 37 °C in 5 % CO_2. Cells were detached using a trypsin-EDTA solution for sub-culture production and neutralized with full medium.

The oesophageal non-cancerous cell line EPC2 was cultured in Keratinocyte Serum Free Medium (KSFM) supplemented with Bovine Pituitary Extract (50 µg/ml), human epidermal growth factor (1 ng/ml), and 100 units/ml P/S. When cells reached a confluency of 80 % cells were sub-cultured with 5 ml trypsin-EDTA and neutralized with 5 ml soybean trypsin inhibitor. Cells were pelleted by centrifugation and re-suspended in supplemented KSFM medium before being re-seeded.

For long term storage, cells were re-suspended in cell freezing medium (10 % DMSO in DMEM) and subjected to slow freezing at 4 °C then transferred to a lower temperature of -80 °C for 48 hours. Thereafter cells were placed in storage in liquid nitrogen.

2.3.3 Mycoplasma testing

Mycoplasma is a microbial contaminant not visible to the naked eye. It is a contaminant commonly present in tissue culture and is therefore tested for 2-3 times a year. The cells are cultured in P/S-free DMEM for 4 days. Cells are transferred onto coverslips and incubated for an additional 24 hours. The cells are then fixed and stained with 0.5µg/ml Hoechst fluorescent DNA-binding stain, then mounted and visualised by fluorescent microscopy.

2.4 siRNA

For the inhibition of ADAM10 expression, short-interference RNA (siRNA) was used (Santa Cruz Biotechnology, sc-41410). Control siRNA consisting of a scrambled sequence (SIGMA-Aldrich, SIC-001) was used a non-silencing control. siRNA was provided in a lyophilised form and resuspended to 10µM according to the manufacturer's instructions.

2.5 Small molecule inhibitor GI254023X

GI254023X (fig C1), an inhibitor of ADAM10 activity, was kindly donated to us by Prof. Andreas Ludwig (Germany) (Hundhausen et al. 2003). Samples were supplied as two 10 µM stock aliquots in DMSO.

2.6 Plasmids

ADAM10 shRNA plasmid was purchased from Addgene (Addgene, Cambridge, MA, USA, 19140) (fig C3) and the pGFP-V-RS plasmid was used as negative control shRNA plasmid (OriGene Technologies, MD, USA, TR30007) (fig C2), which was available in our laboratory. Plasmid stocks were stored in nuclease-free H_2O at -20 °C at concentrations of 0.4 µg/µl.

2.7 Transient inhibition of ADAM10 expression and activity

2.7.1 Short interference RNA (siRNA) transfection

Cell were plated in full medium to a density of $5x10^4$ cells in a 35 mm dish and incubated overnight to allow cells to settle. Transfection mixtures were prepared as shown in table 1 below. Transfection mixtures were added dropwise to cells in 1 ml full medium and incubated at 37 °C overnight. After incubation the transfection medium was removed and replaced with 2 ml full medium. Cells were incubated for 48 hours, after which protein or RNA was harvested for analysis of the knockdown efficiency.

Table 1: Transfection Reaction Preparation Procedure:

Components and procedure	35mm dish (µl)
Full Medium	50
Transfectin (BioRAD)	1.25
Incubate at room temperature for 5 minutes	
siRNA (Control or ADAM10, 10µM, final concentration: 40nM)	4
Incubate at room temperature for 20 minutes	

2.7.2 Small-hairpin RNA (shRNA) transfection

Cells were seeded to a density of $15x10^4$ cells per 35mm dish or $3x10^5$ cells per 60mm dish and incubated overnight to allow cells to settle. In a 60 mm dish, transfection mixtures were prepared by adding 1 µg plasmid DNA to 50 µl full medium followed by 4 µl of GeneCellin (BioCellChallenge, Paris, France). The mixture was vortexed briefly and incubated at room temperature for 15 minutes. The mixture was added dropwise into 2.6 ml full medium on the cells. Treated cells were incubated at 37 °C for 5-6 hours. The treated medium was removed and replaced with fresh full medium and incubated overnight at 37 °C.

2.7.3 ADAM10-specific small molecule inhibitor (GI254023X) treatment

Cells were treated separately with various concentrations (0 µM, 3 µM, 5 µM, or 10 µM) of the small molecule inhibitor for 4-6 hours, after which biological assays including the MTT cell proliferation assay and the adhesion assay were performed.

2.8 RNA analysis

2.8.1 RNA extraction

RNA was harvested from biopsies and cell cultures grown to a confluency of 80 %, using QIAzol (Qiagen) as per the manufacturer's instructions. Purified RNA was re-suspended in diethylpyrocarbonate (DEPC) - treated dH_2O.

2.8.2 RNA Quantification

RNA was quantified by the Nanodrop 2000c. The absorbance ration of 260nm to 280nm was obtained by the program and the concentration calculated was indicated in ng/µl which was converted to µg/µl for future processing.

2.8.3 RNA agarose gel electrophoresis

To determine the integrity of the extracted RNA 1 µg of RNA was electrophoresed on a 1 % formaldehyde-agarose gel, containing 0.5 µg/µl ethidium bromide. RNA was re-suspended in loading dye and resolved for 20 minutes at 80 V in the gel.

2.8.4 cDNA synthesis

Reverse transcription of RNA into cDNA was performed using 500 ng of oligoT7-$(dT)_{20}$ or Random Hexomer primer added to 2 µg RNA and incubated at 70 °C for 10 minutes. The following reagents were added per reaction; 5x first strand buffer, 1.5 mM M_gCl_2, 0.5 mM dNTPs, 1 µl Reverse Transcriptase (Promega), 1 µl RNAsin inhibitor (Promega), and DEPC H_2O to a reaction volume of 20 µl. The mixture was incubated at 42 °C for 2 hours. Deactivation of reverse transcriptase was performed by incubation at 70 °C for 10 minutes. To produce a final concentration of approximately 0.1 µg/µl cDNA 30 µl DEPC-treated water was added. cDNA was aliquoted into 5 µl volumes and stored at -80 °C.

2.8.5 Primers

Primers were synthesized by the UCT oligonucleotide synthesis facility, and diluted to a working stock concentration of 20 µM per primer. Primers utilized are listed below in table 2.

Table 2: Sequences of Primers used for real-time PCR

Target	Forward Primer	Reverse Primer	Ta(°C)
Cyclophillin D	TGA GAC AGC AGA TAG AGC CAA GC	TCC CTGCCA ATT TGA CAT CTT C	60
β-glucuronidase (GusB)	CTC ATT TGG AAT TTT GCC CAT T	CCG AGT GAA GAT CCC CTT TTTA	55
GAPDH	GGC TCT CCA GAA CAT CAT CC	GCC TGC TTC ACC ACC TTC	55
ADAM10	TGG ATT GTG GCT TCA TTG GTG	TGC AGT TAG CGT CTC ATG TGT C	60

2.8.6 Quantitative Real-time PCR (qRT-PCR)

Quantitative real-time RT-PCR is a technique intended for quantifying the mRNA expression of a gene of interest, relative to a control, such as housekeeping genes (Provenzano and Mocellin 2007). The mechanism of SYBR Green I dye is to intercalate with the dsDNA causing fluorescence to be emitted relative to the amount of intercalation. By monitoring a melt curve for each analysed sample, it is possible to confirm the specificity of products amplified. Quantitative RT-PCR was performed using 2 µl cDNA or d.H_2O (negative control), and 0.5 µM specific forward and reverse primers. The PCR reaction was performed using the KAPA SYBR qPCR Master Mix (KAPA Biosystems, Cape Town, South Africa) and analysed by

the StepOne Real-Time PCR machine (Applied Bioscience, Life Technologies, South Africa). The reaction parameters for a 40 cycle reaction are listed in table 3 below.

Table 3: QRT-PCR experimental parameters

stage	Time	Temperature (°C)
Initial denaturation	3 min	95
Denaturation	5 sec	95
Annealing	20 sec	55-60 (adjusts per primer set)
Hold 1	15 sec	95
Hold 2	1 min	68
Hold 3	15 sec	95

The amplified PCR product was monitored by the cycle threshold (C_T) emitted by the SYBR Green I dye. The target product C_T value was chosen based on a chosen threshold distinguishable from background C_T values provided by amplified housekeeping genes, cyclophillin D and GusB. The relative expression of target mRNA was calculated and normalised to the average of the two housekeeping genes C_T value.

2.8.7 Agarose gel electrophoresis

QRT-PCR products were electrophoresed on a 2 % agarose gel, and visualized by ethidium bromide staining. This allows for the identification of the size of the PCR product produced, as well as to identify the presence or absence of non-specific amplified products and contaminants. PCR products were combined with 6X loading dye (Fermentas Life Science, Burlington, Ontario, Canada) and electrophoresed alongside a 1 kb DNA ladder (O'GeneRuler 1 kb DNA Ladder, Fermentus) at 70 V for approximately 1 hour.

2.9 Protein analysis

2.9.1 Protein extraction and quantification

Cells were cultured to a confluency of 80 %. Cells were then washed with 3 ml 1xPBS 3 times. Cells were lysed and proteins were harvested in radioimmunoprecipitation assay (RIPA) buffer with 1x complete protease inhibitor cocktail (Roche, Mannheim, Germany), 50 mM NaF, and 2 mM Na_3VO_4 Phosphatase inhibitor. Cells were scraped together and collected in a 1.5 ml eppindorf tube. Cell lysates were sonicated for 30 seconds and centrifuged to remove all cell debris. The supernatant was stored at -80 °C for future use. The protein concentration in each lysate was quantified using the Bicinchoninic Acid (BCA) Pierce Protein assay kit according to the manufacturer's instructions.

2.9.2 Western Blot Analysis

All gels were cast in 1.5 mm glass plates using the BioRAD MiniProtean Western blotting system. Separating and stacking gels were prepared as stipulated in table 4 below. The separating gel was cast first and allowed to set, followed by the stacking gel with a 10 well comb insert. Protein extracts were thawed on ice and prepared according to the calculated concentration. 4x loading dye was added followed by heating at 95 °C for 5 minutes and a quick spin for 10 seconds. Proteins were loaded alongside a protein ladder (Fermentus, Spectra Broad Range Protein Ladder) to detect protein sizes. Gels were electrophoresed in 1x running buffer at 100 V for 20 minutes after which the voltage was increased to 160 V for an additional 30-45 minutes until complete separation was achieved. Once optimal

separation was reached, proteins were transferred onto a nitrocellulose membrane (Amersham, Hybond ECL). Tanks were filled with 1x transfer buffer and transfer performed at 100 V for 80 minutes.

Table 4: Solutions with specified Volume as per gel for Western blot analysis

4% Stacking Gel		**10% Separating Gel**	
Solutions	**Volume**	**Solutions**	**Volume**
1M Tris pH6.8	312.5µl	1M Tris pH8.8	3750µl
10% SDS	25µl	10% SDS	100µl
Acrylamide	325µl	Acrylamide	3330µl
d.H_2O	1675µl	d.H_2O	2640µl
10% APS (Ammonium Persulfate)	30µl	10% APS (Ammonium Persulfate)	180µl
TEMED (N,N,N′,N′tetramethylethylenediamine)	5µl	TEMED (N,N,N′,N′tetramethylethylenediamine)	15µl

When transfer was complete, the membrane was rinsed in 1x TBS-T and blocked for 1 hour at room temperature with shaking. Primary antibodies were added specific to the protein of interest (shown in table 5) and incubated overnight 4 °C with shaking. The next day the membrane was washed 3 x for 10 min with 1xTBS-T followed by incubation with the secondary antibody for 1 hour at room temperature with shaking. The membrane was then washed 3x with 1xTBS-T for 10min. Detection was performed by the addition of a chemiluminescence detection substrate (SuperSignal West Pico, Pierce, Thermo Scientific, South Africa) to the membrane and exposure to X-ray film in a dark room. Exposed film was developed, rinsed in H_2O and fixed.

2.9.3 Antibodies

All Primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) listed in table 5 below, and secondary antibodies were purchased from BioRAD (BioRAD, Richmond, CA, USA).

Table 5: Primary and secondary antibody usage for Western Blot analysis.

Primary Antibodies (Cat. #)	Dilution/diluent(TBST)	Secondary antibodies	Dilution/diluent (5% milk)
ADAM10 (Sc-28358)	1:1000	Goat anti-mouse IgG (H+L)HRP conjugate	1:1000
β-tubulin (Sc-9104)	1:1000	Goat anti-rabbit IgG-HRP-conjugate	1:2000

2.10 Immunofluorescent analysis

2.10.1 Immunocytochemistry detection in cell lines

Cells were plated onto coverslips at $4x10^4$ cells per 35 mm dish and transfected with 1 μg ADAM10 shRNA plasmid or control shRNA plasmid, and cultured for 48 hours. Cells were rinsed in 1xPBS and fixed onto coverslip with 4 % paraformaldehyde for 15 minutes at room temperature. Cell were then washed 2x in ice cold PBS. To induce permeability cells were incubated in 0.5 % Saponin for 10 minutes. The cells were then washed 3x in PBS. Cells were blocked in 1 % BSA blocking solution for 30 minutes at room temperature. The cells were then incubated with primary antibody in 1 % BSA for 1 hour at room temperature (refer to

table 6 below), followed by washing cells 3x in PBS for 3 minutes. Secondary antibody was added in 1 % BSA and incubated in the dark for 1 hour. Cells were then washed 3x with PBS in the dark and stained with 0.1 µg/ml DAPI nuclear stain for 1 minute. Cell containing coverslips were rinsed with dH_2O and mounted with Mowiol onto glass slides. Slides were visualized on a Zeiss Axiovert 200 fluorescent microscope and analysed by monochrome Zeiss HR and Axiovision 4.8 software.

