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An investigation into the role of Lamin A in cell motility and Epithelial to Mesenchymal Transition in Colorectal Cancer

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Degree in Master of Science in Cell Biology
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Abstract

The Lamin A protein has a number of structural and gene-regulatory roles. Recently novel functions for Lamin A have been established in colorectal cancer, where expression of Lamin A is a biomarker of poor patient prognosis. Colorectal cancer is the fourth most frequent cause of cancer-related death world-wide, therefore understanding the molecular mechanisms behind the disease is of importance in the development of future therapeutic strategies to reduce mortality rates. SW480 colorectal cancer cells that artificially express Lamin A (SW480/Lamin A cells) demonstrate increased cell motility and a mesenchymal-like morphology, suggesting a more aggressive cancer cell phenotype in the presence of Lamin A expression. The aims of this project were to further explore how Lamin A expression contributes to a more aggressive cancer cell phenotype in SW480 cells. Herein cell density experiments show that at low cell density, SW480/Lamin A cells express the mesenchymal markers Slug and Vimentin. Scratch wounding assays of SW480/Lamin A cells show that cell junctions are absent from these cells at high cell density and the cytoskeletons of SW480/Lamin A cells are able to form motile structures. Finally silencing of Lamin A in SW480/Lamin A cells caused some of these changes to reversed. Together this data indicate that transfection of SW480 cells with Lamin A permits a signalling environment conducive to EMT and thus patients with high levels of Lamin A in the cells in their tumours may have a poorer prognosis due to increased cellular metastatic potential as a result of EMT.

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Chapter 1- Introduction

1.1 Background

As human life expectancy continues to increase, the global burden of age related disease must be addressed. Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females (Jemal et al, 2011) and is the fourth most frequent cause of cancer-related deaths worldwide (Weitz et al, 2005). The primary cause of this mortality is the formation of metastatic tumours that allow the malignant cells to spread to secondary sites within the body (Yilmaz and Christofori 2009). The molecular mechanisms underlying uncontrolled cellular proliferation, along with the gene mutations responsible for the formation of a neoplasm have been studied intensively, yet the cellular changes that allow metastasis, the ability for malignant cells to invade cardiovascular and lymphatic systems and extravasate into other sites in the body, are less well known (Kumar and Hall, 2009). The process postulated to be responsible for metastasis is known as Epithelial to Mesenchymal Transition (EMT) and has been the recent focus of many studies involving breast cancer (Iwatsuki et al. 2009). The process of EMT in colorectal cancer has been less well studied, but recent findings have suggested a relationship between the expression of the Lamin A gene in SW480 colorectal cancer cells and EMT (Foster et al, 2011;Willis et al, 2008b). Expression of Lamin A in SW480 cells causes them to be significantly more motile and display some morphological, genetic and proteomic characteristics of mesenchymal cells. The aim of the following studies was to further elucidate the contribution of Lamin A to EMTs in SW480 colorectal cancer cells.

1.2 Colorectal Cancer and adenocarcinoma formation.

The epithelial lining of the colon is renewed once every 5-7 days. The structure of the colon is such that epithelial stem cells, that allow this renewal process to occur, reside in the crypts of the colon. Crypts are bottle shaped invaginations that form proliferative compartments (McDonald et al. 2006) and transit amplifying cells, that are created when a stem cell divides, migrate upwards and form the fully differentiated epithelial cells that reside at the top of the crypt and form the lining of the colon (Figure 1). Lamin A expression has been observed in the stem cells of these crypts, suggesting a role for the Lamin A protein in the maintenance of the stem cell niche and hence indicating the importance of Lamin A expression in epithelial tissue in the colon (Willis et al 2008a).
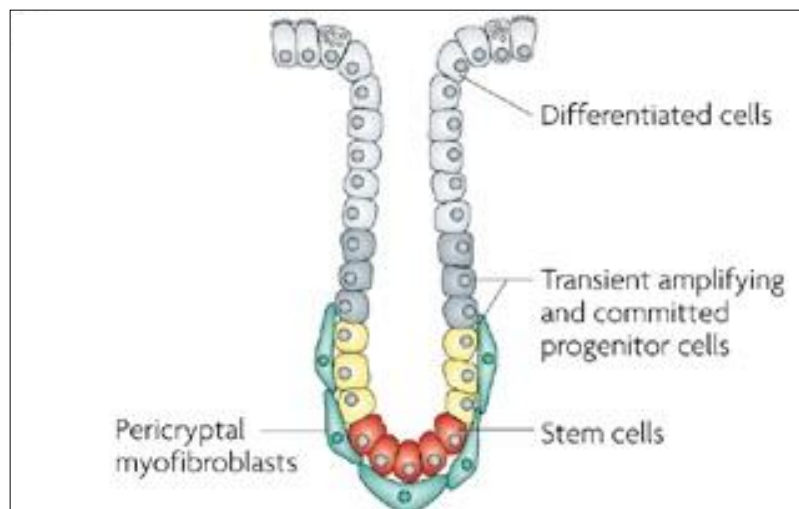
Figure 1: The structure of the colonic crypt with stem cells labeled in red (Taken from Humphries and Wright, 2008).

A benign tumour, known as an adenoma, is the result of uncontrolled proliferation from a cell that would normally migrate out of the crypt compartment to become terminally differentiated. This is the first step towards the formation of a malignant adenocarcinoma. In the case of sporadic colorectal cancer, a genetic mutation or deletion in a tumour suppressor gene or a mutation in an oncogene causes the cell to behave aberrantly within the crypt (McDonald et al, 2006). One of the most common genetic changes that can occur within the cell is an inactivating mutation in the APC tumour suppressor gene that controls the cell cycle. This accounts for 90% of cases with the other approximate 10% being caused by heterozygous activating mutations in the β-catenin gene. Mutations in this gene cause nuclear accumulation of the transcription factor β-catenin, which activates the Wnt signaling pathway resulting in increased cellular proliferation due to aberrant cell cycle control (Pinto and Clevers, 2005).

The transition of a benign adenoma into a malignant adenocarcinoma is thought to involve further activating genetic mutation in the proto-oncogene K-Ras, inactivating mutations in the tumour suppressor gene Smad4 and inactivating mutations in the tumour suppressor gene p53 (Pinto and Clevers 2005). In response to further genetic mutation, cells undergo uncontrolled proliferation and penetrate through the crypts into the underlying muscularis. When the cells subsequently metastasize, they disseminate into cardiovascular or lymphatic transport systems (Fearon and Vogelstein, 1990). The transition from healthy epithelial cell to malignant adenocarcinoma is demonstrated in Figure 2.
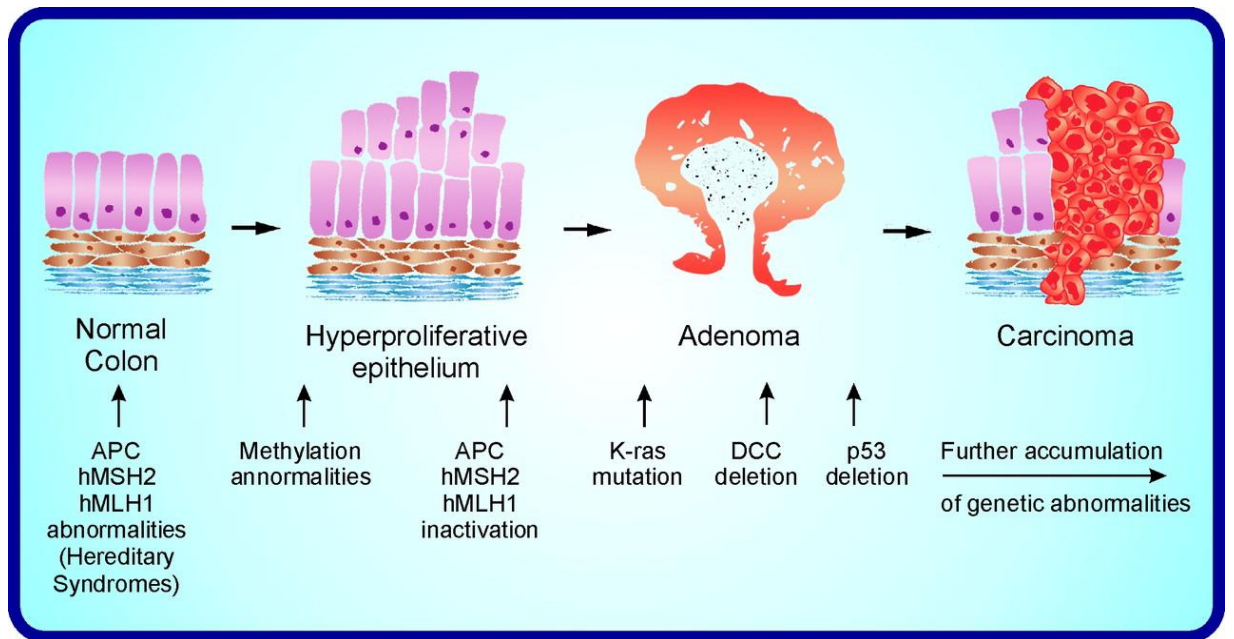
Figure.2- The series of genetic alterations involved in the adenoma-carcinoma sequence. Mutations in the APC, K-ras, and p53 ultimately result in colorectal cancer (taken from http://clinicalvignettes.blogspot.co.uk/2010/12/old-man-with-colonoscopic-findings-of.html)

1.3 Colorectal Cancer- Hereditary forms and global incidence rates.

The vast majority of studies into colorectal cancer have been based on the hereditary forms of the condition. These are classified into two groups, depending on the formation of colonic adenomas. In 80% of cases, Familial adenomatous polyposis (FAP) syndrome occurs when there is a germline mutation in the APC tumour suppressor gene, which results in the formation of thousands of colonic adenomas in the third and fourth decades of life (Weitz et al, 2005). The presence of these polyps increases the likelihood of adenocarcinoma formation by almost 100%. Hereditary non-polyposis colon cancer (HNPCC) (Kinzler and Vogelstein, 1996) arises as a result of a germline mutation in one of the DNA Mismatch Repair (MMR) genes. The resulting defect in DNA repair mechanisms leaves the patient susceptible to a mutation

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in a tumour suppressor gene or an oncogene that will eventually lead to the uncontrolled cellular proliferation responsible for the formation of an adenocarcinoma (Eshleman et al, 1995). These cases account for only around 10% of the global incidence, with the rest being sporadic forms of the disease (Lynch and de la Chapelle, 2003).