Table 6: Antibody conditions for Immunofluorescence

Primary Antibody	Dilution	Secondary antibody	Dilution
ADAM10 (Sc-28358)	1:100	Alexa anti-mouse	1:300
Phalloidin (P1951)	50µg/ml in 1%BSA	No secondary antibody required	

2.11 Cell biology assays

2.11.1 Cell proliferation assays

Cells were plated in sextuplet at $1x10^3$ cells per well of a 96 well plate. Cells were incubated at 37 °C for 24 hours. Full medium of 50 µl was added to each well, and each well was transfected with 2 µl transfection mixture, containing either siRNA or shRNA. Transfection mixtures were added dropwise to the cells and incubated for 24 hours at 37 °C, after which media was removed and replaced with 150 µl full medium and incubated at 37 °C with 5 % CO_2.

Ten microlitres of 5 mg/ml of MTT [3-(4,5-Dimethylhtiazol-z-yl)-2,5-diphenyltetrazolium bromide] reagent (Sigma), was added to each well and was incubated for 4 hours, followed by the addition of solubilisation buffer (10 % SDS, 10 mM HCl) and incubated overnight. The following day the colourometric change of each well was measured at OD 595 nm using a microplate reader (Beckman). MTT analysis was repeated for the remaining days.

2.11.2 Migration assays

Cells were plated at $3x10^5$ cells per 60mm dish and transfected with 1 µg shRNA ADAM10 or control shRNA plasmid. Cells were cultured for 24 hours thereafter cells were trypsinized and counted. A total of $25x10^4$ cells were re-plated in duplicate in a 24 well plate for 100 % cell confluency for both treatments, representing 0 hour and 10 hour time points. Cells were cultured for an additional 24 hours to allow cells to settle. The following day a scratch of equal size was introduced to each well. Each well was washed with 1 x PBS to remove all unbound cells. The 2 wells representatives of 0 hours were fixed in 200 µl 4% paraformaldehyde for 15 minutes and stained with 100 µl 1 % crystal violet for 1 minute. Stained cells were washed 3x with 500 µl 1x PBS and allowed to dry. After 10 hours the remaining 2 wells representative of the 10 hr time points were also fixed and stained. Stained wells were allowed to dry completely overnight. Wound closure was captured by a microscope camera and the migration of cells was analysed.

For migratory analysis of cells treated with the small molecule ADAM10 inhibitor (GI254023X) cells were plated in 4 wells to 100 % confluency and allowed to settle overnight. The next day a wound of equal size was introduced in all 4 wells and unbound cells were removed by 1x PBS washes. Cells were treated with 10 µM GI254023X in 2 representative wells for time 0 hours and 10 hours. Fixing and staining were performed as mentioned above.

2.11.3 Adhesion assays

2.11.3.1 Un-coated adhesion assay

Cells were pre-treated with shRNA plasmid or GI254023X ADAM10 inhibitor. Thereafter of $5x10^4$ cells of each treatment condition was added to 8 wells of a 24-well plate and incubated for 1 hour at 37 °C. The medium was removed from all the wells and discarded. Half of the wells for each treatment were washed with 3x with 300 µl 1x PBS to remove non-adherent cells and the unwashed wells were essential for a total cell control. Both washed and unwashed wells were then fixed with 100 µl 4 % paraformaldehyde for 15 minutes and stained with crystal violet for 5 minutes. All the wells were washed 3x with dH_2O to remove excess crystal violet and allowed to dry overnight at room temperature. The number of cells in various fields of view were counted for each treatment and calculated in relation to the number of cells in the unwashed wells accordingly.

2.11.3.2 Fibronectin-coated adhesion assay

Cells were pre-treated with shRNA plasmid or GI254023X ADAM10 inhibitor at concentrations of 0 µM, 5 µM, and 10 µM. In a 96 well plate 40 µl 20 µg/ml fibronectin was coated on the base of each well and incubated at 37°C for 1 hour. Wells were then washed 2x with 200 µl wash buffer and blocked in 200 µl binding block buffer for 1 hour at 37 °C. The binding block buffer was washed off with 200 µl wash buffer and incubated on ice for 10 minutes. A total of $8x10^4$ cells were plated into 6 wells per treatment and incubated at 37 °C for 30 minutes, with gentle tapping every 3 minutes. Thereafter unbound cells were washed 3x with 200 µl wash buffer. Bound cells were fixed in 50 µl 4 % paraformaldehyde for 15 minutes and stained with 60 µl crystal violet for 10 minutes at room temperature. Excess crystal violet was washed 3x with 100 µl d.H_2O and allowed to dry overnight in a fumehood. An amount of 50 µl 2 % SDS was added to solubilise stained cells and colourmetric intensity was analysed on a microplate reader at 550 µm. Data was calculated and illustrated by graphical representation.

2.11.4 Invasion assay

Cells were pre-treated with shRNA plasmid or GI254023X ADAM10 inhibitor at concentrations of 0 µM and 10 µM. In a 12 well plate 12 well chambers were coated with 120 µl of 1:3 of Matrigel to serum-free medium. The chambers were incubated in a 37 °C incubator for 4 hours to allow the matrigel to solidify. Thereafter 1.4 ml full medium was added to each of the 12 wells. Then $5x10^5$ serum-starved and treated cells were added to the chamber in a volume of 700 µl serum-free medium. Cells were incubated at 37 °C for 48

hours, followed by fixing and staining with crystal violet. Various fields of invaded cells were counted and analysed accordingly. Data was graphical representation.

2.12 Statistical analysis

Experiments were performed in triplicate and repeated at least two times where possible. Results are represented as the mean value ± standard deviation. The Student's *t*-test was applied to calculate statistical significant differences between samples. The 2-tailed distribution was used, showing either equal variance for matched treated-untreated samples or unequal variance for unmatched samples. Results were determined to be statistically significant if the p-value was < 0.05. Statistical significance is marked on the graphs with asterisks, with $p < 0.05$ represented by *, $p < 0.005$ represented by **, and $p < 0.0001$ represented by ***. Calculations were performed using either Microsoft Excel or GraphPad Prism.

Chapter 3: ADAM10 expression in cervical and oesophageal cancer

3.1 Introduction

Recent studies have shown that ADAM10 is overexpressed in various types of cancer (Ko et al. 2007). In prostate cancer ADAM10 expression has been shown to be regulated by growth factors such as the insulin-like growth factor 1 (IGF1) and epidermal growth factor (EGF) (McCulloch et al. 2004). Dihydrotestosterone (DHT) regulation of ADAM10 expression has also been associated with the proliferation, motility and invasion of cancer calls (McCulloch et al. 2000). Increased ADAM10 expression has been associated with the increased activation of amyloid precursor protein (APP), and its association with the proliferation of oral squamous cell carcinomas (Ko et al. 2007). In human colon cancer ADAM10 has been shown to be over-expressed and associated with the activation of L1-CAM which leads to metastasis and invasion of cancer cells in the colon (Gavert et al. 2007). In these cancers the misregulation of ADAM10 has proven to assist in the progression of cancer, either directly or indirectly.

ADAM10 has been implicated in the development of various cancers, and found to be pertinent for the proliferation, migration and invasion of these cancers (Doberstein et al. 2011; Guo et al. 2012). Very little however is known regarding ADAM10 expression in cervical and oesophageal cancer cells.

In our laboratory, gene expression analysis using cDNA microarrays to compare the gene expression patterns of normal and cervical cancer identified genes that have increased expression in cancer patient material compared to normal. Amongst these genes ADAM10 was identified to have significantly increased expression in cervical cancer tissue compared to normal (Ward et al. 2011).

The objective of this study was therefore to independently validate ADAM10 expression levels in both cervical and oesophageal cancer. The expression of ADAM10 was analysed in both patient material and cancer cell lines.

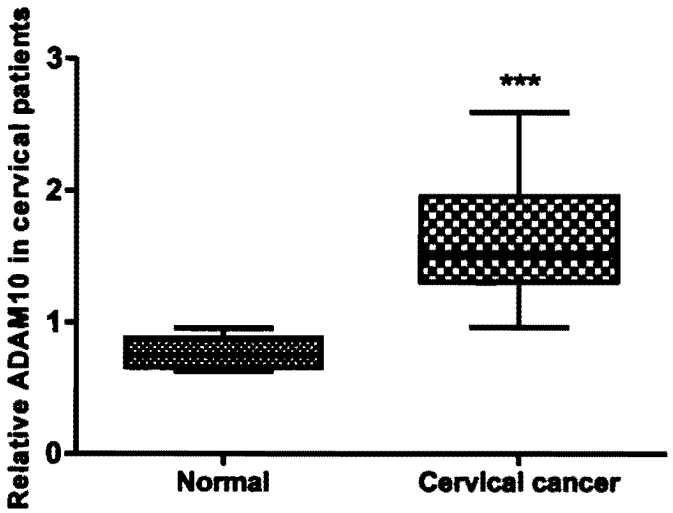
3.2 Results

3.2.1 ADAM10 expression in cancer patient material

3.2.1.1 Expression of ADAM10 in cervical patient specimens

To compare the gene expression patterns of normal and tumour tissue of the cervix, a microarray analysis was performed in our laboratory, using RNA isolated from ten normal and sixteen cervical cancer tissue biopsies (obtained from the Department of Obstetrics and Gynaecology, Groote Schuur Hospital, University of Cape Town, South Africa). Numerous genes were found to be differentially expressed between the normal and cancer samples, and amongst the most significantly upregulated in the cancer group was ADAM10 (with a p value of < 0.00001) (fig 3.1A).

A



B

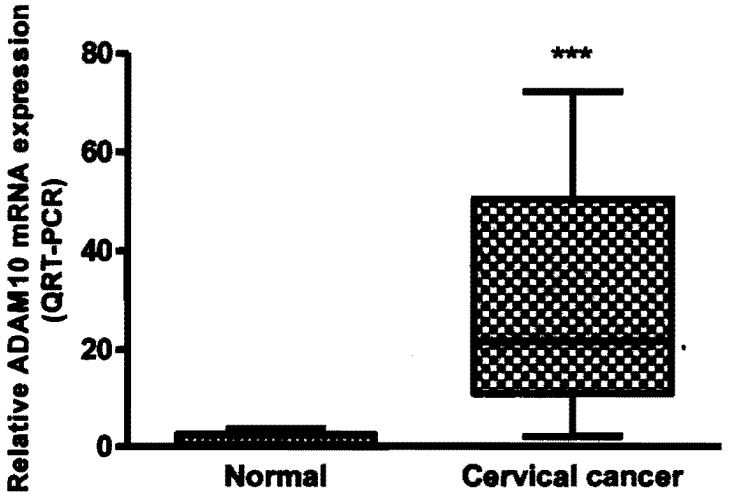


Figure 3.1 Relative mRNA expression levels of ADAM10 in normal and cancer cervical tissues. (A) ADAM10 mRNA expression levels determined by microarray analysis (adapted from van der Watt. PHD thesis, 2009, unpublished data) (normal; n=5, cancer; n=15). **(B)** Relative ADAM10 expression determined by QRT-PCR analysis (normal; n=6, cancer; n=14). GusB and Cyclophillin D were used as normalisers for this data. Results shown represent the mean ± SEM (***, p-value = 0.0004).

To independently confirm ADAM10 expression in normal and cervical cancer tissue, quantitative real-time PCR was performed where GusB and cyclophillin D were used as housekeeping normalisers. To provide a comparative analysis between cancer tissue and normal tissue samples Box and whiskers plot was used (McGill et al. 1978).The results obtained confirmed a significant increase in ADAM10 expression in cervical cancer patient material compared to normal cancer tissue (fig 3.1B). These findings support the microarray results and confirm elevated ADAM10 expression in cervical cancer

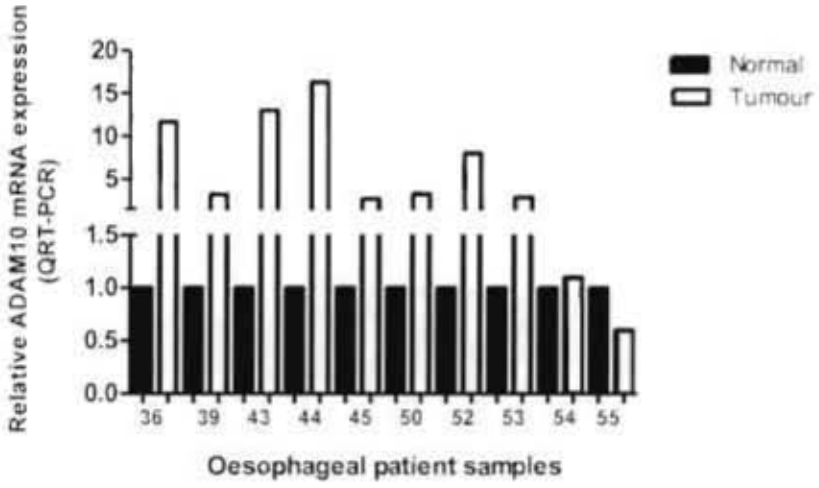
3.2.1.2 Expression of ADAM10 in oesophageal patient specimens

ADAM10 expression was also analysed in oesophageal cancer tissue. Biopsies of surgically removed paired normal and oesophageal cancer tissues were used to isolate mRNA, reverse transcribed into cDNA by RT-PCR for further analysis by QRT-PCR for ADAM10 expression. The endogenous mRNA expression of ADAM10 was quantified by QRT-PCR and GusB and Cyclophillin D were again used as housekeeping genes for normalisation to provide the relative mRNA expression in oesophageal cancer patient material compared to normal.