Statistics on the global incidence rates of the disease were carried out by Jemal et al (2011) using GLOBOCAN, a standard set of worldwide estimates of cancer incidence and mortality. High incidence rates were found in the more economically developed areas of Australia, New Zealand, Europe and North America whereas rates were low in less developed countries, such as Africa and south central Asia. The statistics demonstrate how a lifestyle involving alcohol and red meat consumption, which is more typical in developed countries increased colorectal cancer incidence. The reduced levels of physically active individuals in these populations are also thought to increase incidence of colorectal cancer. This lifestyle is also responsible for the high incidence rates seen in eastern Asia.

Another factor to be considered is the global availability of treatment. Recent improvements in awareness of the disease and screening strategies in the USA have allowed a significant decrease in incidence rates due to patient removal of precancerous lesions before they develop into malignant carcinomas. This is also reflected by higher incidence rates in areas where health infrastructures are less established and resources are limited, such as central and Southern America and Eastern Europe (Jemal et al 2011)
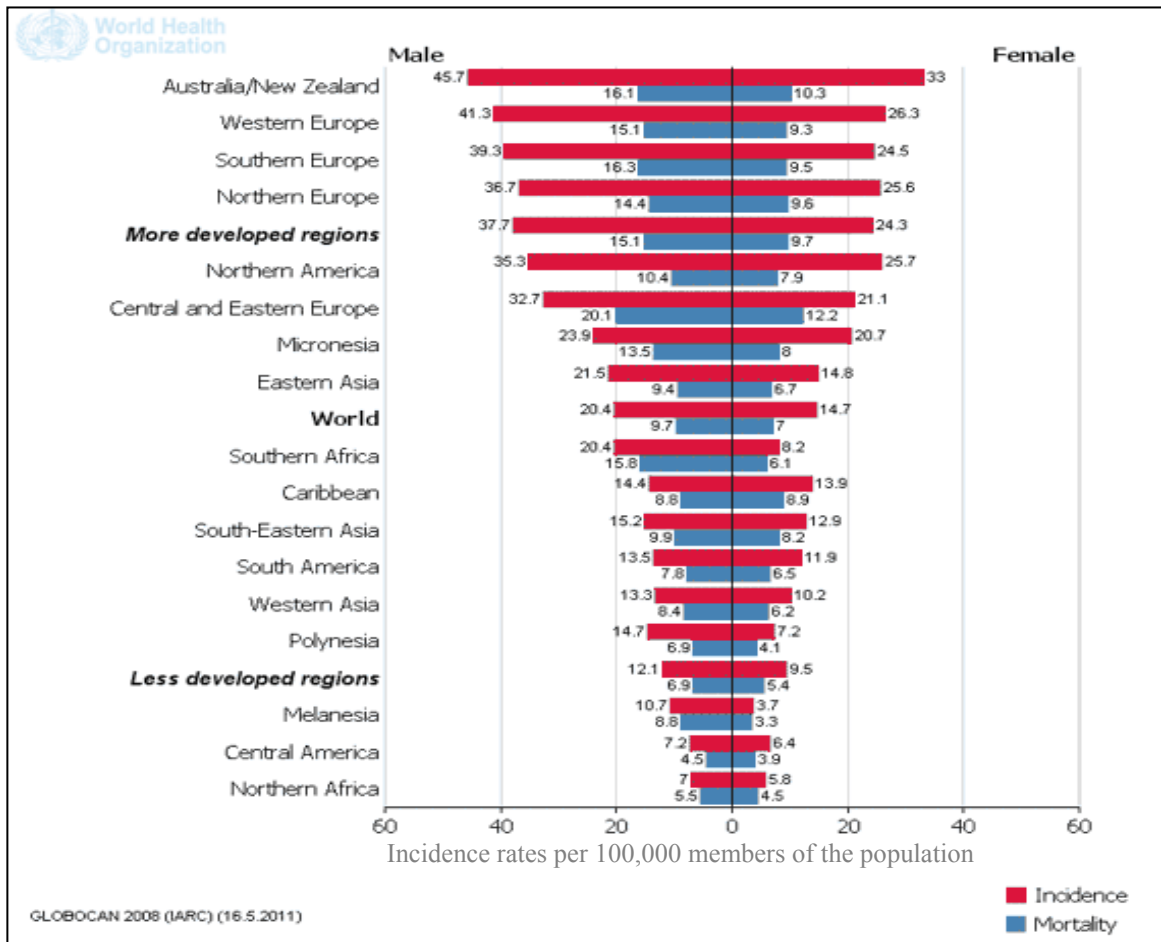
Figure 3- Worldwide colorectal cancer incidence rates are dramatically higher in developed countries compared to less developed countries (Taken from the INDOX cancer research network website- originally published in the 2008 GLOBOCAN study).

1.4 Lamin Proteins

The Lamin proteins are type V intermediate filament proteins with a well-conserved domain structure. This consists of a NH2 globular head domain, a central α-helical rod domain and a globular COOH terminus. The central portion of the filament is composed of linker regions interconnecting coiled-coil regions. These linker regions are highly conserved across species, which is suggestive of their important role in Lamin structure and function (Fisher et al, 1986). The Lamin proteins form the lattice-shaped nuclear lamina, situated just

below the inner nuclear membrane of the nucleus therefore forming a structural cage surrounding the cell's genetic material (Hutchison, 2002).
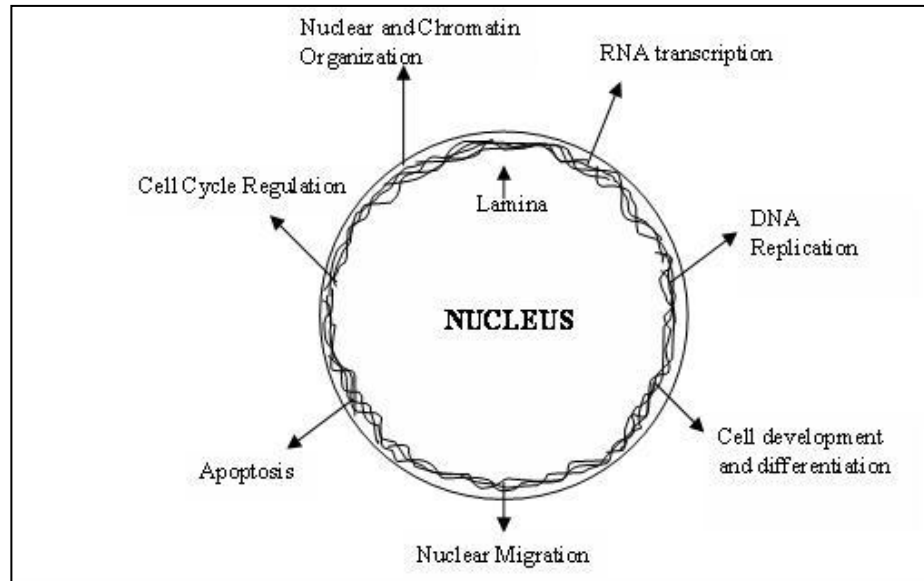
Figure 4- Location, structure and functions of the nuclear lamina. (Taken from Clark et al ,2008).

The location of the lamina, and the spectrum of diseases that result through the acquisition of mutations in the Lamin A gene are highly suggestive of a multifunctional role for these proteins in both genetic and structural aspects of cell physiology, including tumour progression, control of nuclear architecture, regulation of gene expression, apoptosis, senescence and chromatin organization and segregation (Foster et al, 2010).

Although the Lamin A protein has multiple functions, its expression is limited to differentiated cells within post embryonic tissues, unlike the B-type Lamins, whose expression is crucial for cellular development and survival. This has led to the conclusion that B-type Lamins constitute the fundaments of the nuclear lamina whereas the A-type lamins play more specialized roles (Sullivan et al

1999). Elucidation of these roles is commonly studied through observation of the Laminopathies- the spectrum of diseases that arise through mutations in the LMNA gene. The structural role that Lamin proteins play in cellular integrity is demonstrated through mutations in the LMNA gene, which can give rise to striated muscle disorders known as Muscular Dystrophies. The Lamin proteins form part of the LINC complex, which binds the nucleus to the cytoskeleton through a series of proteins (Figure 5). Mutations in Lamin A will compromise the structural integrity within this complex, resulting in aberrant cytoskeletal organization and positioning of the nucleus that causes disease (Meinke et al 2011).
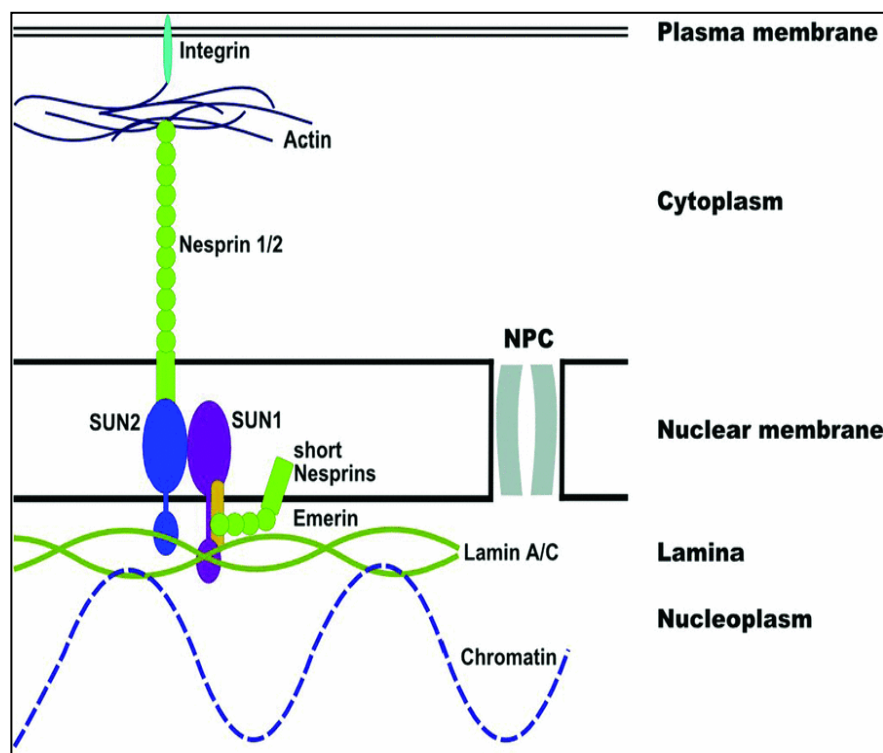
Figure 5- The LINC complex links the nuclear lamina to the actin cytoskeleton via a series of proteins (taken from Meinke et al 2011).

1.5 Lamin Proteins- Structural and gene regulatory roles

Lamins A and C have number of binding partners that allow a bridge from the inner nuclear membrane to the cytoskeleton. The Lamina, situated on the inner nuclear membrane is bound to SUN proteins that traverse the inner nuclear membrane. Sun proteins bind to Nesprin proteins, that traverse the outer nuclear membrane. Outside of the nucleus, Nesprins bind to the actin cytoskeleton (Figure 5). Nesprin 3 also binds to intermediate filaments via plectin and Nesprin 4 binds to microtubules via kinesins (Schneider et al, 2010)(Not shown in figure). The structural links made by Lamins, SUNS and Nesprins tether the nucleus to the actin cytoskeleton thus allowing mechano-transduction of the nucleus. This allows nuclear re-orientation and cell migration (Luxton et al., 2010). As the nucleus is the largest and most rigid organelle, harbouring precious genetic material, its physical properties contribute critically to the biomechanical behavior of motile, contractile cells. This is reflected in the spectrum of disorders that arise as a result of mutation in the nuclear envelope proteins, including many forms of muscular dystrophy (Meinke et al., 2011).

The gene-regulatory roles of the Lamin A protein have been indicated by its interaction with a number of binding partners situated at the nuclear membrane. These include the proteins LAP2α, FACE1, MAN1 and emerin (Maraldi et al., 2011). Genetic or epigenetic variations in both Lamin A/C and its binding partners results in changes in cellular signaling pathways. When signalling proteins normally binding to nuclear membrane proteins are unable to do so, aberrant mis-localisation of signal transducers can occur, resulting in the de-regulation of signalling pathways that control cellular proliferation and

apoptosis. An example of this is the TGFβ signaling pathway, where mutations in Lamin A can cause altered interaction with PPA2 phosphatase. This results in aberrant phosphorylation of Smad transcription factors thus preventing their activation of target genes within the nucleus (Van Berlo et al., 2005). The ultimate result of aberrant cellular signaling is disease, and given the diverse number of binding partners and signaling pathways regulated by Lamins A/C, the resultant Laminopathies constitute a wide spectrum of disorders affecting muscular and fat tissues, insulin regulation and cellular ageing (Broers et al., 2006).

1.6 Lamin Proteins and Cancer

As highlighted, Lamin proteins have both structural and gene regulatory roles. Both of these functions of Lamin proteins have implications in tumour cell progression and metastasis. Mutations in the Lamin proteins and their binding partners leads to alterations in nuclear plasticity, cell polarity cues and cell motility- all of which play a role in invasion and metastasis (Chow et al., 2012). There are also a number of signaling pathways that are commonly found to be de-regulated in cancer cells that are also known to be modulated directly or in-directly by Lamin A/C or its binding partners. Examples include TGFβ, Epidermal Growth Factor, WNT and NOTCH (Chow et al., 2012). More recently, the LMNA gene has become a prognostic biomarker in both colorectal (Willis et al., 2008b) and prostate (Kong et al., 2012) cancers, where it is indicative of poor patient prognosis. Lamin A expression within colorectal cancer cells causes them to adopt a more motile, aggressive phenotype , which is indicative of a multifunctional and highly complex role for Lamin A within the gene expression and cytoskeletal organisation of neoplastic cells (Foster et al 2009).

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1.7 Epithelial to Mesenchymal Transition.

Metastasis of cancerous tumours is responsible for the majority of cancer-related death due to the difficulty in localizing and removing secondary tumours that have been seeded at distant sites (Kumar and Hall, 2009). The process by which cells are able to become detached from an epithelial sheet, invade the basement membrane of an epithelial tissue and intravasate into the cardiovascular or lymphatic system is known as Epithelial to mesenchymal transition (EMT). This is thought to be the cause of cancer metastasis and chemotherapeutic resistance (Iwatsuki et al 2010). EMT's may also act to allow the continued process of uncontrolled cellular proliferation seen in a carcinoma by preventing cellular senescence. The overriding of cell cycle arrest is achieved by inducing the expression of certain oncogenes. As a result of continued proliferation beyond normal limits, subsequent aggressive dissemination of cells from the primary tumour is permissible, thus allowing the invasion of cancer cells into the blood and lymph (Kalluri and Weinberg, 2009).

 The process of EMT occurs during normal embryonic development, during gastrulation and segment formation, which formed the basis of early study into this phenomenon (Bates and Mercurio, 2005). More recently important differences have been outlined in this process occurring within metastatic cells, where oncogenic activation of signaling molecules is responsible for the process. This haphazard signaling is un-like the distinct spatial and temporal signaling of certain molecules programmed to orchestrate cell migration within a developing embryo (Kalluri and Weinberg, 2009).

Of the biological molecules identified in cancerous EMT, NF-κB and TGFβ signaling partners and their down-stream transcriptional effectors, Twist and Snail have been implicated in oncogenic EMT, where they repress the expression of the epithelial cell junctions via repression of E-cadherin expression and induce the expression of mesenchymal markers such as vimentin (Kumar and Hall, 2009)(Figure 6)
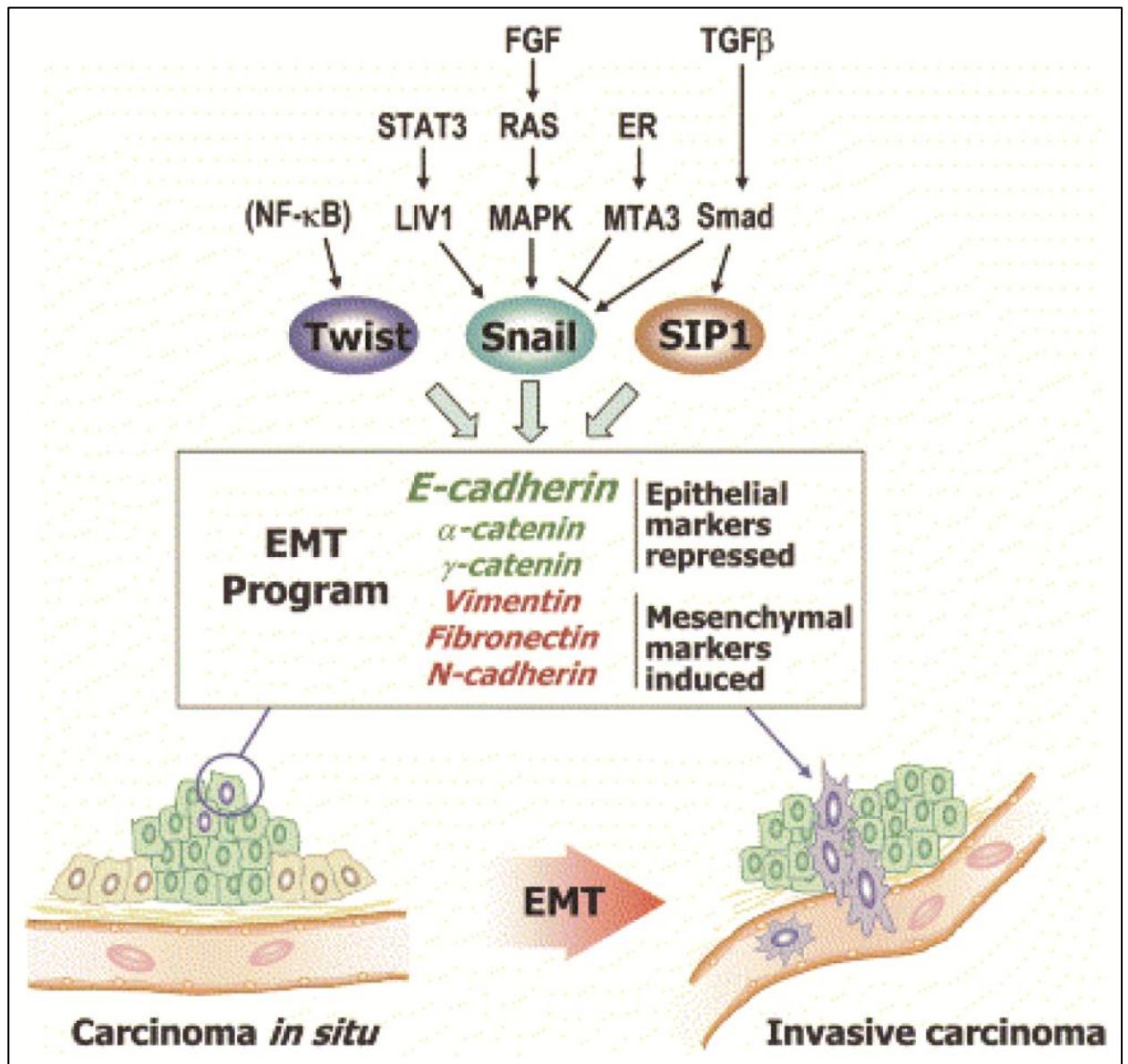
Figure 6- A representation of some of the signaling events causing EMT in cancer (taken from Zvaifler, N J (2006).

1.8 Inducing EMT's

Signals originating from the stroma have been shown to be the initial inducers of EMT. Early experiments identified growth factors as inducers of EMT by the observation that conditioned fibroblast medium, containing HGF, could induce polarized epithelial cells to scatter into separate single cells (Stoker and Perryman, 1985). In this way EMT is induced by extracellular stimuli through activation of kinase-dependent signalling cascades. Other EMT-inducing signals that have been identified are EGF, PDGF and TGFß (Kalluri and Weinberg, 2009).

TGFß in particular has been extensively studied and has been identified as an important mediator of tumour progression. The role of TGFB as both a suppressor of epithelial cell proliferation and a positive regulator of tumour progression and metastasis is dependent on the cellular and environmental context. In pre-malignant cells, it plays an anti-oncogenic role by functioning as a tumour suppressor, regulating senescence and autophagy. However, in concert with other signalling pathways, TGFβ signaling can promote epithelial cancer once initiation of the transformation into a malignant cell has occurred. Furthermore, binding of TGFß to its receptors can activate many non-canonical signaling pathways, such as phosphoinositol-3-kinase, mitogen-activated protein kinase and small guanosine-triphosphate pathways that are implicated in EMT (Grady, 2007). The downstream result of activation of one of the protein kinase pathways- the serine/threonine kinase signaling pathway, is the ubiquitination and subsequent degradation of RhoA which is a small GTPase responsible for the maintenance of apico-basal polarity and junctional stability. In this way it causes the dissolution of the cell-cell contacts at E-cadherin based

cell junctions which allows the production of metastatic cells from an epithelial sheet (Ozdamar et al, 2005).

In order for cancerous cells to accomplish EMT, they must undergo a number of molecular changes that causes them to firstly dissolve their cell-cell junctions. This process initially involves the down regulation of the homophillic cell adhesion molecule E-cadherin (Bates and Mercurio, 2005). This protein normally functions in the maintenance of the integrity of epithelial cell layers, where it forms adherens junctions that join adjacent cells into a polarized epithelial sheet. Its replacement with an increase in N-cadherin expression is known as the "cadherin switch" and is a prominent characteristic of cells undergoing EMT (Yilmaz and Christofori, 2009). N-cadherin expression occurs when cells undertake a fibroblastic phenotype, and has shown to be correlated with motile and metastatic cells (Nieman, 1999).