A total of 10 matched normal and oesophageal cancer patient specimens were analysed and the results obtained showed that eight out of ten paired patient samples investigated had elevated ADAM10 expression in the cancer material compared to normal (fig 3.2A). Of the paired samples tested, one showed no change in ADAM10 expression (patient 54) and one sample (patient 55) showed a reduction in ADAM10 expression, and these results indicate variation in patients. Further analysis using box and whisker plots comparing total cancer samples to total normal samples showed a significant overall increase of ADAM10

expression in cancer group compared to normal (fig 3.2B). These findings indicated a significantly higher expression of ADAM10 in oesophageal cancer patient material compared to normal.

A



B

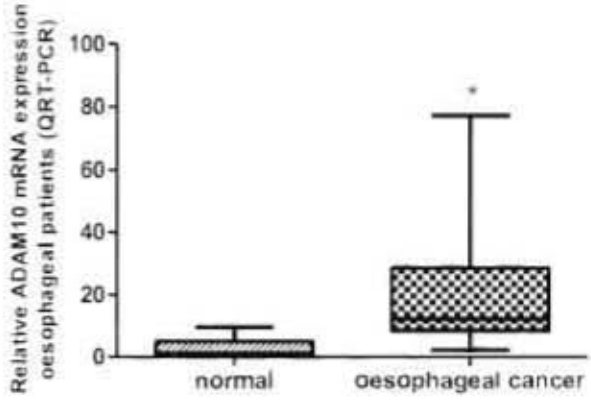


Figure 3.2 Relative mRNA expression levels of ADAM10 in normal and cancer oesophageal tissues. **(A)** Relative ADAM10 expression in paired normal and cancer oesophageal tissue specimens, determined by QRT-PCR analysis. GusB and Cyclophillin D were used as normalisers for this data. **(B)** Cumulative analysis of cancer and normal oesophageal patient material, indicating a significant increase in ADAM10 mRNA expression in cancer compared to normal patient samples. Results shown represent the mean ± SEM (*, p-value < 0.05).

3.2.2 ADAM10 expression in human cell model systems

3.2.2.1 ADAM10 mRNA expression in cervical and oesophageal cancer cell lines

Having established elevated expression of ADAM10 in cancer using patient material, we next investigated its expression in cancer cell lines of cervical and oesophageal origin. For this purpose a panel of cervical (HeLa, CaSki, Me180, SiHa, and Ms751) and oesophageal (WHCO1, WHCO5, WHCO6, and KYSE70) cancer cells lines were cultured and RNA isolated for ADAM10 QRT-PCR analysis. ADAM10 expression was calculated relative to two control genes, GusB and cyclophillin D and the results showed ADAM10 expression across the different cancer cell lines albeit at varying levels. (fig 3.3).

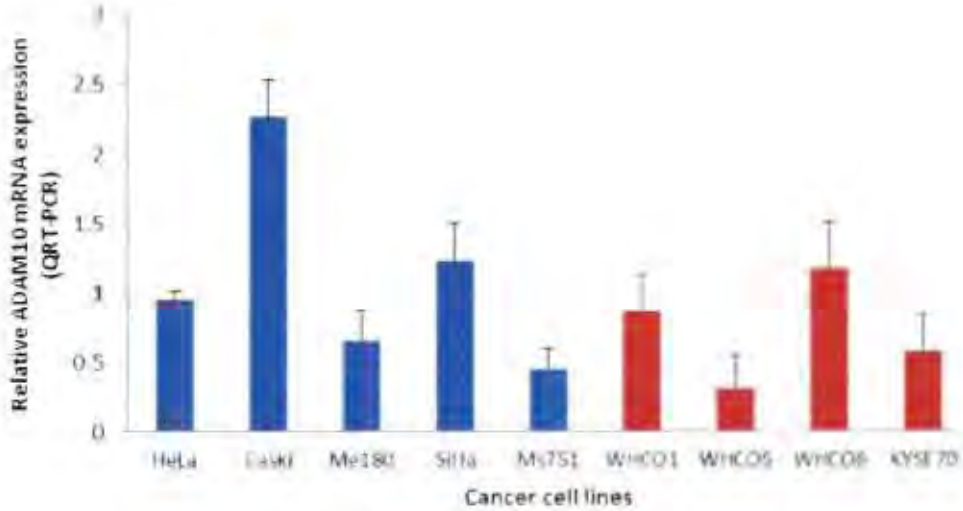


Figure 3.3 Relative mRNA expression levels of ADAM10 in Cervical and oesophageal cancer cell lines. Relative ADAM10 expression determined by QRT-PCR analysis. Cervical cancer cell lines: HeLa, CaSki, Me180, SiHa, and Ms751, and oesophageal cancer cell lines include: WHCO1, WHCO5, WHCO6, and KYSE 70. GusB and Cyclophillin D were used as normalisers for this data. Results shown are the mean ±SEM of experiments performed in triplicate.

3.2.2.2 ADAM10 protein expression in cervical and oesophageal cancer cell lines.

After determining that ADAM10 mRNA was expressed at elevated levels in cancer patient material and is present in cancer cell lines, its expression was next examined at the protein level. For this purpose a panel of cells were used; JurKat cells (immortalized T-lymphocyte) were used a positive control for its high expression of both the ~84kDa and ~66kDa isoforms of ADAM10 (Condon et al. 2001); WI38 and EPC2 as representative non-cancer cell lines, of fibroblast and epithelial origin, respectively; oesophageal cancer cell lines WHCO1, WHCO5, WHCO6, and KYSE410, KYSE70, KYSE520; and cervical cancer cell lines; CaSki, HeLa, and Me180 were used. All cell cultures were grown to 90% confluency, after which cells were lysed and protein samples were isolated and purified. Protein isolates were quantified and ADAM10 expression analysed by western blot analysed. Two bands were detected for ADAM10, corresponding to 84kDa and 66kDa (fig 3.4A). While some ADAM10 protein was detected in the normal cell lines, WI38 and EPC2, its expression was elevated in the majority of the cancer cell lines. Densitometry analysis of the ADAM10 band intensities showed an increase in ADAM10 protein expression in cancer cell lines compared to non-cancer cell lines (fig 3.4B).This result supports our earlier observation of high ADAM10 expression in cervical and oesophageal cancer.

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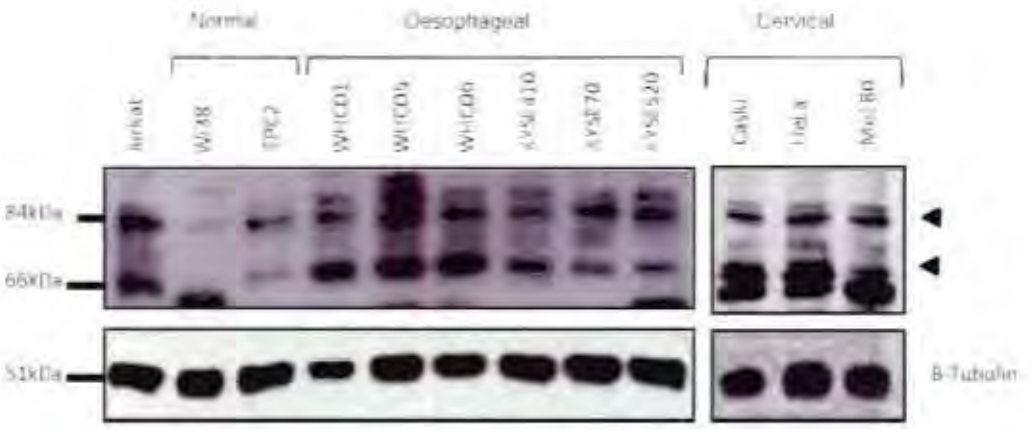

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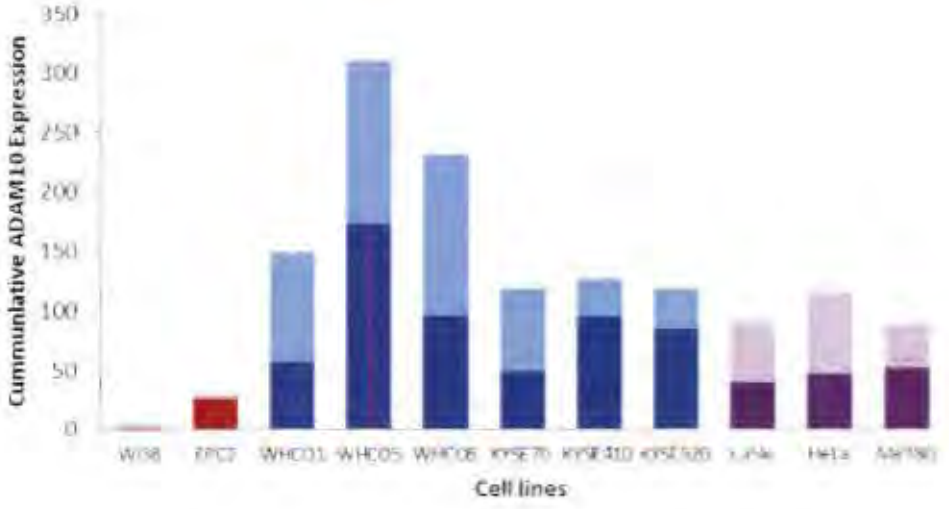

Figure 3.4 Endogenous expression of ADAM10 protein in cervical and oesophageal cancer cell lines **(A)** Western blot analysis of ADAM10 protein expression in a panel of normal and cancer cell lines, in which JurKat was used as a positive control. The ~84kDa and ~66kDa isoforms of ADAM10 were detected. Wi38 and EPC2 were representatives of normal cell lines, WHCO1, WHCO5, WHCO6, KYSE 410, KYSE70, and KYSE520 were representatives of oesophageal cancer cell lines, and HeLa, CaSki, and Me180 were representatives of cervical cancer cell lines. **(B)** Densitometric analysis of the active (top bar) and inactive (bottom bar) form of ADAM10 depicted by graphical representation, indicating a higher amount of active ADAM10 protein in cancer cell lines compared to normal.

3.2.2.3 Immunocytochemical analysis of ADAM10 expression

Having established the increased ADAM10 protein expression in cancer cell lines by Western blot analysis, its expression was independently validated by immunocytochemistry. Immunocytochemistry was performed on the following cell lines; WI38 and FG_0 cell lines were used as representative normal cell lines and HeLa and WHCO5 were used as representative cervical and oesophageal cancer cell lines, respectively. Cells were cultured on coverslips and then fixed and blocked, followed by immunocytochemistry using an ADAM10 antibody and Alexa488-conjugated secondary antibody to detect ADAM10 expression (fig 3.5A). Immunofluorescent analysis for ADAM10 expression showed very low levels of expression in the two normal cell lines, WI38 and FG_0, and increased fluorescence in the cancer cell lines, HeLa and WHCO5 (fig 3.5A). Densitometric analysis quantified the fluorescence intensity of ADAM10 expression in cancers compared to normal cell lines (fig 3.5B). These results provide further evidence for elevated ADAM10 expression in cancer cells compared to normal.