Upregulation of Vimentin, which is an intermediate protein that forms the cytoskeleton in mesenchymal cells, has been shown to play an important role in cancer cell metastasis and motility (Ivaska et al, 2007). Mice with targeted removal of the Vimentin gene have impaired wound healing, which is indicative of the effect a lack of Vimentin has on cell migration. This is thought to be due to the fact Vimentin intermediate filaments form part of the cytoskeleton in mesenchymal cells, and hence play a role in mechanotransduction within the cell that has a direct effect on cellular motility.

The Vimentin cytoskeleton has been found to associate with a6b4 integrin proteins, that are located at cell-cell junctions. This is indicative of a further function for Vimentin in the regulation of the change in the endothelial barrier

seen during the EMT process as it suggests Vimentin may communicate with cell junction proteins in order to dissolve cell-cell contacts. The protein is thought to be a regulator of a number of key genes that govern the cellular motility responsible for metastasis, such as the tyrosine kinase protein AXL. Knockdown of Vimentin within breast cancer cell lines has demonstrated down regulation of this gene, along with other genes linked to cancer cell migration and invasiveness (Vuoriluoto et al, 2011).

Other important governors of this phenotypic change are transcription factors that induce the expression of key genes related to the acquisition of a more motile cellular phenotype. These transcription factors are also commonly found to be employed in non-oncogenic embryonic development, hence underlining how oncogenic EMT maintains some similarities with developmental EMT (Kalluri and Weinberg 2009). The Snail family of transcription factors have been shown to be important inducers of EMT through their powerful ability to suppress E-cadherin expression. Snail and Slug are thought to promote the formation of ß-catenin T-cell transcription complexes that promote the expression of TGFß by binding to the promoter of TGFß3 gene to increase its transcription (Hoshino et al, 2009). The downstream effects, as previously discussed, result in the promotion of EMT.

A study by Camp et al (2011), has demonstrated that expression of the Slug transcription factor resulted in phenotypic and molecular changes characteristic of EMT within in a non-invasive rectal cancer cell model. The DLD-1 cell lines used were transfected with Slug to allow overexpression to occur. The resulting genetic changes gave the cells a significantly enhanced ability to migrate through a matrigel matrix along with morphogenetic changes to the cells, such

as an increased spindle shape and the formation of motile cytoskeletal structures such as pseudopodia. Slug expression inversely correlated with e-cadherin expression and when the genetically transformed cells were transplanted into mice a significant tumour formation rate was recorded, where 90% of the mice transplanted with the Slug-expressing cells formed tumours whereas only 11% of the mice transplanted with the non- Slug cells showed tumour formation.

1.9 Recent work leading up to this project and aims

Mutation and aberrant expression of the LMNA gene, which codes for the A-type Lamin protein, is known to give rise to a spectrum of diseases where abnormal expression, distribution and structural organisation of the protein predominantly affects cells of a mesenchymal lineage. Aside from these laminopathies (Broers et al 2006), a new role for Lamin A has been postulated within epithelial cancer. It has been shown that patients with colorectal adenocarcinomas have a poorer prognosis when Lamin A expression was found within their tumours (Willis et al, 2008). In order to explore this, experiments were conducted to elucidate how Lamin A expression affects cell motility and invasiveness in a well characterised pre-metastatic colorectal cancer cell line SW480. The SW480 cells, which were originally established from a primary adenocarcinoma of the colon, were stably transfected with DNA constructs encoding a fusion protein of EGFP-lamin A full-length (SW480 Lamin A cells) (gift from Dr M. Izumi, Institute of Physical and Chemical Research, Saitama, Japan) and EGFP (SW480 control cells) The transfected cells were selected depending on their successful increase in expression of Lamin A and viability. (detail in Willis et al., 2008).

This model revealed that Lamin A expression causes a change in cell phenotype that allowed the cells to become more motile- demonstrated by their ability to close the gap created by wounding a layer of confluent cells (Foster et al, 2011). In order to explain this phenomenon, RT PCR was carried out to investigate genetic changes in the actin-bundling family of proteins, known as the plastins. These proteins play key roles in the organization of the actin cytoskeleton, which has a direct effect on cellular motility. Furthermore, their expression has

been associated with aggressive and radiation- resistant tumour cells. Analysis revealed that T-plastin was up regulated in the GFP-Lamin A transfected cells compared to the control cells that were transfected with GFP only. This up-regulation was coupled with a down regulation in the expression of the cell adhesion molecule E-cadherin, which is an indicator of invasive cellular behaviour in neoplastic tissue and development of a carcinoma (Willis et al 2008b). This data, along with the wounding assay gave a strong indication of the role that Lamin A expression may play in cytoskeletal re-organization that allows cells to adopt a more motile and aggressive phenotype (Foster et al. 2011).

This thesis looks to extend our knowledge of the contribution of Lamin A in the ability of SW480 cells to undergo EMT. Cell density experiments, wounding assays and Lamin A gene silencing were used to study classical EMT markers in relation to Lamin A gene expression in SW480 cells.

Chapter 2- Materials and Methods

2.1 General Chemicals and Materials

All chemicals were supplied by Sigma-Aldrich (Poole, UK).

2.1.1 Mammalian Cell Lines

The initial SW480 cell line obtained for use was derived from a Broders' grade 4, Dukes B primary colon adenocarcinoma (Leibovitz et al., 1976) and was supplied by the European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, UK). Stable transfection of the cells with DNA constructs encoding for EGFP-lamin A and EGFP was performed by Dr Naomi Willis (Willis et al., 2008) hence creating the SW480/Lamin A (Stable GFP Lamin A expressing) and SW480/Control (Stable GFP expressing) cell lines respectively.

2.1.2 Mammalian Cell Culture

Cell culture was performed in a CL2 suite (Containment Level 2 laboratory) under a sterile flow hood using aseptic technique. Cells were grown in a CO_2 free, humidified incubator at 37°C.

2.1.3 Subculture

Cells were grown in Leibovitz's L15 media with Glutamine and Amino Acids (supplied by Fischer) and supplemented with 100units/ml of both penicillin and streptomycin (supplied by Invitrogen). Media was further supplemented with

10% foetal bovine serum lot 057K3395. Cells were grown in both 75cm^2 and 25cm^2 flasks supplied by Greiner Bio-One Ltd (Gloucestershire UK).

Cells were washed at 70%-80% confluency with Versene [137mM NaCl, 2.7mM KCl, 8mM Na$_2$HPO$_4$, 1.5mM KH$_2$PO$_4$, 1.5mM EDTA pH 7.4]. Cells were then detached from flasks by incubating them in 10% Trypsin in Versene for 4-5 minutes at 37°C. The cell suspension obtained was neutralised with medium and centrifuged (Eppendorf 5810R- programme 4) at 200g for 5 minutes. The resulting supernatant was removed using an aspirator and cell pellets were re-suspended in fresh medium and seeded into new flasks. The cells were split according to growth rate, SW480/Lamin A cells were split 1:4-1:6 and SW480/Control cells were split 1:3-1:4.

Versene, Versene with 10% Trypsin and L-15 Media were stored at 4°C and heated to 37°C in a chemically treated water bath before use on cells.

2.1.4 Cryopreservation

Cells pellets were obtained as above. Pellets were then re-suspended in 1ml of a mixture containing 90% medium and 10% DMSO (dimethylsulfoxide- added dropwise to the cells after resuspension in 0.9ml media). Cells were then transferred to cryovials that were stored at -150°C.

To re-establish cultures after freezing, cells were thawed at 37°C and re-suspended in L-15 medium. Cells were then centrifuged (Eppendorf 5810R- programme 4) at 200g for 5 minutes. The supernatant was removed by an aspirator and the cell pellets were re-suspended in fresh media before seeding cells into 25cm^2 flasks. Cells were subsequently passaged into 75cm^2 flasks and some were cryopreserved to maintain frozen stocks of cells.

2.2 Immunoblotting

Immunoblotting was performed to investigate the effect that cell confluency has on the expression of proteins in relation to cell motility.

2.2.1 Preparation of whole cell extracts

Cell cultures were grown to varying stages of confluency (50%-90%) before washing in Versene at 37°C. Cells were detached from culture flasks using cell scrapers (supplied by Greiner Bio-One). Cell suspensions obtained were centrifuged at 200g for 5 minutes. In order to generate equal sized pellets at various levels of cell confluency, multiple flasks of the same cells were scraped at the lower levels of confluence (50-60%) and the suspensions from each flask were combined before centrifugation. The resulting supernatant was aspirated away from the cell pellet before resuspension in PBS stored at 4°C. The resuspension was centrifuged at 200g for 5 minutes (Eppendorf 5810R). The supernatant was aspirated away and the resulting cell pellet was snap-frozen in a flask of liquid nitrogen before storage at -80°C. Cell samples produced are shown in Table 1.

Table1: Cell types and densities used to make samples for immunoblotting

Cell confluency	Cell Type
50%	SW480/Lamin A
100%	SW480/Lamin A
50%	SW480/Control
100%	SW480/Control

Cellular lysis was performed by thawing then incubating pellets on ice in 500 μl of a detergent-containing hypotonic buffer [10mM Tris-HCl pH7.4, 10mM KCl, 3mM MgCl$_2$,0.1 (v/v) Triton X-100] plus 2μl DNase (5units/μl), 2 μl protease inhibitor cocktail (Sigma- AEBSF,2 mM ,Aprotinin, 0.3 μM, Bestatin, 130 μM EDTA, 1 mM E-64, 14 μM Leupeptin, 1 μM) and 50μl NEM (n-ethylmaleimide)].2-10 μl of each sample was then removed in order to calculate protein concentration. 500 μl of 2x sample buffer [125mM Tris-HCl pH 6.8, 2% (v/v) SDS, 2mM DTT, 20% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.25% (w/v) bromophenol blue] was then added. Samples were stored at ---80°C when not in use.

2.2.2 Bradford assay

In order to make accurate comparisons of the levels of protein shown on an immunoblot, equal levels of protein must be loaded onto the original resolving gel. In order to accomplish this, the level of protein within each individual sample must be quantified. The Bradford assay uses colorimetry in order to do this. Bradford reagent (Sigma-Aldrich®) contains a Brilliant Blue G dye reagent that forms a complex with the available protein in solution. The dye undergoes an absorbance shift from 465nm to 595nm upon protein binding therefore measuring the absorbance of samples at 595nm prepared with reagent will give an accurate quantification of the amount of protein available.
Protein standards were prepared using BSA (bovine serum albumin) according to Table 2 in order to produce a standard curve for use in determination of the unknown protein concentration of whole cell extracts. The standards were made up in triplicate and their absorbance value was measured at 595nm using

a spectrophotometer (). A standard curve- Figure 7, was produced from the obtained absorbance values using Microsoft Excel ®.

Table 2 Reagents used for preparation of protein standards for Bradford Assay

1mg/ml BSA (µl)	0	1	2	5	10	15
0.1M HCl (µl)	10	10	10	10	10	10
Lysis Buffer (µl)	2	2	2	2	2	2
H_2O (µl)	88	87	86	83	78	73
¼ Bradford Reagent (µl)	900	900	900	900	900	900
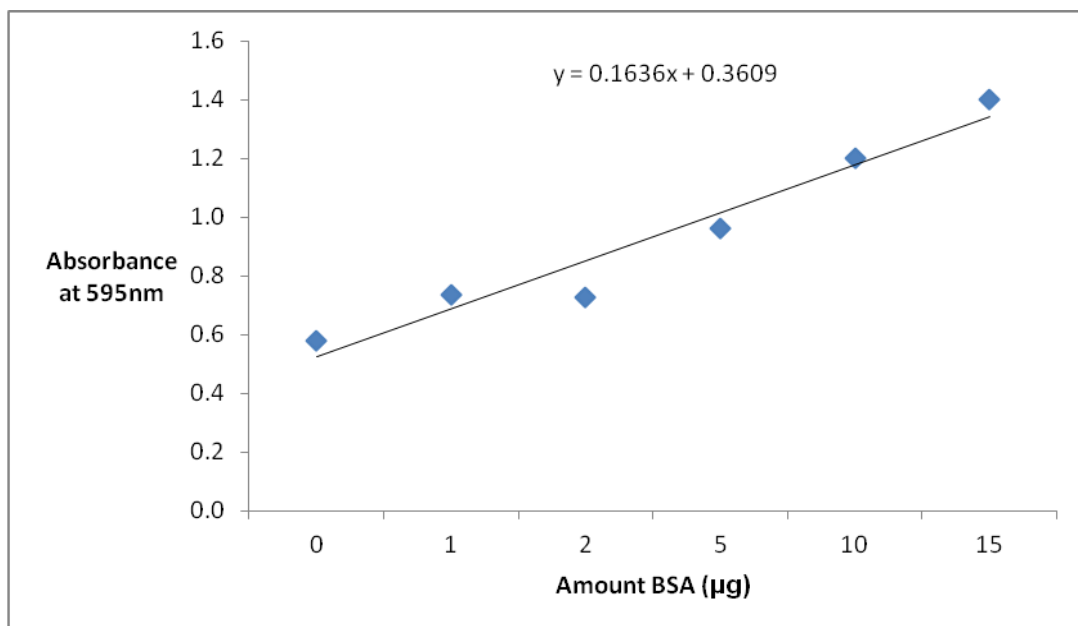
Figure 7: Standard curve produced from the obtained absorbance values in Bradford Assay

To measure unknown protein concentration of samples, 2µl aliquots of protein sample were added to 10µl of 0.1M HCl, 88µl of H_2O and 900µl of ¼ Bradford Reagent (diluted in Milli-Q H_2O). Samples were produced in triplicate and the

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obtained absorbance values were used to extrapolate from the pre-produced standard curve in order to determine unknown protein concentration.

2.2.3 One-Dimensional SDS-PAGE

The procedure of Laemmli (Laemmli, 1970) was used to separate proteins according to their molecular weight (M_r) on an acrylamide-based gel. Gels were cast using the Mini-Protean Electrophoresis System (Biorad). The resolving gel used depended on the molecular weight of the proteins of interest. 10% gels were used to probe for E-Cadherin (135kDa), GFP-Lamin A (135kDa) Lamin A (75kDa). 12% gels were used to probe for Slug (30kDa) and Vimentin (55kDa). Gel mixture was made up according to Table 3, where the given quantities are suitable to make 2 gels. All solutions were prepared with Milli-Q™ H_2O. Gels were pipetted between pre-assembled plates and then butan-1-ol was pipetted on top of the gel mixture to ensure to top of the gel was flat. The gel was left for 40minutes to set before pouring off the butan-1-ol and pipetting on stacking gel mix (Table 3). In order to form wells a teflon comb was inserted into the stacking gel, between the gel plates and removed once the gels had set after 15 minutes.

Table 3- Quantities of Reagents for Acrylamide Gel Preparation

Amount Reagent	Resolving Gel- 10%	Resolving Gel-12%	Stacking Gel
ProSieve® 50 acrylamide gel solution (Cambrex BioScience Wokingham, Ltd., UK), (ml)	2.00	2.40	0.50
Milli-Q™ H$_2$0 (ml)	5.30	4.90	3.96
1.5M Tris-HCl pH 8.8 (ml)	2.50	2.50	0.50
10% SDS solution (µl)	100	100	50
10% Ammonium persulphate solution (µl) (Fisher Biosciences)	100	100	50
TEMED (µl) ((N,N,N',N'-Tetramethylethylenediamine).	4	4	5

Tank buffer [25mM Tris pH 8.3, 192mM Glycine, 0.1% (v/v) SDS] was used to submerge gels that were subsequently loaded with samples containing equal concentrations of protein using a fine tipped pipette. A molecular weight marker (PageRuler™ Prestained Protein Ladder Plus (Fermentas)) allowed molecular weights of visualised proteins to be determined and also demonstrated successful transfer of proteins onto a nitrocellulose membrane. Gel electrophoresis was performed at 100V and 40mAmp until samples migrated to the bottom of the resolving gel. This took around 2 hours.

2.2.4 Transfer of protein onto a nitrocellulose membrane.

Proteins that had been separated on gels were subsequently transferred onto nitrocellulose membrane (Protran®, grade BA85, Schleicher and Schuell Bioscience Inc., Keene, NH) using the Mini Transblot System (Bio-Rad) in transfer buffer [192mM Glycine, 25mM Tris HCl pH9.2, 20% (v/v) methanol, 0.1% (v/v) SDS] at 100V, 250mAmp for 1.5 hours at room temperature (25°C).

2.2.5 Antibody Incubations

Membranes were subsequently blocked in 4% (w/v) non fat dry milk in 2x Blot Rinse Buffer/Tween-20® (BRB/T) [150mM NaCl, 10mM Tris pH 7.4, 1mM EDTA, 0.1% (v/v) Tween-20®] at 4°C overnight on an orbital shaker. Primary and secondary antibodies were diluted in 2X BRB/T and 1% (v/v) NCS. Dilution and incubation time with primary antibody was dependent on the antibody and is listed in Table 2.5. All secondary antibodies were Polyclonal HRP-conjugated (Stratech Scientific Limited, UK) and were diluted to 1:2000 in BRB/T + 1% (v/v) NCS. All secondary antibodies were incubated for 1hr at 25°C.
AC-40 and GAPDH were used as loading controls as the levels of these proteins remain constant independently of cell type and cell density. Primary and secondary antibody incubations were separated by washing membranes for 3 X 5 minutes in BRB/T on an orbital shaker, changing the solution after each wash. After the secondary incubation membranes were washed again as above.

Table 4- Primary Antibodies used for Immunoblotting

Antibody	Working Dilution	Incubation Time on orbital shaker at 25°C (min)
Jol 2 (produced onsite)	1:200	45
Jol 4 (produced onsite)	1:200	45
Vimentin (Sigma)	1:1000	60
Slug (Cell Signalling Technologies)	1:500	75
AC-40 (Anti-β actin) (Sigma)	1:1000	60
GAPDH (abcam)	1:800	60 or overnight at 4°C

2.2.6 ECL Detection

Approximately 400µl per 5cm^2 of enhanced chemiluminescence (ECL) reagent (Amersham Biosciences) was added to each membrane for 2 minutes before detecting bands in a Dark Room using Hyperfilm™ ECL films (Amersham Biosciences) and a Compact X4 Automatic X-ray Film Processor (Xograph Imaging Systems Ltd., Gloucestershire, UK).

2.2.7 Densitometry

As the size of the dark band on an immunoblot is representative of the level of the targeted protein in the sample, amounts of protein were quantified using Densitometry, which calculates the optical density of band. Developed X-ray films were scanned onto a computer before quantification using Image J (Rasband, W.S., ImageJ, U.S National Institutes of Health, Bethesda, Maryland, USA http://rsb.info.nih.gov.ij/, 1997-2009.

2.3 Scratch wound assays

Scratch wound assays were performed to investigate the levels of expression of proteins in relation to cell motility in both the confluent cells at the centre of the scratch wounds and at the wound edges.

2.3.1: Cell Seeding

Glass coverslips with a circumference of 1cm were prepared in 6 well plates in a sterile flow cabinet. 3 coverslips were placed in each well using tweezers sterilised with ethanol. 200µl of 0.01mg/ml of Poly-D-Lysine (Sigma) was pipetted onto each coverslip and left to dry in the hood for 24h. Cellular suspensions for seeding were prepared when cells were at 60% confluence. Growth flasks of cells were washed in Versene before undergoing trypsinisation and pelleting as described in section 1.1 above. Cells were then counted by sucking a small sample of suspension into a 10ml pipette then dropping the liquid on to the chambers of an improved Neubauer haemocytometer by capillary action. Counting grids were viewed under a Zeiss Televal 31

microscope and cells that fell within the 16 square grid area were counted. Cells that fell on bottom and left sides of the grid were included and cells that fell on the top and right hand sides of the grid were ignored.

As the SW480/Control cells displayed a slightly reduced growth rate in comparison to SW480/Lamin A cells, SW480/Control cells were seeded at a density of 1x 10^6 cells per well 24h before seeding 1.1 x 10^6 of the SW480/Lamin A cells in L15 media that has been supplemented as described in section 1.1. The cells were then left to grow to 100% confluence for 2-5 days. If the growth period required lasted more than 2 days the cells were given fresh media on the 3rd day. Once confluent, cells were scratched using a p10 RNAase free pipette tip (Star Lab, UK). The media was then changed twice to remove any floating cell debris. Cells were then left for 4hr in a CO_2 free, humidified incubator at 37°C before the wounding reaction was stopped by replacing the media with 1x PBS.

2.3.2 Processing for immunofluorescence

Cover Slips were incubated in 500µl per well of 4% Paraformaldehyde (PFA) (Agar Scientific) at a pH of 7.4 at 25°C on an orbital shaker for 15min. PFA was then removed and the coverslips were washed for 3 x 5min in 1x PBS to remove any residual PFA. Cells were then incubated in 400µl per well of Permeabilisation buffer [1% (w/v) PBS, 10% (v/v) NCS, 0.1% TritonX-100 (Sigma)] for 15min at 25°C before washing for 2x 5min in 1x PBS. To avoid non-specific anti-body binding cells were subsequently blocked in 1% PBS containing 10% NCS for 1hr on an orbital shaker at 25°C.