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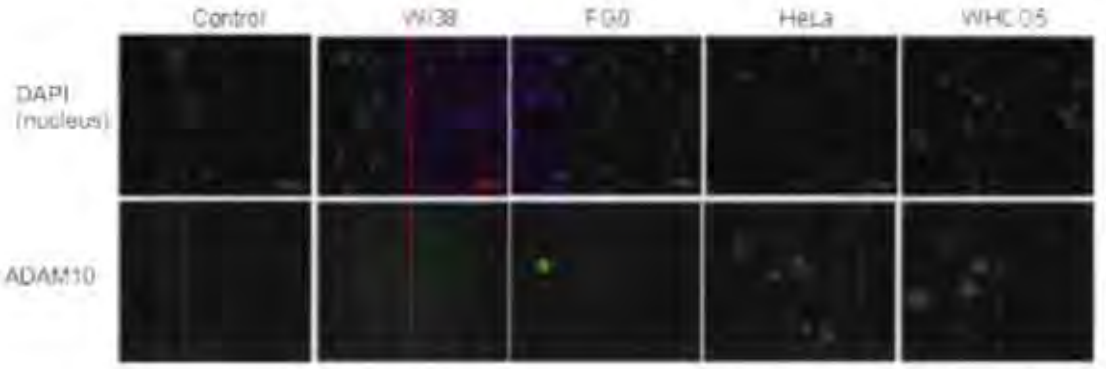

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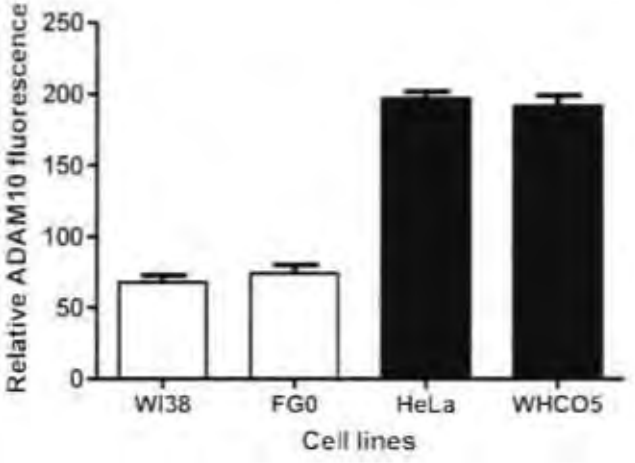


Figure 3.5 Immunofluorescent analysis of endogenous ADAM10 expression in cell lines. **(A)** Immunocytochemistry was performed to detect ADAM10 expression in 4 cell lines. Wi38 and FG0 were representative of normal cell lines, and HeLa and WHCO5 representative of cervical and oesophageal cancer cell lines, respectively. Cells were cultured and fixed to glass slides followed by fluorescent detection specific for ADAM10 expression. Nuclear staining (Blue) was done by DAPI staining, ADAM10 was detected by Alexa488 fluorescently labelled secondary specifically binding to ADAM10 primary antibody. **(B)** Densitometric analysis of fluorescent intensity representing the expression of ADAM10 in the cell lines. 40 cells were quantified for analysis.

3.3 Discussion

Expression profiling has become a useful tool that allows for the identification of genetic changes associated with cancer development. The data generated from global expression pattern can lead to the discovery of tumour-specific biomarkers and therapeutic targets. Previous microarray analysis in our laboratory identified a significant up-regulation of ADAM10 expression in cervical cancer patient material compared to normal (van de Watt, PHD thesis, 2009). This study was aimed at confirming ADAM10 expression at both mRNA and protein levels, and analysing its expression in cervical and oesophageal cancer material.

To validate the results found previously by microarray analysis, we initially analysed the endogenous expression of ADAM10 in cervical and oesophageal patient material. Our results indicated a significant up-regulation of ADAM10 expression at mRNA levels in both cervical and oesophageal cancer patient material compared to the normal. In gastric cancer patient material, ADAM10 expression has been identified to be up-regulated in cancer patient material compared to normal (Wang et al. 2011). In addition, in mantle cell lymphoma, ADAM10 was identified to have an increased expression of 87% of tumour samples tested (Armanious et al. 2011). Increased ADAM10 expression has been shown to be associated with the progression of both gastric and mantle cell lymphoma patient material. Our data showing increased expression of ADAM10 in patient material, suggest that ADAM10 may be associated with the progression of cervical and oesophageal cancer.

Although analysis of mRNA is important for the investigation of gene expression (Helm 2006), the functional analysis of protein and proteolytic cleaved enzymes such as the ADAMs family of proteins can only be validated at protein levels post-activation (Huovila et al. 2005). In understanding how protein abundances are related to mRNA, it is essential to identifu transcription levels for interpreting gene expression, protein interactions, structure and function in a cellular system (Greenbaum et al. 2003).

To fully evaluate the overall expression of the ADAM10 protein, we investigated its protein expression in cervical and oesophageal cancer cell lines. Our results indicated a relatively similar expression pattern of the inactive form of ADAM10 in both normal and cancer cell lines. However when analysing the expression patterns of the active form of ADAM10 we identified an increased number of active ADAM10 in cancer compared to normal cell lines. ADAM10 protein expression in cancers such as melanomas proved to be associated with the metastasis and proliferation of cancer cells (Lee et al. 2010). Furthermore, increased expression of active ADAM10 was associated with invasion, metastasis and proliferation of adenoid cystic carcinoma cells (Xu et al. 2010).

Our immunocytochemistry analysis detecting for the expression of ADAM10 in a representative cell line from each cancer and compared their fluorescence to two normal cell lines independently confirmed higher ADAM10 expression in the cancer cell lines. These results also showed the localization of ADAM10 to be in the nucleus, cytoplasm and cell membrane. Localization of ADAM10 has predominantly been associated with the cell

surface, where it readily cleaves and activated various cell surface molecules (Gutwein et al. 2002; McCulloch et al. 2004b; Murai et al. 2006). However, some literature identifies localization of ADAM10 in the nucleus of mantle cell lymphoma cells (Armanious et al. 2011), and others suggest that based on the intended use of ADAM10 the localization may occur in intracellular components specific to the cell type it is expressed in (Seals & Courtneidge 2003a).

Previously, ADAM10 has been identified to be up-regulated in gastric (Wang et al. 2011), prostate (Saftig & Reiss 2011), breast (P. Liu et al. 2006), and other cancers (Mochizuki & Okada 2007). ADAM10 has been associated with the activation of proteins involved in cell migration, invasion, and cell-cell communication (Gavert et al. 2007). The adhesive molecule E-cadherin has been shown to be up-regulated and its activation mediated by increased expression of ADAM10 in the migration and cell-cell adhesion in breast cancer cells (Berx & Van Roy 2001). As we have successfully identified the increased expression of ADAM10 in both cervical and oesophageal cancer compared to normal, we proceeded to investigate the potential biological relevance of the increased activation and expression of ADAM10 in both these cancers.

Chapter 4: Effect of ADAM10 inhibition on cancer cell biology

4.1 Introduction

Cancer progression has been associated with misregulated protein expression patterns which have resulted in the identification of many potentially useful targets for future treatment. The misregulation of protein involves either the up-regulation or down-regulation of this protein in cancer compared to normal. In identifying these proteins knowledge is gained regarding their role in cancer survival and progression (Westermarck & Kähäri 1999). Different approaches can be used to investigate the requirement of a protein to cell biology. Traditionally, for proteins that have elevated expression in eg. cancer, the approach has been to inhibit either its expression or activity, followed by investigating the effect of this inhibition on cancer cell biology.

The classic approach used to inhibit gene expression is the use of small interference RNA's. Small interference RNA are short strands of mRNA complimentary sequences that bind to RISC to form a complex. This complex then binds to a specific region of mRNA of the targeted gene, and inhibits the binding of complimentary sequences delivered by tRNA to form double stranded DNA (dsDNA) (Paddison et al. 2002). The inhibited mRNA sequence is degraded enabling posttranscriptional silencing of the target gene (fig 4.1A). Short hairpin RNA (shRNA) has a similar method of action as siRNA, however shRNA is dsRNA sequence that is transported into the nucleus as part of a plasmid construct. The shRNA is transcribed using a U6 promoter producing shRNAs repeatedly (Kern et al. 2005). These shRNAs require

cleavage by the endogenous cytoplasmic nuclease Dicer to yield siRNAs which are 21 to 27 nucleotides long (fig 4.1B) (He et al. 2009).

To investigate the biological relevance of ADAM10 in cervical and oesophageal cancers, its expression and or activity was inhibited using 3 methods; inhibit ADAM10 expression using siRNA and shRNA approaches, and activity inhibition using a molecule inhibitor, GI254023X. Previous studies have shown that GI254023X binds specifically, as well as preferentially to the active pocket of ADAM10 (Tippmann et al. 2009). GI254023X has a 100-fold selectivity for ADAM10 over another ADAM10 family member, ADAM17, even though these metalloproteases share several structural and functional homologies (Saftig & Reiss 2011). This inhibitor of ADAM10 has been shown to inhibit cell growth and migration of stomach, ovary, and oral cavity cancers (Mochizuki & Okada 2007). In addition, by inhibiting ADAM10 activity with GI254023X; E-cadherin shedding is reduced, as well as cell-cell communication and adhesion (Reiss et al. 2005), and β-catenin translocation (Maretzky et al. 2005).

In this study, we predominantly used and shRNA to inhibit ADAM10 expression in cervical and oesophageal cancer. Our results suggest that ADAM10 expression is necessary for Actin organization, migration and invasion of cervical and oesophageal cancer cells.

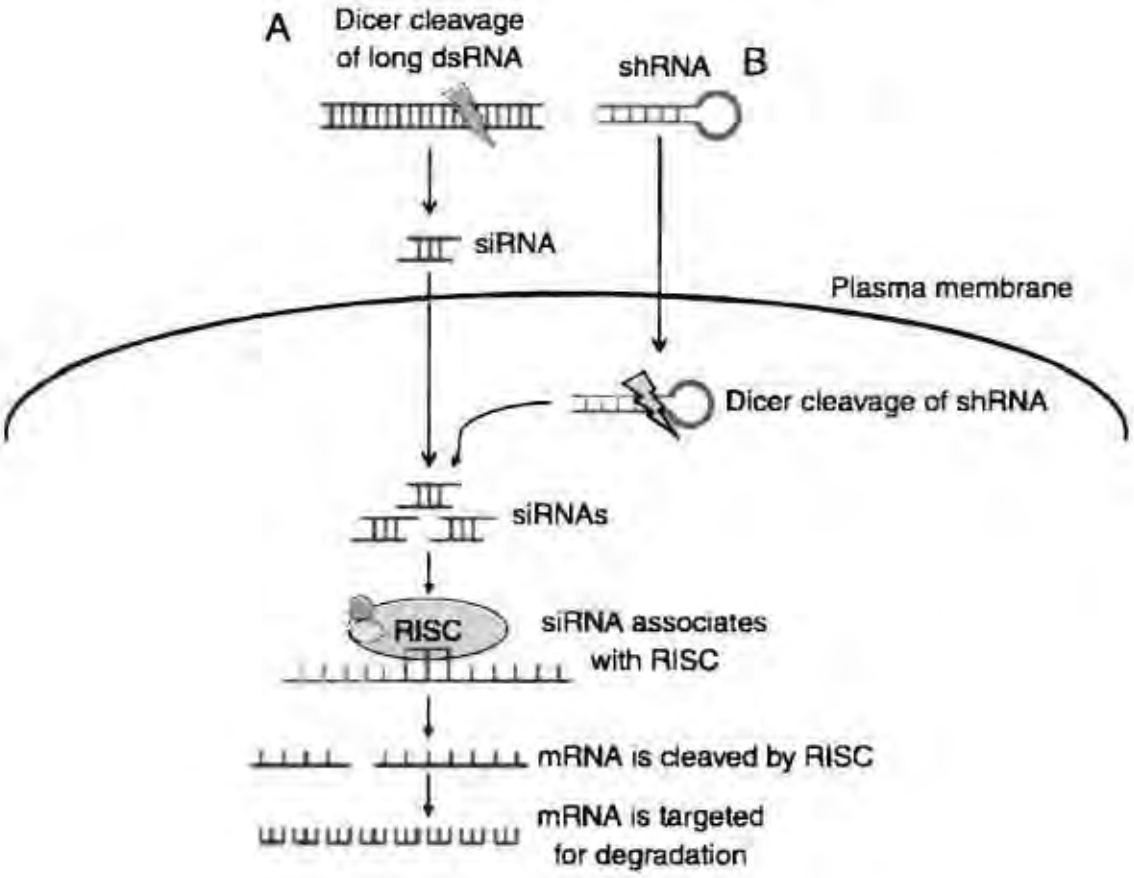


Figure 4.1 Diagrammatic representations of siRNA and shRNA silencing in a mammalian system. **(A)** Double-stranded DNA fragments enter the cell and are cleaved by the Dicer to form single stranded fragment. The fragment separates into single strand forming a complex with RISC and binds to the targeted mRNA sequence targeted for degradation. **(B)** Plasmid clone containing shRNA enters the cell and the nucleus. In the nucleus shRNA fragments are produced and converted into siRNA by the Dicer enzyme. The siRNA forms a complex with the RISC molecule and binds to the target mRNA for degradation (He et al. 2009).

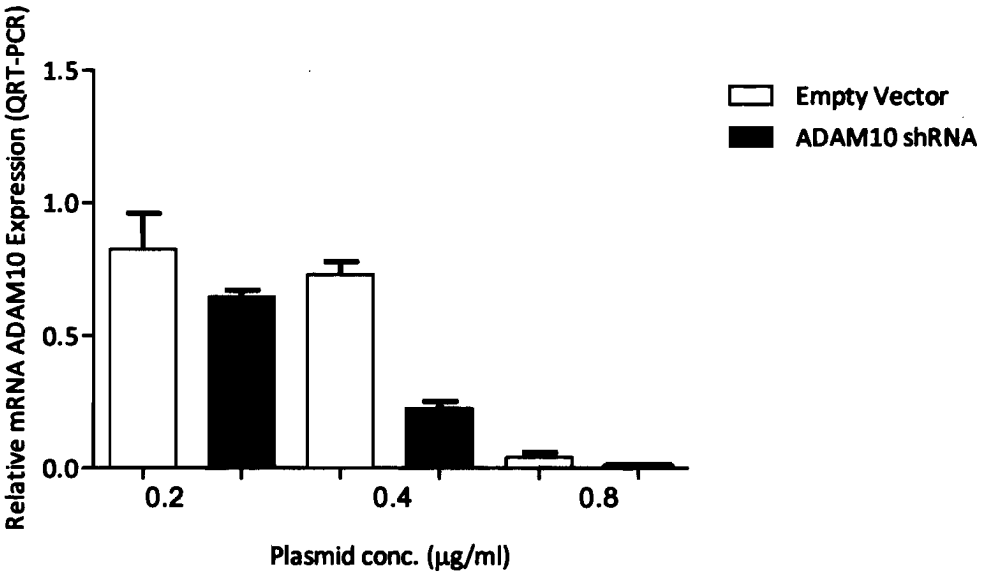
4.2 Results

4.2.1 The effects caused by ADAM10 inhibition on cancer cell biology.

Two cell lines representative of cervical and oesophageal cancer were used to investigate the biological relevance of ADAM10. HeLa and WHCO5 cells were selected as representative cell lines of cervical and oesophageal cancer respectively. ADAM10 expression was transiently inhibited using a hADAM10 shRNA containing plasmid construct.