2.3.3 Primary Antibody Incubations.

Primary Antibodies were prepared in a solution of 1% PBS containing 1%NCS and primary antibodies at the dilutions shown in Table 3.3. A number of dilutions were experimented with. 50µl of antibody solution was used per coverslip. Coverslips were removed from 6 well plates using sterile, fine tweezers and incubated in moist, dark staining chambers prepared using absorbent paper soaked in 1% PBS covered with aluminium foil and left for 1hr at 25°C.

Table 5- List of Antibodies used in immunofluorescent experiments

Antibody	Dilution Ranges Tried	Optimal Dilution Achieved
Vimentin (Sigma)	1:50, 1:100, 1:150, 1:200	1:200
Slug (Cell Signalling Tech)	1:50, 1:100, 1:150, 1:200	1:100
E-Cadherin (Abcam)	1:100, 1:150	1:150
TRITC Phalloidin (No secondary antibody)	1:100, 1:200, 1:400, 1:600	1:400

2.3.4 Secondary Antibody Incubations

Before application of secondary antibody coverslips were returned to 6 well plates and washed for 3x 5min in 1% PBS. All secondary antibodies used were TRITC (Tetramethyl Rhodamine Isothiocyanate) conjugated. 50µl of secondary

antibody was applied to each coverslip once the washing steps were complete and incubated in moist, dark staining chambers as described above for 1hr at 25°C.

2.3.5 Mounting and Imaging.

Coverslips were inverted and mounted onto glass slides in 30% (v/v) glycerol containing 12% (v/v) Mowiol (Sigma), 2mg/ml 4,6-diamindino-2-phenylindole (DAPI) and 2.5% (v/v) 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma) and left to dry overnight before imaging using a Zeiss Aksioskopp 40 fluorescent microscope (Carl Zeiss GmbH, Germany) fitted with a 40X Oil lens and a Leica SP5 Confocal Laser Scanning Microscope fitted with a 40X oil lens. Images were viewed and saved in the corresponding software (Zeiss Axiovision) and (Leica LAS) before collation in Adobe Photoshop.

2.4. siRNA knockdown of Lamin A and GFP-Lamin A.

siRNA knockdown was performed in order to investigate whether the proteins studied in the wounding assays were linked to LMNA gene expression.

2.4.1: Oligonucleotide sequences

Silencer® Select Custom Designed siRNA (Ambion) that targeted sequences in exons 11 or 12 of the LMNA gene was used. This allowed us to use an oligonucleotide that was specific for lamin A but not lamin C. Previous optimisation of sequences had been performed by Foster et al., (2011) and therefore the following sequence was used:

Sequence Name	Sequence ID number	Sequence (5'-3')
Si-1	S238117	Sense: UCAUCUAUCUCAAUCCUAAtt Antisense: UUAGGAUUGAGAUAGAUGAga

Silencer® Select Negative Control #1 siRNA (si-c) was used as a negative control.

2.4.2 Transfection in 6 well plates

24 Hours before transfection cells were washed and trypsinized as described in section 2.1 before pelleting and counting using a haemocytometer as described in section 3.1. Cells were seeded at a density of 4×10^5 cells per well using antibiotic-free L15 media + 10% FBS. Media was changed immediately preceding transfection using 1.5ml of antiobiotic free L15 media with 10% FBS. In a 2ml RNAse free tube 10µl Oligofectamine™ (Invitrogen) reagent was prepared in 160µl serum and antibiotic free L-15 medium for each well and incubated for 10min. 10 µl [20µM] Lamin A siRNA or 10µl scrambled siRNA that had been prepared in 40µl of serum and antibiotic-free media for each well was added to the tube and left for 20min.The total amount of reaction mixture for each well (220 µl) was then added to the well before rocking the plates gently to ensure even mixing. Plates of cells were then grown in a CO_2 free, humidified incubator at 37°C. Media was changed using antibiotic-free L15 with 10% FBS after 24h and subsequently every 48h as was necessary.

After 48h cells were subcultured as described in section 2.1. The cells were passaged 1:3 so that a cellular suspension that comprised 1/3 of the original pellet was seeded onto pre-prepared coverslips that were treated as described in section 3.1. Antiobiotic free L15 media supplemented with 10% FBS was then added to each well to create a total volume of 2ml per well. Cells were then left for a further 48h, 62h or 96h as shown in Table 6 before the Media was replaced with 1% PBS thus stopping the knockdown reaction. The coverslips were then immediately processed for immunofluorescence as described in section 2.3.2, 2.3.3,2.3.4 and 2.3.5 using the antibodies shown in Table 7.

Table 6- Tabulated overview of siRNA experiments performed in 6 well plates.

Experiment	0h	24h	48h	96h	120h	144h
siRNA 1	Transfect Cells	Change Media	Subculture on to coverslips	Add 1% PBS process for immuno-fluorescence		
siRNA 2	Transfect Cells	Change Media	Subculture on to coverslips	Change Media	Add 1% PBS process for immuno-fluorescence	
siRNA 3	Transfect Cells	Change Media	Subculture on to coverslips	Change Media		Add 1% PBS process for immuno-fluorescence

Table 7-Primary Antibodies used in siRNA knockdown experiments

Antibody	Dilution
Slug (Cell Signalling Technologies)	1:150
E-Cadherin (Abcam)	1:150
Vimentin (Sigma)	1:200
EGFR (Santa Cruz Biotechnology)	1:150
Fibronectin (Abcam)	1:150
T-Plastin (Abcam)	1:150
Phalloidin	1:400

Coverslips were subsequently viewed and Imaged using a Leica SP5 Confocal Laser Scanning Microscope fitted with a 40X oil lens. Images were viewed and saved in the corresponding software Leica LAS before collation in Adobe Photoshop.

2.4.3 Transfection in T25 culture flasks.

24 Hours before transfection cells were washed and trypsinized as described in section 2.1 before pelleting and counting using a haemocytometer as described in section 3.1. Cells were seeded at a density of 3.5×10^5 cells per flask using 5ml of antibiotic-free L15 media + 10% FBS per flask.

In a 2ml RNAse free tube, 20µl Oligofectamine™ (Invitrogen) reagent was prepared with 320µl serum and antibiotic free L-15 medium for each flask. The mixture was mixed gently using a pipette and left for 10min. 30 µl [20µM] Lamin A siRNA or 30µl scrambled siRNA that had been prepared in 80µl of serum and antibiotic-free media was needed per flask and was subsequently

added to the Oligofectamine™ mixture. This mixture was then left for 20min. The total amount of reaction mixture per flask (400μl) was subsequently added to each flask and mixed gently. Flasks were left to grow in a CO_2 free, humidified incubator at 37°C. The media was changed after 24h using anti-biotic free L15 media supplemented with 10% FBS.

After 48h, cells were subcultured into T75 flasks using a versene wash and trypsinization as described in section 1.1. The entire pellet obtained by centrifugation was re-suspended in 12ml of fresh antibiotic free L15 media supplemented with 10% FBS. Cells were then harvested for lysate preparation using a plastic cell scraper as described in section 2.1. Samples were taken every 24h as demonstrated in Table 8.

The cell pellets obtained were then stored at -80°C before being processed for immunoblotting as described in sections 2- 2.7. The only primary antibody used in this experiment was Jol2, which was incubated as described in Section 2.2.5.

Experiment	0h	24h	48h	62h	96h	120h	144h
siRNA 1	Transfect Cells (3x T25 flask for control siRNA and si-1)	Change Media	Harvest 1 flask for immunoblotting Subculture remaining flasks → T75cm^3	Harvest 1 flask for immunoblotting	Harvest 1 flask for immunoblotting		
siRNA 2	Transfect Cells (4x T25 flask for control siRNA and si-1)	Change Media	Harvest 1 flask for immunoblotting Subculture remaining flasks → T75cm^3	Harvest 1 flask for immunoblotting	Harvest 1 flask for immunoblotting	Harvest 1 flask for immunoblotting	
siRNA 3	Transfect Cells (5x T25 flask for control siRNA and si-1)	Change Media	Harvest 1 flask for immunoblotting Subculture remaining flasks → T75cm^3	Harvest 1 flask for immunoblotting	Harvest 1 flask for immunoblotting	Harvest 1 flask for immunoblotting	Harvest 1 flask for immunoblotting

Table 8: Tabulated overview of siRNA experiments in T25cm^3 cell culture flask

Chapter 3: Cell density experiment results

3.1 Background and experiment aims

Studies by Foster, 2011 (PhD thesis) have explored the effect that Lamin A over-expression has on sub-confluent and confluent SW480 cell cultures. The endogenous expression of Lamin A was shown to be higher in sub-confluent SW480 cultures that have been transfected with GFP- Lamin A in comparison to their confluent counterparts. This is thought to be directly related to the motile behaviour of the cells, whereby at sub-confluence, when the cells are free to move, the Lamin A protein expression is up-regulated. The reasons behind this phenomenon are currently not well known but it has been established using proteomic techniques that the transfection of SW480 cells with Lamin A also leads to changes in the proteins situated within the cytoskeletal fraction of the cells in relation to cell motility, including an up-regulation in the intermediate filament Vimentin (Foster et al., 2011). Microarray data generated using SW480 cells transfected with GFP Lamin A also revealed an up-regulation in the transcription factor Slug compared to the same cell line that was transfected with GFP only (Foster, 2011). Ingenuity Pathways Analysis (IPA) carried out on this microarray data indicated the up-regulation of Slug with Vimentin may form part of a genetic regulatory network that may contribute to Epithelial to Mesenchymal Transition (EMT) (Foster 2011, PhD thesis). It has also been documented in the literature that cells undergoing EMT may demonstrate high expression levels of the Transcription factor Slug along with an up-regulation in Vimentin (Yilmaz and Christofori, 2009). This shows that Vimentin and Slug are important mediators of an increase in cell motility as part of EMT. The aim of

these immunoblotting experiments was therefore to assess the levels of Vimentin and Slug in SW480/Lamin A cells in sparse and confluent culture conditions to determine if there are differences at low cell density. The extent to which Lamin A contributes to differences in protein expression was determined by comparing SW480 cells that have been transfected with GFP Lamin A (in this thesis referenced as "SW480/Lamin A") and Control cells that have been transfected with GFP only (in this thesis referenced as "SW480/Control"). Both kinds of cell cultures were collected at 50% confluency where none of the cells formed contacts with one another (sparse culture- low cell density) and 100% confluency where all of the cells formed contacts with one another (high cell density). These two conditions were chosen as the ability for cells to form contacts in culture has been shown to affect their expression of mesenchymal markers (Conacci- Sorrell et al, 2003).