Experiments were performed to determine the optimal concentration of ADAM10 shRNA required to inhibit ADAM10 expression. Cell were transfected with increasing concentrations of either a control plasmid or a plasmid containing ADAM10 shRNA. Twenty-four hours after transfection, RNA was extracted and its quality analysed by agarose gel electrophoresis. After cDNA preparation, quantitative real-time RT-PCR analysis for ADAM10 expression was performed. Our results showed a reduction in ADAM10 mRNA expression in both HeLa and WHCO5 cancer cell lines, after transfection with varying concentrations of ADAM10 shRNA plasmid (fig 4.2). The 0.2μg/ml concentration had minimal knockdown effect and 0.8μg/ml proved to be toxic, killing a high percentage of the cell population as seen when viewing cell effects microscopically. A concentration of 0.4μg/ml was the optimal concentration, showing ADAM10 knockdown at a concentration where the control plasmid had little or no effect. This concentration was thus used for further experiments.

A



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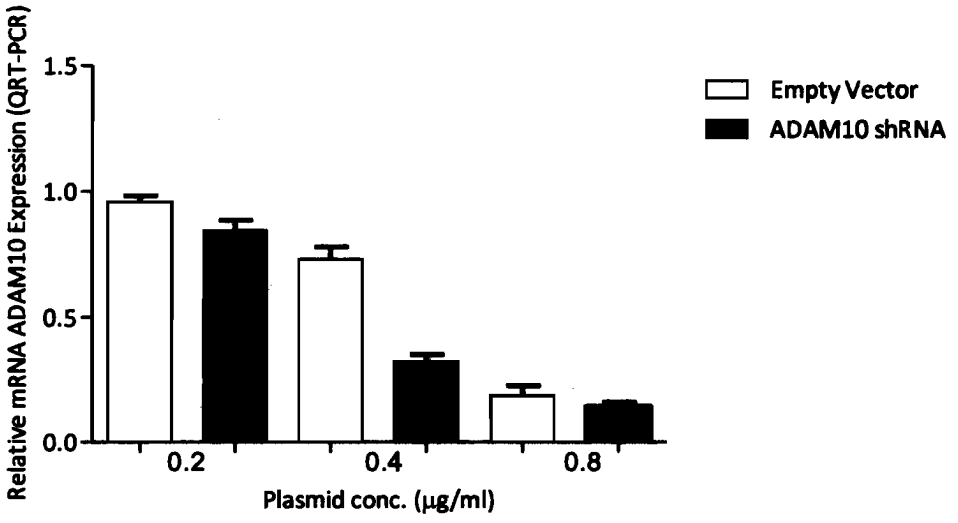

Figure 4.2 Plasmid Concentration Gradient analysis. (A). Transfection with increasing concentration, 0.2μg/ml, 0.4μg/ml, and 0.8μg/ml of either a control plasmid or ADAM10 shRNA containing plasmid into HeLa cervical cancer cell line, **(B).** and into WHCO5 oesophageal cell line.

Following optimization of ADAM10 shRNA concentration, we next investigated the time period of ADAM10 inhibition. ADAM10 expression was monitored at mRNA expression levels by qRT-PCR (fig 4.3A and B) and at protein expression levels by western blot (fig 4.4A and B). The shRNA's knockdown effect on ADAM10 mRNA expression was found to be maintained over a period of six days post-transfection at RNA levels, with substantial inhibition of ADAM10 expression observed 72-120 hrs after transfection in both HeLa and WHCO5 cells (fig 3.3 A and B). Our results also showed that ADAM10 expression increased over the time period assayed, likely a result due to an increase in cell confluency. ADAM10 shRNA suppressed ADAM10 expression over the time period. ADAM10 protein expression levels were also monitored after transfection with the shRNA-containing plasmid. In HeLa cells the 84kDa protein was substantially decreased by 120 hrs after transfection and in the WHCO5 oesophageal cancer cells inhibition was seen at both 96 hrs and 120 hrs (fig 4.4). Due to the combination of transient inhibition and 72 hr half-life of ADAM10, expression was observed throughout the RNA levels and and protein reduction only noted after 5 days. The control empty plasmid showed no effect on the expression of ADAM10 at the mRNA level.

ADAM10 expression was also transiently knocked-down using an ADAM10 siRNA (Santa Cruz Biotechnology. QRT-PCR analysis showed that the inhibition of ADAM10 mRNA expression was achieved after 24 hours and maintained for up to 120 hours after transfection (appendix A1). While inhibition of ADAM10 mRNA expression with ADAM10 siRNA was observed within 24hrs, ADAM10 protein levels only decreased at 5 days post-transfection (appendix A1). Our results therefore showed that both ADAM10 shRNA and

ADAM10 siRNA approaches showed inhibition of ADAM10 expression. Further investigation continued using the hADAM10 shRNA plasmid as the primary method of inhibition, with independent validation of some expression using shRNA and the small molecule inhibitor, GI254023X reported in the appendix section of this report.

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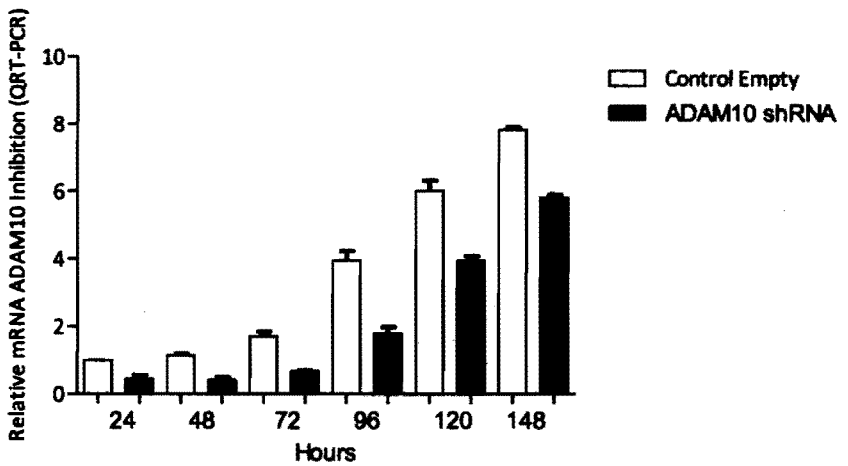

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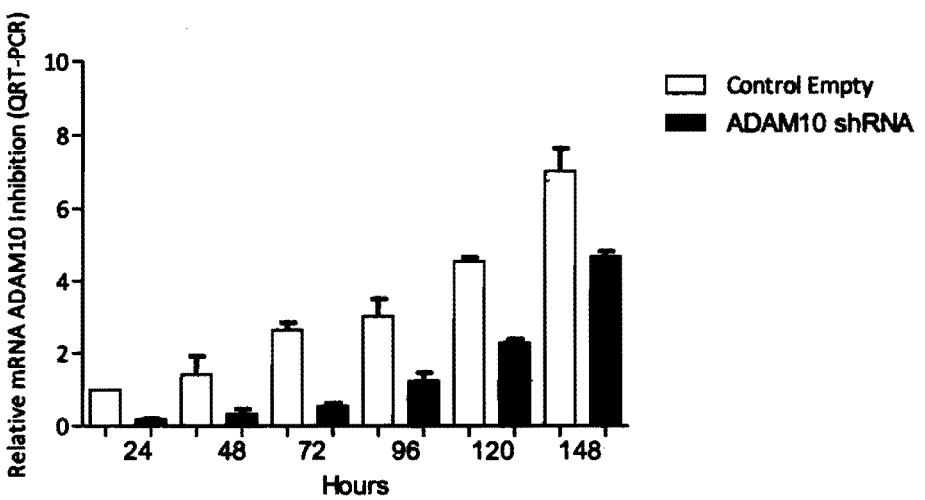

Figure 4.3 Time course analysis of ADAM10 expression after shRNA transfection. Control and ADAM10 shRNA containing plasmid was transfected into HeLa **(A)** and WHCO5 **(B)** cells. RNA was extracted from qRT-PCR analysis up to 148 hrs after transfection. Results shown of triplicate qRT-PCR analysis for qRT-PCR analysis for ADAM10 expression relative to GusB as an internal control.

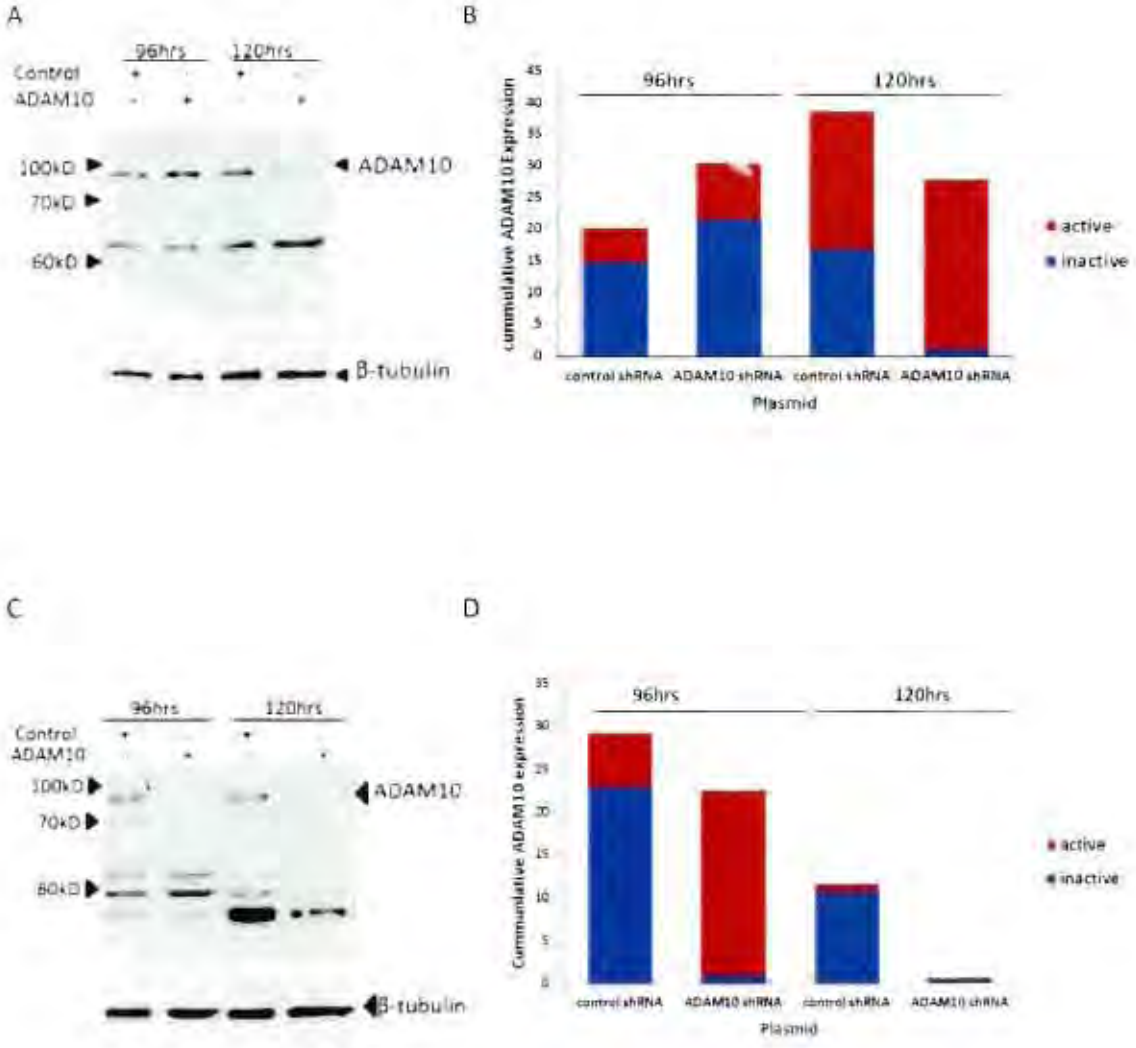

Figure 4.4 shRNA inhibition of ADAM10 protein expression over time. (A) Protein expression pattern analysis of ADAM10 over the duration of 5 days in HeLa, **(C)** and WHCO5 cancer cells, after transfection with either control or ADAM10 shRNA containing plasmid.**(B)** Densitometric analysis of the active (red) and inactive (blue) form of ADAM10 depicted by graphical representation in HeLa, **(D)** and WHCO5 cells indicating a higher amount of active ADAM10 protein in cancer cell lines compared to normal.

4.2.2 Biological effects of ADAM10 inhibition.

To identify the effects caused by the inhibition of ADAM10 expression, the following biological endpoints were assayed; cell proliferation, cell adhesion, cell migration, and cell invasion. These are some of the key characteristics of cancer progression.

4.2.2.1 Effects of ADAM10 inhibition on cancer cell proliferation.

Uncontrollable proliferation is one of the biological phenotypes of cancer cell progression. The role of ADAM10 in the proliferation of HeLa and WHCO5 cells was therefore investigated. Cells were transfected with 0.4μg/ml control or ADAM10 shRNA plasmid and cell proliferation was analysed using the MTT assay for a period of six days (fig 4.5). This assay measures the metabolic activity of living cells, enabling a quantitation of cell number. Our results showed that the inhibition of ADAM10 had no significant effect on cell proliferation in either HeLa or WHCO5 cells (fig 4.5A and B). This result suggests that ADAM10 is not required for the proliferation of HeLa cervical cancer or WHCO5 oesophageal cancer cells.

Similar results were also observed using 40nM control or ADAM10 siRNA or GI254023X shown in appendix A2. These experiments independently confirmed that ADAM10 inhibition had no effect on cell proliferation.

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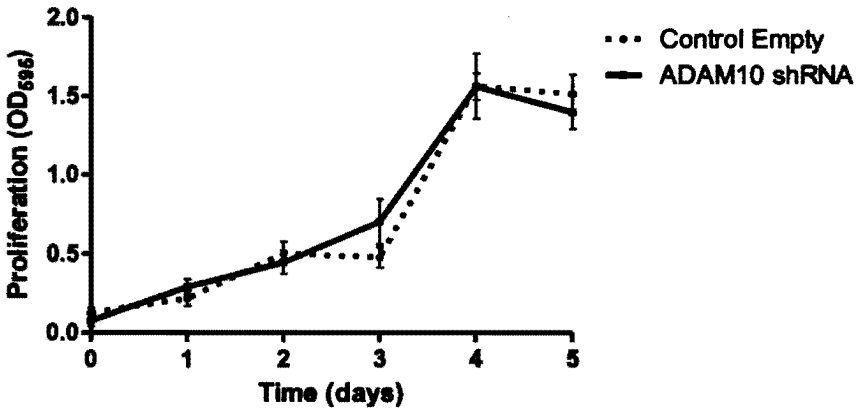

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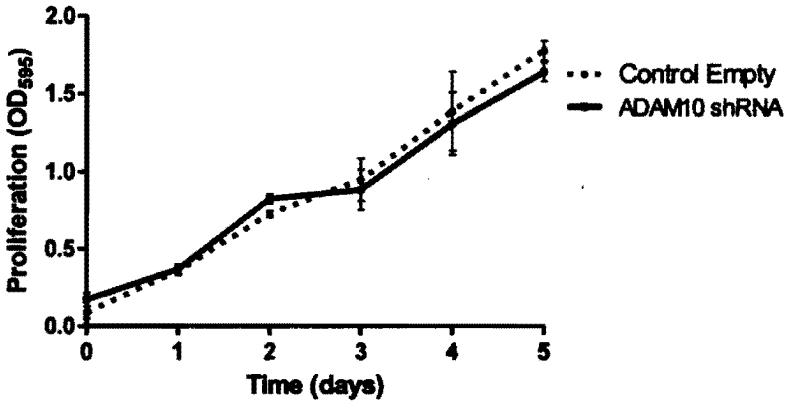


Figure 4.5 MTT analyses of HeLa and WHCO5 cells after the inhibition of ADAM10 expression. Cell proliferation assays using MTT analysis in cancer cell lines **(A)** HeLa cervical cancer cell line, **(B)** and WHCO5 oesophageal cancer cell line, transfected with control or ADAM10 shRNA plasmid. Experiments were performed in triplicate and repeated at least three independent times. Results shown represent the mean ±SD.

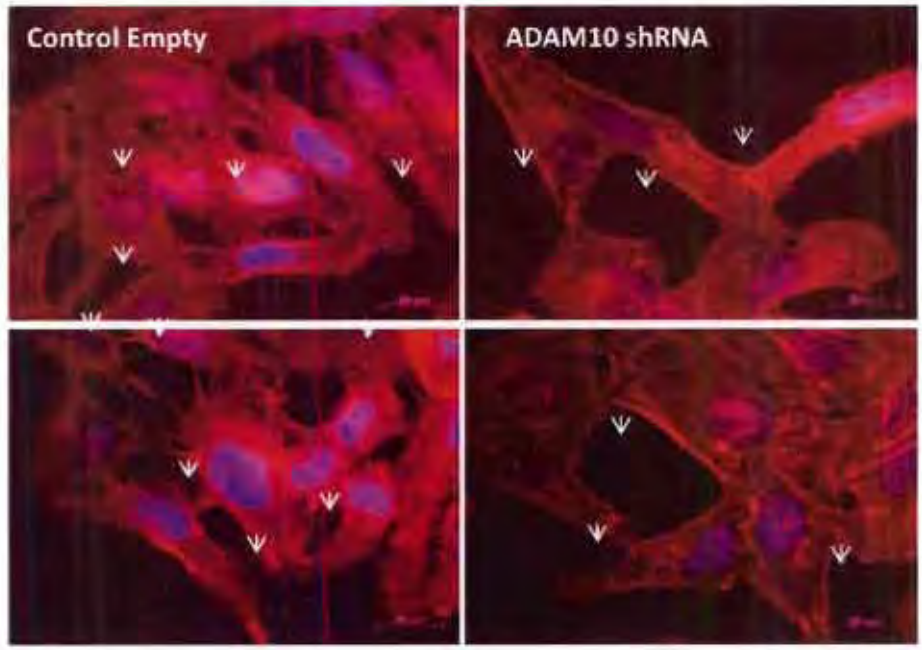
4.2.2.2 Effects of ADAM10 inhibition on cancer cell morphology and actin organisation.

ADAM10 has been associated with many adhesion factors relating to cell-cell adhesion and cell-surface binding (Maretzky et al. 2005). These factors can influence cell shape, size and cytoskeletal organization. To investigate the effects of ADAM10 inhibition on HeLa and WHCO5 cell shape, size and cytoskeletal organization, Phalloidin was used. Phalloidin stains F-actin and can be viewed using fluorescence microscopy (Wehland et al. 1977).

HeLa and WHCO5 cells were cultured on coverslips and transfected with 1μg control or ADAM10 shRNA. After 24 hours cells were fixed onto coverslips and actin filaments detected using Phalloidin. Phalliodin staining revealed the actin distribution (shown in red), and DAPI was used to stain for cell nuclei (shown in blue).

Our results indicated actin reorganization in cells where ADAM10 expression was inhibited using shRNA (fig 4.6). HeLa control cells showed clear indication of numerous cell extensions whereas ADAM10 inhibited cells formed islands of cells showing reduced extensions (fig 4.6A). Quantification of the number of protrusions in control and ADAM10 knockdown cells revealed a significant decrease in the ADAM10 inhibited HeLa cells (fig 4.6C). HeLa cells in which ADAM10 expression was inhibited were more tightly packed compared to control cells and showed fewer cytoplasmic extensions. WHCO5 cells, while different in cell shape to HeLa cells, showed similar results (fig 4.6B). It was however not possible to quantify the changes in cytoplasmic extensions in WHCO5 cells due to their tight cell shape.

A



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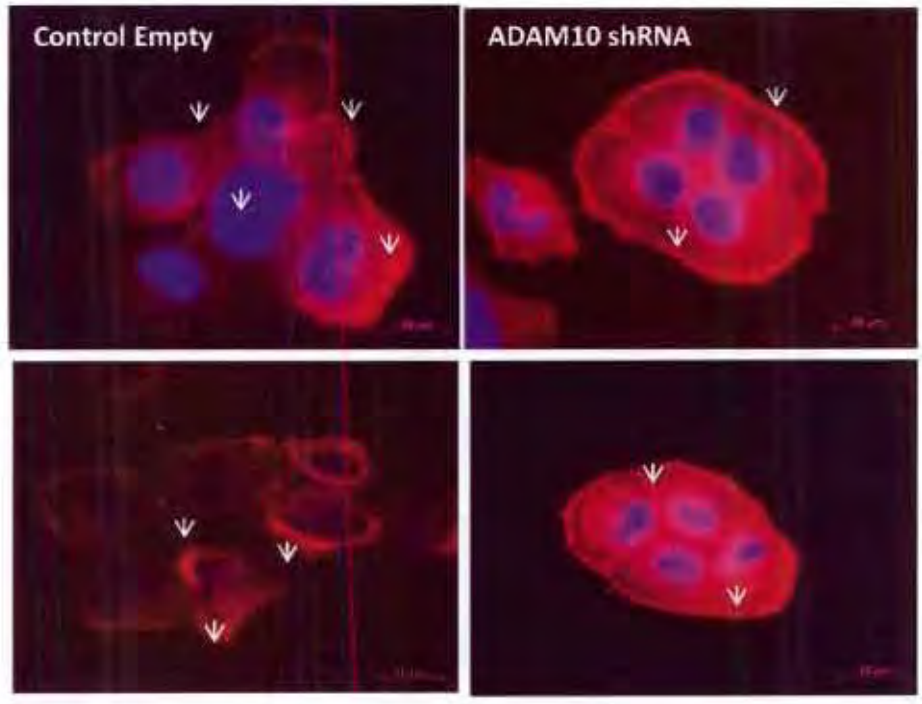
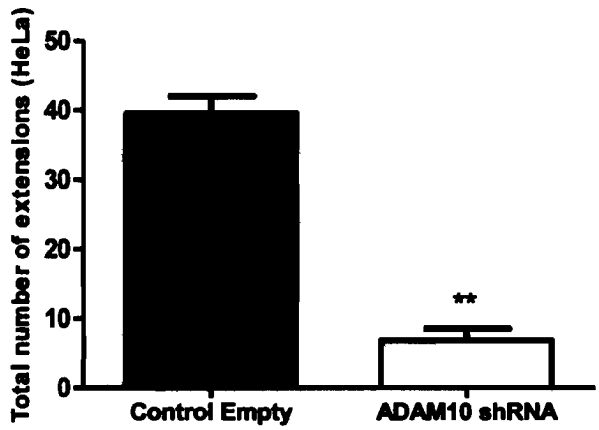


Figure 4.6 Actin organization in control and ADAM10 inhibited cells. Fluorescent staining of polymeric F-actin in HeLa **(A)** and WHCO5 **(B)** control and ADAM10 shRNA transfected cells. Phalloidin was used to stain F-actin. Arrows piont to cytoplasmic extensions.

C



Fugure 4.6 (C) Quantification of the changes in the number of cytoplasmic extensions per cell from the capture fluorescentimages. The results shown are the mean ±SEM over six fields of view. ** $p<0.05$

Results indicated that in both HeLa and WHCO5 cells, there was a reorganization of actin in cells with reduced ADAM10 expression (fig 4.6). The actin cell linkers, indicated by arrows, are visible as extended filaments and connection in the control cells and these protrusions are absent in the cells with reduced ADAM10 expression. The cells with reduced ADAM10 expression were found primarily in islands of cells with reduced actin connections between cells. The reorganization of actin in these cells suggested mesenchymal to epithelial like cells after ADAM10 inhibition, as actin redistribution is associated with EMT (Savagner 2001). This result suggested that there may be an effect on cell adhesion, cell motility, and cell invasion with ADAM10 inhibition.

4.2.2.3 Effects of ADAM10 inhibition on cancer cell adhesion

The morphology and Actin organization results suggested there may be an effect on cell adhesion when ADAM10 expression is inhibited. Filamentous actin cytoskeleton is associated with numerous physical cellular processes, including cell division, cell adhesion and cell migration (Stricker et al. 2010). This led us to investigate the effects on cell adhesion when ADAM10 expression was reduced. When cells are motile they are less adhesive, and are referred to as being mesenchymal. Conversely, when cells are epithelial in nature they are more adhesive. As our cells morphology results suggested mesenchymal-like to epithelial-like changes when ADAM10 expression was inhibited, we next investigated ADAM10 inhibition on cell adhesion.

To investigate the effects of ADAM10 inhibition on HeLa and WHCO5 cell adhesion, two types of adhesion assays were performed: non-coated and fibronectin-coated adhesion assays. These two conditions were used to analyse two types of adhesion, non-coated indicative of cell-surface adhesion, and fibronectin-coated indicative of cell-cell adhesion. Fibronectin is a high molecular weight glycoprotein of the pericellular matrix, and is found on the cell surface (Tarone et al. 1982). Both HeLa and WHCO5 cells were transfected with control or ADAM10 shRNA plasmids and adhesion assays performed using non-coated (fig 4.7A and B) and fibronectin coated (fig 4.7C and D) 96 well dishes and assays performed as described in materials and methods above . Adherent cells were expressed relative to total cell number.