3.2 Controls

Experimental normalization was performed through the use of protein loading controls, whereby the expression of the house-keeping proteins GAPDH and β-actin were determined using the relevant antibodies. The level of expression of protein of interest was then expressed as a ratio to the level of loading control protein. This standardized the protein levels between the different cellular samples and thus any up-regulation of proteins of interest could be determined as a result of cell confluency and the presence of Lamin A overexpression, instead of being an artifact of the experimental design.

3.3 Statistical analysis

Experiments were carried out in triplicate. Results were analysed using Densitometric analysis, on Image J software, where the total internal density of the protein bands was calculated for each replicate and its corresponding loading control. Statistical analysis used the average relative value of amount of protein for each sample type. Sub-confluent versus confluent SW480/Lamin A cell cultures were compared using a one-tailed, paired T-test. Sub-confluent versus confluent SW480/Control cells were also compared using a one-tailed, paired T-test. Sub-confluent SW480/Lamin A cells were then compared to sub-confluent SW480/Control cells using an one-tailed, un-paired T-test. Confluent SW480/Lamin A cells versus confluent SW480/Control cells were also compared using an un-paired T-test.

3.4 Lamin A expression.

The level of GFP-Lamin A(95kDa) has not been included in this data analysis as the cell-density dependent changes observed in the expression of endogenous Lamin A (75kDa) were more significant than those observed for GFP-Lamin A (95kDa). This may be due to the fact that the initial transfection and characterization of SW480 cells with GFP-Lamin A also lead to an increase in the levels of endogenous lamin A, possibly due to increased protein stabilization due to the presence of GFP-Lamin A in the transfected SW480 cells (Willis-2005- e thesis gives more details and Foster, 2011 e-thesis confirms endogenous expression of Lamin A (75kDa) is increased in the SW480 cells transfected with GFP-Lamin A).
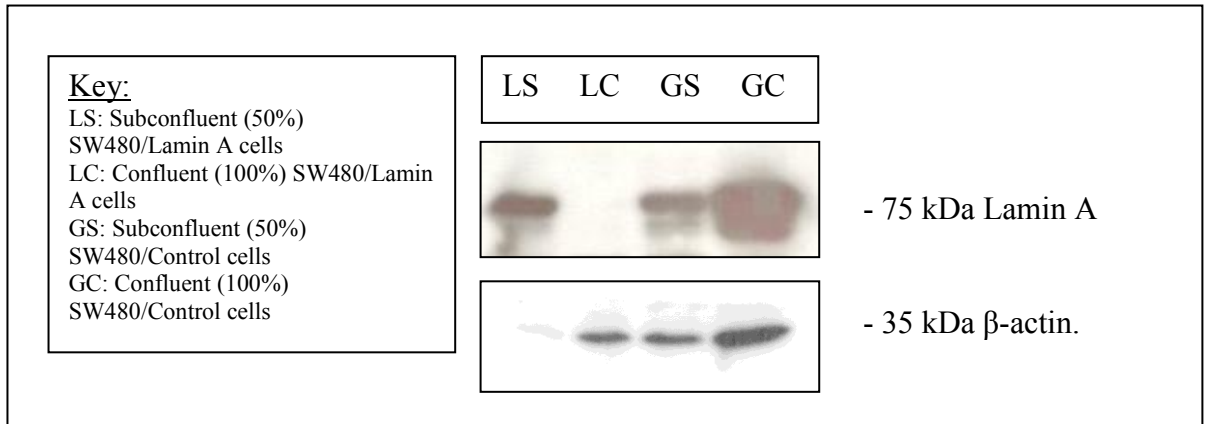
Figure 8: Immunoblotting of Lamin A using a Jol2 (anti Lamin A/C) antibody with β-Actin as a loading control.

In the sub-confluent samples of SW480/Lamin A cells (LS- column 1) a strong band was observed at the 75kDa marker, indicating the expression of endogenous Lamin A whereas is the confluent samples of SW480/Lamin A cells (LC- column 2) only a faint protein band was observed at 75kDa, indicating the relative levels of endogenous Lamin A protein expression decreased at confluence in the SW480/Lamin A cells. In the SW480/Control cultures a protein band was observed in both the sub-confluent (GS-column 3) and the confluent (GC-column 4) samples at 75kDa. Both of these samples had a greater level of protein overall, shown by the more dense protein bands obtained when the samples GS and GC samples were probed for β-Actin expression as a loading control in comparison to the LS and LC samples.

Figure 9: A graph showing the average relative levels of Lamin A protein (to a β-actin loading control) across 3 experimental immunoblotting replicates. Lines indicate where a T-test has demonstrated a statistically significant difference in protein level. *= P <0.05 **= P < 0.005. Samples are in the same order left to right as in figure 8

As Figure 9 shows, once protein levels were standardized against the β-Actin loading control, Lamin A expression was highest in the sub-confluent SW480 cell sample (LS). This level of expression was statistically significantly higher than both the confluent SW480/Lamin A cells (LC) (P=0.011) and the sub-confluent SW480/Control cells (GS)(P=0.001) and thus the highest levels of Lamin A expression occurred in the sub-confluent SW480/Lamin A sample. The change in Lamin A expression in sub-confluent (GS) versus confluent (GC) SW480/Control cells was not significant.

3.5: Vimentin expression
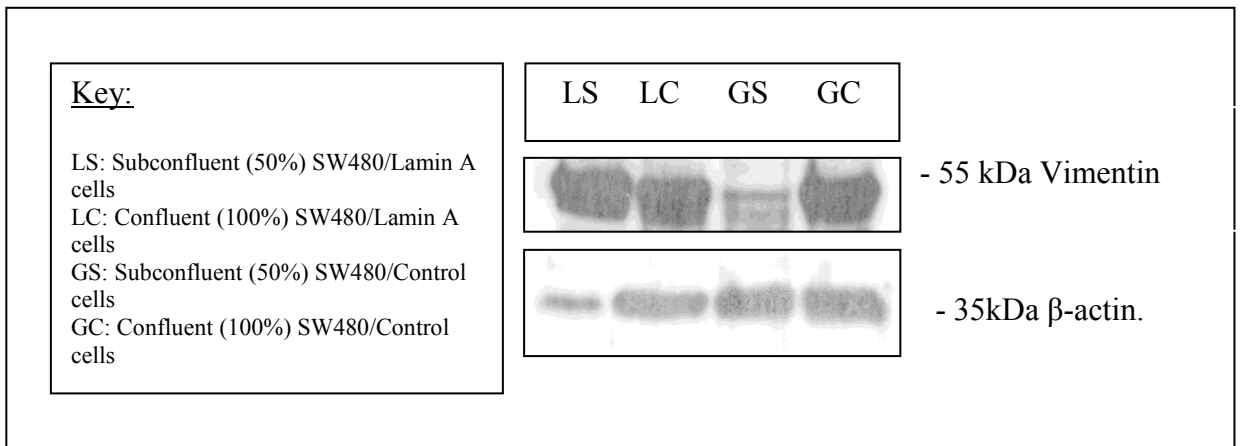
Figure 10: Immunoblotting of the intermediate filament protein Vimentin(molecular weight 55kDa) using a Vimentin antibody with β-Actin as a loading control

In the sub-confluent SW480/Lamin A cells (LS-column 1) a strong protein band was observed for Vimentin at 55kDa. In the confluent SW480/Lamin A samples (LC- column 2) a band at 55kDa was of similar density to the LS band, but there was more protein present overall in the confluent SW480/Lamin A cells as shown by a denser band for the loading control (LC- column 2- β-actin). This indicates that the relative Vimentin expression was reduced in the confluent SW480/Lamin A samples (LC) compared to the sub-confluent SW480/Lamin A samples (LS). In the SW480/Control samples a faint band at 55kDa was seen in the sub-confluent cells (GS- column 3) and a more dense band at 55kDa was seen in the confluent cells (GC- column 4). As more protein was present in the confluent SW480/Control sample(column 4) compared to the sub-confluent sample (column 3), which was shown by the denser loading control band in column 4, this suggests little or no change in the relative expression of Vimentin in sub-confluent versus confluent SW480/Control cells.
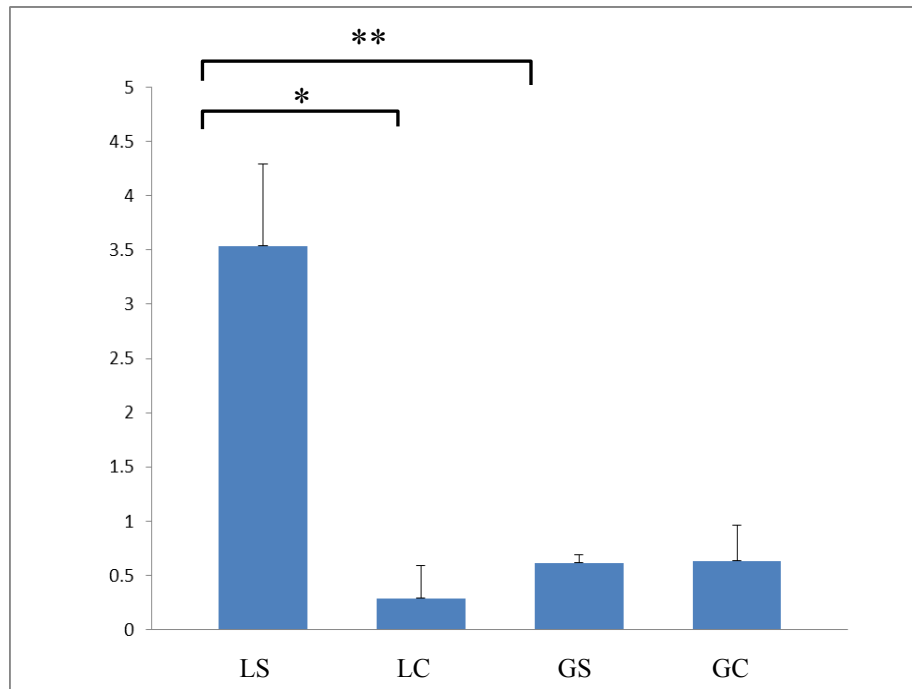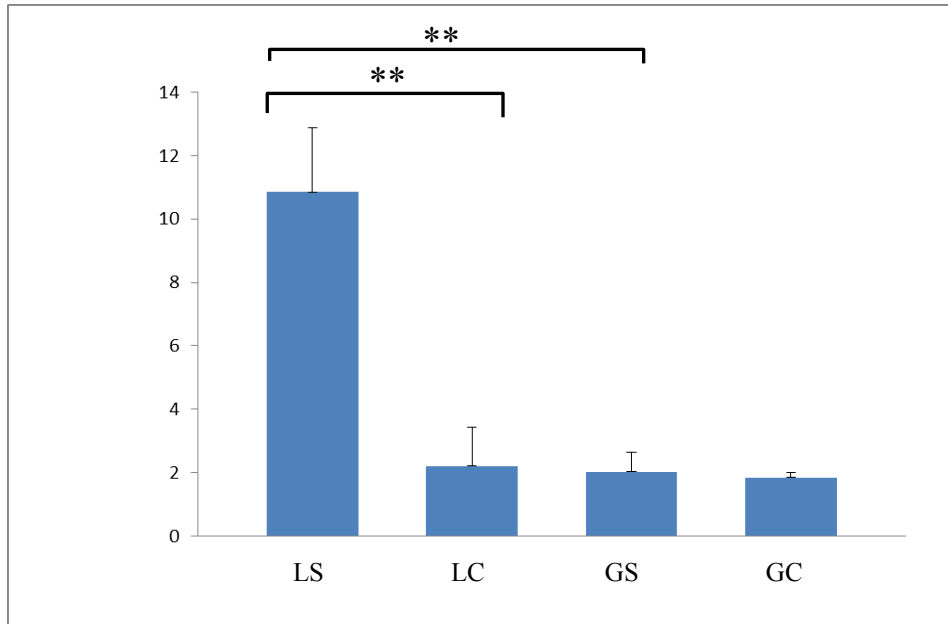
Figure 11: A Graph showing the average relative level of Vimentin protein (to a β-actin loading control) across 3 experimental immunoblotting replicates. .Lines indicate where a T-test has demonstrated a statistically significant difference in protein level. ** = P < 0.005.