The results show that using both non-coated and fibronectin-coated adhesion conditions, inhibition of ADAM10 expression results in significantly increased amount of cell adhesion (fig 4.6). This was observed in both HeLa (fig4.6A and C) and WHCO5 cells (fig 4.6 Band D). These results suggest that elevated ADAM10 expression in cancer cells reduces cell-cell adhesion and cell-surface adhesion. The adhesion assay was also performed using the ADAM10 small molecule inhibitor, GI254023X, and similar results were obtained as that using ADAM10 shRNA (appendix A3).

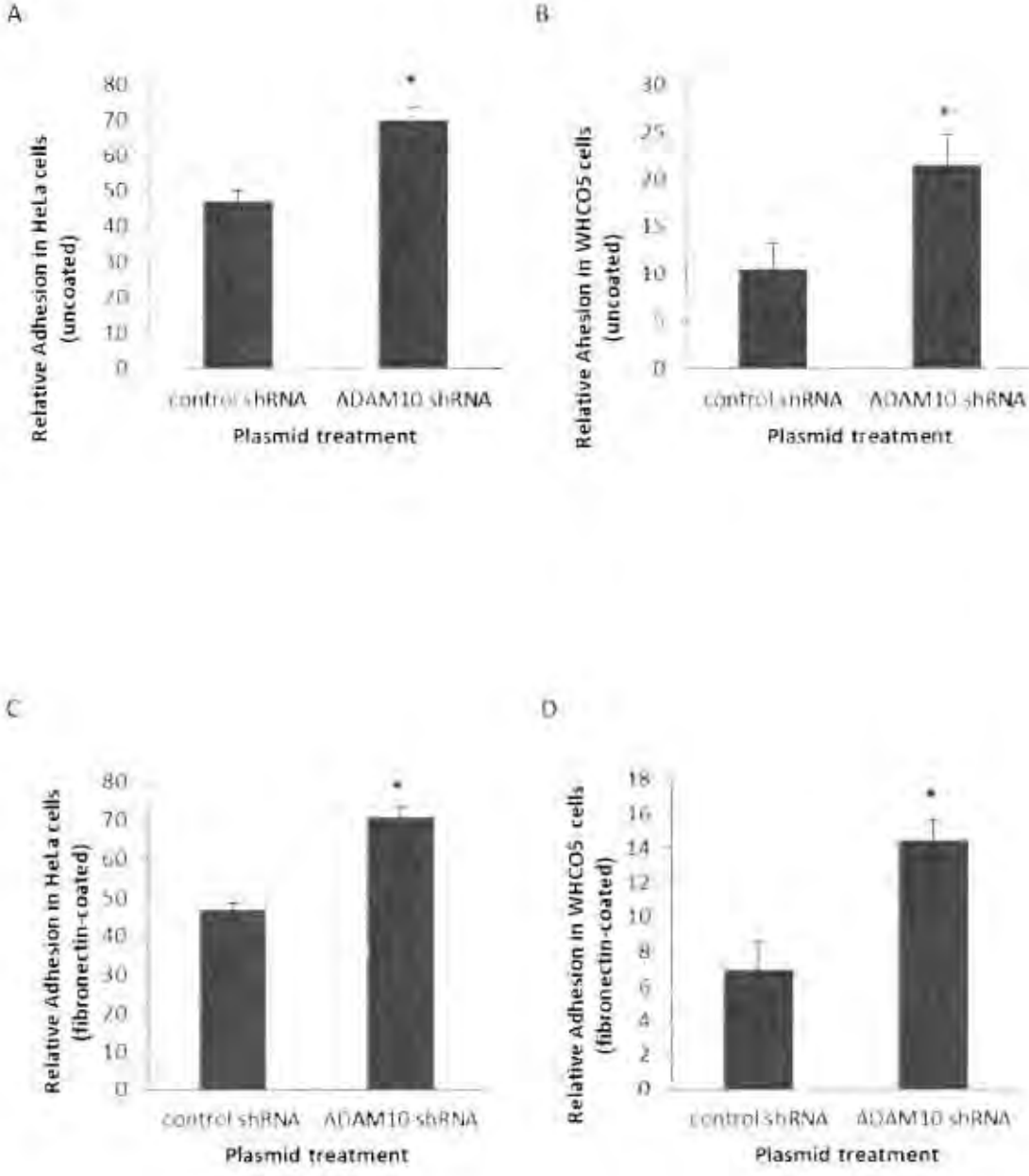


Figure 4.7 Adhesion assay analysis of cancer cells in fibronectin-coated and non-coated conditions. **(A,B)** Adhesion analysis in non-coated conditions of **(A)** Hela and **(B)** WHCO5 cells indicated a significant increase in cell adhesion in both cell lines with reduced ADAM10 expression. **(C, D)** Adhesion analysis in fibronectin-coated conditions with **(C)** HeLa and **(D)** WHCO5 cancer cell indicated a significant increase in cell adhesion in both cell lines. (*,p-value< 0.05)

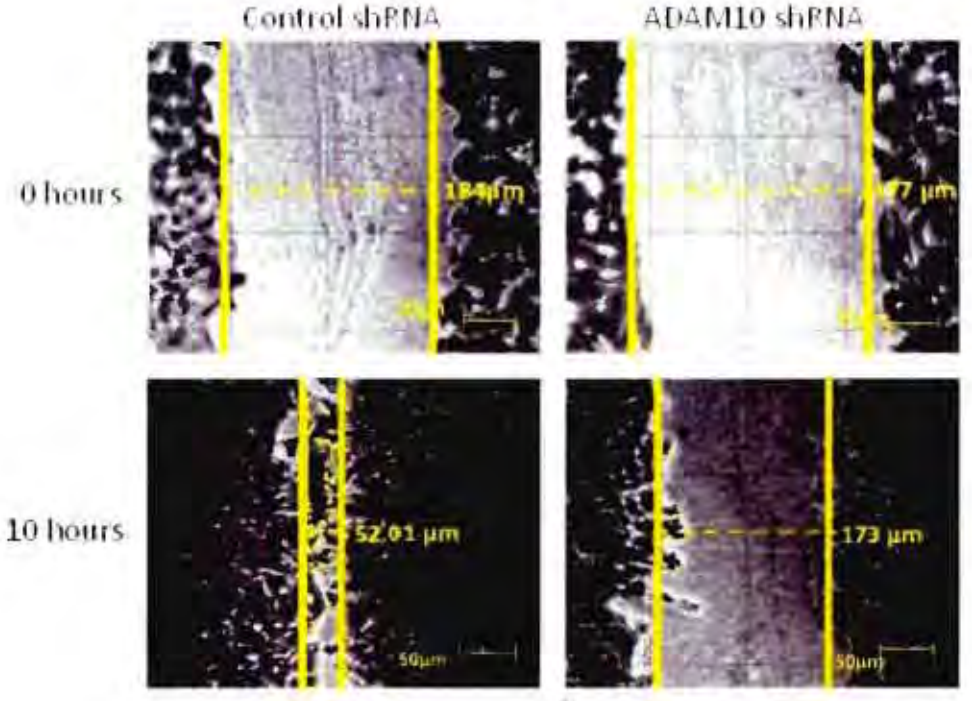
4.2.2.4 Effects of ADAM10 inhibition on cancer cell migration

Cell migration is a process involving multiple steps that arranges embryonic morphogenesis (Thiery et al. 1985); contributes to tissue repair and regeneration (Schmidt & Leach 2003); and drives disease progression in cancer (Arribas et al. 2006).

In cell migration, polarization is often accompanied by sensitization of receptors at the leading edge, therefore supporting continued movement in the one direction. Adhesion molecules communicate propelling forces and serve as traction points during cell movement. The migration cycle is accomplished as adhesions detach and the rear retracts (Lauffenburger & Horwitz 1996). The detachment of adhesions is controlled by pathways that include FAK, ERK, Src, and the protease calpain (Webb et al. 2004). The release of adhesions at the rear and front appear to share some common mechanisms and are coupled to the formation of protrusions at the front (Ridley et al. 2003).

To investigate the requirement of ADAM10 in the migration of cervical and oesophageal cancer cells, we performed a migration (scratch) or wound healing assay. In this experiment, HeLa and WHCO5 cells were transfected with control or ADAM10 shRNA plasmids. Forty-eight hours post-transfection, cells were re-plated to 95% confluency. After a wound (or scratch) was introduced, cells were fixed and stained at 0 hour and at 10 hours. Images of the wound size remaining in control and ADAM10 inhibited cells were captured and quantified by measuring the distance between the scratch edges at 0 and 10 hours.

A



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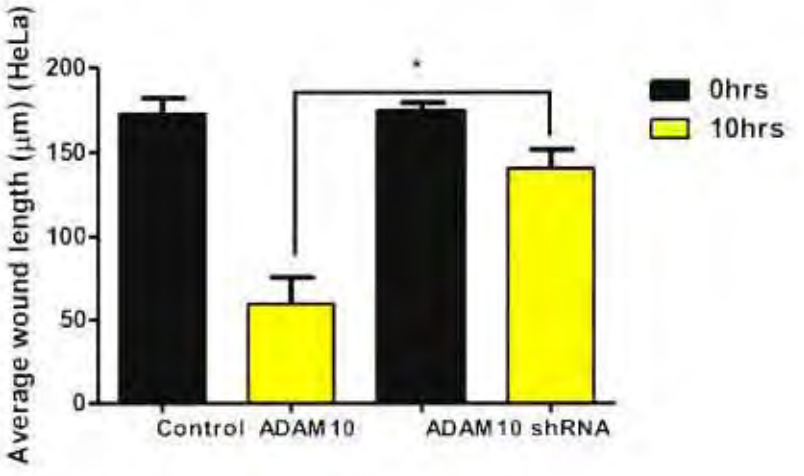
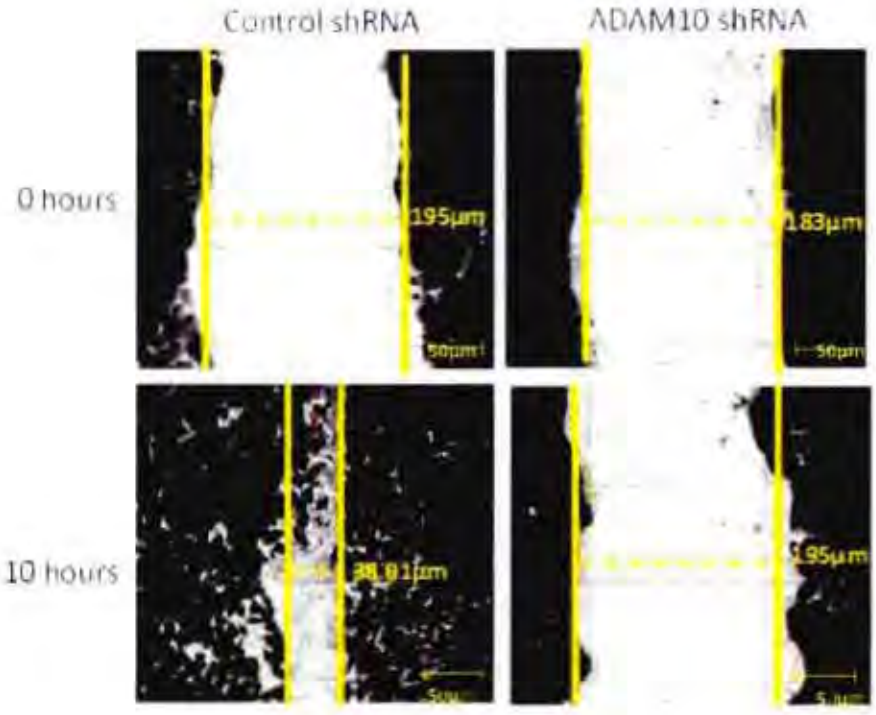


Figure 4.8 Migration assay analysis in HeLa cells with ADAM10 expression inhibition. **(A)** Migration of cells captured at initial time of wound insertion, 0 hours, and after 10 hours or both control and ADAM10 shRNA treated conditions. **(B)** Densitometric analysis of wound size remaining.

A



B

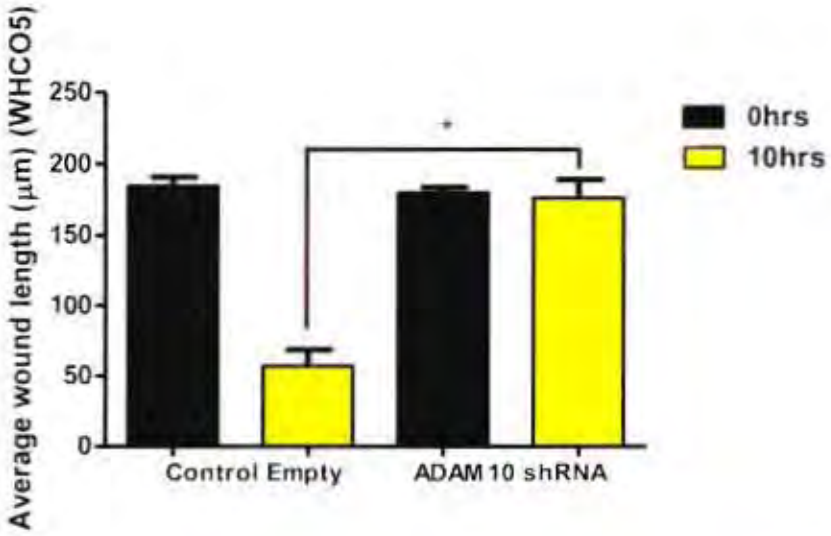


Figure 4.9 Migration assay analysis in WHCO5 cells with ADAM10 expression inhibition. (A) Migration of cells captured at initial time of wound insertion, 0 hours, and after 10 hours or both control and ADAM10 shRNA treated conditions. **(B)** Densitometric analysis of wound size remaining.

Our results indicated that inhibition of ADAM10 expression results in reduced migration of both HeLa (fig 4.8A) and WHCO5 (fig 4.9A) cells. Quantification of the wound (scratch width) remaining in control and ADAM10 inhibited cells showed a significant reduction in both HeLa and WHCO5 cell migration when ADAM10 expression is knocked down (Fig 4.8B and fig 4.9B). These results suggest a role for ADAM10 in cervical and oesophageal cancer cell motility.

4.2.2.5 Effects of ADAM10 inhibition on cancer cell invasion

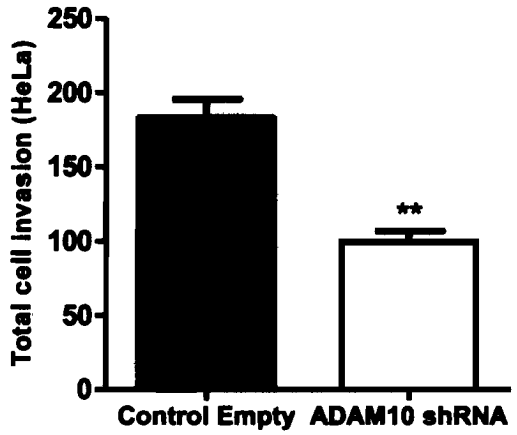
An important feature of metastatic cells is the ability to not only migrate, but also invade to other areas of the body. This involves the movement of cells through the extracellular matrix. As the inhibition of ADAM10 resulted in reduced cell migration, we next investigated if its inhibition would have an effect on the invasiveness in HeLa and WHCO5 cells.

To analyse cancer cell invasion, HeLa and WHCO5 cells were cultured and transfected with control or ADAM10 shRNA. The cells were then starved for 4 hours and plated in serum-free media onto matrigel-coated chambers. Matrigel is a basement membrane preparation that functions as an extracellular matrix. The bottom chamber was filled with complete media contains 10% FCS, and the cells were incubated overnight to allow for invasion of cells through the matrigel to the underside of the thincert chamber towards the 10% serum

containing media. The invaded cells were then fixed and stained with crystal violet and quantified.

The results obtained showed a significant reduction in HeLa and WHCO5 cell invasion when ADAM10 expression is inhibited (fig 4.10A and B). These results in combination with our earlier results showed that ADAM10 is required for Actin organization, adhesion, migration, and invasiveness of HeLa and WHCO5 cancer cells.

A



B

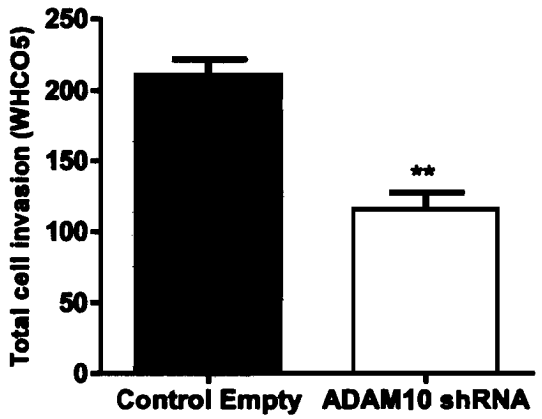


Figure 4.10 Invasion assay analysis in cancer cell lines with reduced ADAM10 expression. (A) The cervical cancer cell line, HeLa, trasnsfected with ADAM10 shRNA expression plasmid, showed a significant decrease in cancer cell invasion. **(B)** The oesohageal cancer cell line,WHCO5, similarly showed a significant decrease in cancer cell invasion after ADAM10 inhibition. Results shown are the mean±SE of expresiments performed three independent times.

4.3 Discussion

In this study we report that inhibition of ADAM10 expression in the cervical cell line, HeLa and oesophageal cancer cell line, WHCO5 has a significant inhibitory effect on cell morphology, actin organization, adhesion migration and invasion, while having no effect on cell proliferation. Our data showing that inhibition of ADAM10 has no effect on the cell proliferation of HeLa and WHCO5 cells is in contrast to other studies. In prostate cancer, LNCaP cells transfected with ADAM10 siRNA indicated a significant decrease in cancer cell proliferation (Arima et al. 2007). In adenoid cystic carcinoma cells, transfection of ADAM 10 siRNA resulted in a significant reduction of cellular growth suggesting that proliferation of adenoid cancer cells demonstrated that ADAM 10 expression is correlated with increased melanoma cell proliferation (Lee et al. 2010).

Our results showing that ADAM10 inhibition has no effect on HeLa and WHCO5 cell proliferation may be explained by the compensatory role of different members of the ADAMs family. Inhibition of ADAM10 has also been shown to activate Notch signalling by suppressing ectodomain shedding of delta-1, which subsequently leads to a strong inhibitory effect on tumour cell proliferation (Jin et al. 2007). However, ADAM10 and ADAM17 have been shown to compensation for the absence of the other (Bzowska et al. 2009). Both ADAM10 and ADAM17, have been linked to Notch1 activation (Bozkulak & Weinmaster 2009). In addition, both proteases has been known to activate epidermal-like growth factor receptor (EGFR), which is strongly associated with the proliferation of cancer

cells (Sahin et al. 2004). It is therefore possible that in the presence of reduced ADAM10 expression in HeLa and WHCO5 cells, ADAM17 may be compensating for the loss, activating EGFR and other growth factors.

ADAM10 has previously been associated with non-small lung cancer cell metastasis (Guo et al. 2012). Features of metastatic cells include changes in cytoskeletal organization, migration and invasion. Our analyses of the cytoskeletal protein, f-actin showed significant cytoskeletal changes in actin reorganization in ADAM0 inhibited cells. Fluorescently labelled phalloidin stains polymer microfilaments called F-actin both of which are essential for such important cellular functions as the mobility and contraction of cells during cell division (Stricker et al. 2010). Our results indicated a reduction in the F-actin protrusions along the cell membrane of cancer cells with reduced ADAM10 expression. This result suggested that ADAM10 may be essential for the migration of cells and the communication and adhesion between adjacent cells. For this reason we investigated the effects caused on adhesion, migration and invasion.

ADAM10 activates adhesion molecules such as L1-CAM (Weidle et al. 2009), N-cadherin (Reiss et al. 2005), and E-cadherin (Maretzky et al. 2005). L1-CAM which is cleaved by ADAM10 leads to the metastasis of colon cancer to the liver (Gavert et al. 2007). E-cadherin is essential for cell-cell adhesion and migration (Maretzky et al. 2005). Our data showing a significant reduction in cell migration in cervical cancer HeLa and in oesophageal WHCO5 cells is in line with other studies (Yue et al. 2003; Nijkamp et al. 2011; Cheng et al. 2012). A

significant increase in the adhesion of HeLa and WHCO5 cancer cells was observed in ADAM10 inhibited cells using fibronectin coated and non-coated conditions. The reduced migration and increased adhesion observed, associated with a significant reduction in the invasion of HeLa and WHCO5 cancer cells with reduced ADAM10 expression. These findings are in alignment with a recent study performed in non-small cell lung cancer (NSLC) cells showing that when ADAM10 expression was reduced by shRNA, it resulted in reduced migration and invasion (Guo et al. 2012).

These results collectively suggest that ADAM10 may be potentially important for the invasiveness of cervical and oesophageal cancer cells during cancer progression. During the late stage of tumour growth, cancer cells become invasive and metastatic and require other sources of nutrition forming new tumour bodies (Yilmaz & Christofori 2009). This induces the transition of epithelial non-motile proliferative cells to mesenchymal-like motile metastatic cells. This process is referred to as epithelial-mesenchymal transition (EMT) (Zavadil et al. 2008). ADAM10 has been identified to regulate the activation of E-cadherin, a marker of epithelial cells, and many other proteins relating to the adhesion of cells as well as cell-cell communication (Maretzky et al. 2005).

Chapter 5: Conclusions

In the identification of a potential biomarker, it is necessary to investigate the expression pattern of the target gene in a cancer and non-cancer environment. The misregulation of a gene leads to the suppression or over-expression of a protein, and therefore may serve an important role in the survival of cancer cells. In a previous study we identified a number of proteins up-regulated in a microarray analysis performed using cervical cancer patient material. ADAM10 showed significant increased expression of more than 2-fold in cancers compared to normal patient samples (Ward 2011) and presented as a target gene that may associate with the biology of this cancer.

In this study, we investigated the up-regulation of ADAM10 in cervical and oesophageal cancel patient material and representative cell line model systems. To validate the increased expression of ADAM10 in patient material as seen in the microarray, we proceeded to analyse the expression pattern of ADAM10 in patient's material isolated from cervical and oesophageal cancer and normal tissue samples. The expression pattern indicated significant up-regulation of ADAM10 in cancer patient material compared to the normal samples. In addition when analysing the expression of ADAM10 protein in various cervical and oesophageal cell lines, ADAM10 expression was readily detected. When a proteins expression pattern is altered in the environment of cancer cells, this suggests that this change is related to the progression of the cancer. ADAM10 expression has been has been found to be up-regulated in gastric patient material compared to normal. These authors

describe that this increased expression was directly linked to the invasion and metastasis of gastric cancer and suggested ADAM10 to be a useful marker for predicting tumour progression (Wang et al. 2011). By immunocytochemistry analysis we also identified the increased expression of ADAM10 in cervical and oesophageal cancer cell lines compared to non-cancer, as well as localization throughout the cell. ADAM10 has been predominantly been identified to be localized on the cell-surface (Gutwein et al. 2002; Stoeck et al. 2006), it has recently been detected in the nucleus and cytoplasm of cells (McCulloch et al. 2004a).

Our data suggest a role for ADAM10 in the migration and invasion of both cervical and oesophageal cancer cell lines. These results suggest too that ADAM10 may be important for the transition of epithelial cells of cervical and oesophageal origin to metastatic mesenchymal cells, since inhibition of ADAM10 expression causes a reversion in cells of a more epithelial-like nature. ADAM10 is associated with epithelial-mesenchymal transition (EMT) of cells leading to metastasis, and the development of new tumours in other parts of the body (Maretzky et al. 2005; Nijkamp et al. 2011)

EMT has been reported to associate with the epidermal growth factor (EGF) and E-cadherin in cervical cancer (Lee et al. 2008). ADAM10 has been identified to be an enzyme responsible for the activation of E-cadherin and HB-EGF, hence ADAM10 may be a useful primary target for reducing metastasis and invasion of cancer cells (Zhang et al. 2008; Maretzky et al. 2005). ADAM10 has also been associated with the activation of many other cytokines (Bzowska et al. 2009), adhesion molecules (Reiss et al. 2005), and cell cycles such

as Notch signalling (Bozkulak & Weinmaster 2009). ADAM10 thus has potential as a target for reducing cancer cell progression.

Although results suggest an association of ADAM10 with aspects of EMT transition such as adhesion, migration, cytoskeletal reorganization and invasion in cervical and oesophageal cancer, the fact that we saw no inhibition in cell proliferation when ADAM10 is inhibited. This suggests that different mechanisms of action may occur depending on cancer and/or tissue type. The ADAMs family of proteins are very closely related, with ADAM10 and ADAM17 sharing many structural and functional similarities (Saftig & Reiss 2011). These metalloproteases also compensate for loss of function of the other (Bozkulak & Weinmaster 2009). It may be important to analyse the expression of different ADAM family members, such as ADAM17, in the absence of ADAM10 expression in cervical and oesophageal cancers. The effect on inhibiting the expression of both ADAM10 and ADAM17 in cancers, such as cervical and oesophageal cancer may yield interesting results in terms of cell proliferation.

In analysis of EMT, epithelial cells require an increased expression of E-cadherin and low expression of vimentin for increased cell-cell adhesion for tumour formation. Alternatively, in mesenchymal cells there is a high expression of vimentin with low expression of E-cadherin, causing cell metastasis (Nijkamp et al. 2011). Decreased expression of E-cadherin and increased expression of vimentin has been associated with the survival of cervical squamous cell carcinomas (Cheng et al. 2012). Thus, to conclusively analyse ADAM10

relevance in EMT in cervical and oesophageal cancer cells, E-cadherin and vimentin relative expression should ideally be analysed in cells with reduced expression of ADAM10. These experiments will be performed as part of future work.

In conclusion, our study using cervical and oesophageal cancer patient material shows that ADAM10 is elevated in the cancers and thus has the potential to serve as a biomarker for these cancer types. Using cell based assays we show that ADAM10 is required for the migration and invasion of cervical and oesophageal cancer cells suggesting that targeted inhibition of ADAM10 in these cancers may have therapeutic potential.

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