As Figure 11 shows, The sub-confluent SW480/Lamin A cells had a statistically significant higher level of expression of Vimentin compared to confluent SW480/Lamin A cells (P=0.002) and also compared to SW480/Control cultures that were sub-confluent (P=0.001). The greatest level of Vimentin expression was seen in the SW480/Lamin A cells at low cell density. The sub-confluent and confluent SW480/control cells expressed a lower level of Vimentin and this expression did not significantly change depending on cell density.

3.6: Slug expression

As the molecular weight of Slug (36 kDa) is similar to β-Actin (35kDa), a GAPDH (25kDa) protein loading control was used instead. Densitometry analysis of samples from both loading controls indicated similar levels of protein were found in the same samples (Table 9), indicating that the use of GAPDH instead of β-actin in this instance should not affect the use of this loading control to standardize for variations in the starting levels of protein in the 4 samples, and in particular in the sub-confluent SW4890/Lamin A cells (LS), where significantly less starting protein was observed in all of the experiments.
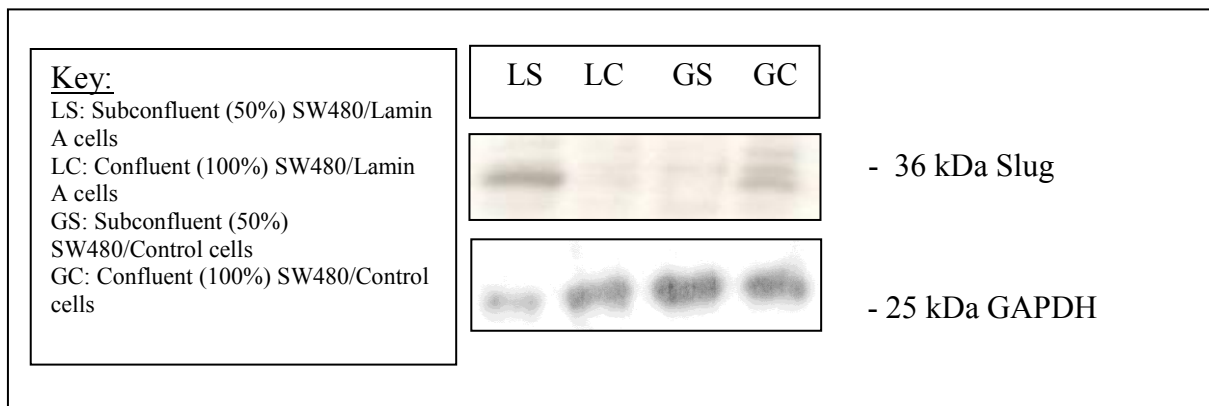
Figure 12: Immunoblotting of the transcription factor Slug using a Slug antibody with GAPDH as a loading control across 3 experimental replicate

As Figure 12 shows, a protein band was seen at 36kDa indicating Slug expression in the sub-confluent SW480/Lamin A cells. A very faint band was seen in the SW480/Lamin A confluent cells. In the SW480/Control cells a faint band was seen in the sub-confluent cultures and a number of slightly darker bands around the 36kDa mark in the confluent SW480/Control cultures. As a number of bands were present in the SW480/Control cultures, this suggests

some non-specific binding of the Slug anti-body to the membrane and thus in the densitometry quantifications only the band at the 36kDa mark was analysed.
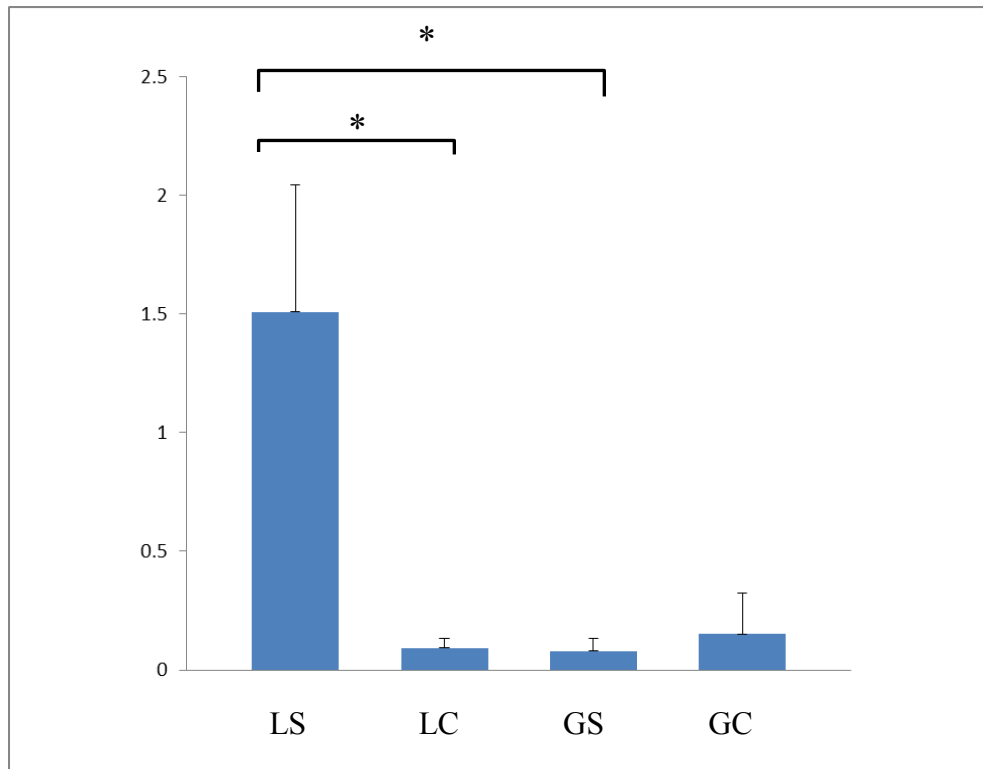
Figure 13: A Graph showing the average relative levels of expression of Slug across 3 experimental immunoblotting replicates. Lines indicate where a T-test has demonstrated a statistically significant difference in protein level.
 * = P < 0.05

As Figure 13 shows, the sub-confluent SW480/Lamin A cells (LS) had statistically significantly more Slug expression than confluent SW480/Lamin cells (P= 0.021) and sub-confluent SW480/Control cells (P=0.005).Once normalized against the β-Actin loading control, the sub-confluent SW480/Lamin A cells had the greatest level of Slug expression overall. In the SW480/Control cultures Slug

expression was marginally increased in the confluent cultures compared to the sub-confluent cultures but this result was not statistically significant.

Table 9: Densitometry analysis of Loading control bands. As in above data, LS- sub-confluent SW480/Lamin A, LC- confluent SW480/Lamin A cells, GS- sub-confluent SW480/control cells and GC- confluent SW480/control cells. Level of protein relative to the largest band (GS) is shown.

Loading Control	Sample LS	Sample LC	Sample GS	Sample GC
B-Actin	0.68	0.96	1.00	0.99
GAPDH	0.68	0.96	1.00	1.00

Table 10: A summary of the P values generated when using a paired T-test to compare protein expression in SW480/Lamin A sub-confluent to SW480/Lamin A confluent cultures.

Protein Expression	P Value
Lamin A	P: 0.011
Vimentin	P: 0.002
Slug	P:0.021

Table 11: A summary of the P values generated when using an un-paired T-test to compare protein expression in sub-confluent SW480/Lamin A cells compared to sub-confluent SW480/Control cells.

Protein Expression	P Value
Lamin A	P:0.001
Vimentin	P:0.001
Slug	P:0.005

As the P Value's in Table's 9 and 10 show, the expression of Lamin A, Vimentin and Slug was significantly higher in the SW480/Lamin A cells when they were

sub-confluent (at low cell density) compared to the same cell line at confluency (Table 9). The level of expression firstly of Lamin A was statistically greater in the sub-confluent SW480/Lamin A cells compared to the sub-confluent SW480/Control cells as were the levels of Slug and Vimentin.

3.7: Chapter Discussion

In a culture environment, the motile behaviour of a cell has been shown to be dependent on whether it is able to form contacts with neighbouring cells or whether it is free to move within the surrounding environment, thus cell confluency, or density, is an important director of cell motility (Persson et al., 2010). The cell lines selected for these experiments, SW480/Lamin A and SW480/Control cells, allowed us to assess how Lamin A expression affected the expression of mesenchymal proteins at varying cellular densities due to the fact the SW480/Lamin A cells had significantly more Lamin A expression than the SW480/Control cells at low cell densities as shown in Figure 9.

At sub-confluence, epithelial cells can employ the use of Vimentin and Actin cytoskeletal filaments in order to move within their environment. As the cells reach confluence, Vimentin expression is down-regulated and replaced by Keratin filament expression that allows the formation of cell junctions. The expression of cytoskeletal filaments has also been shown to be dependent on the density of cells in 2D cultures. In freshly plated bovine pulmonary micro-vascular endothelial cells, cytokeratin expression is initially observed as being disorganized and "hazy" at sub-confluence. With time, the Keratin forms plaques at cell junctions and eventually forms filaments in confluent cells. The assembly of the Vimentin filament cytoskeleton, also present in the cells at sub-

53

confluency, is shown to decrease as the cells reach a more confluent state although the levels of expression of the protein may or may not change. This demonstrates how confluency may dictate the arrangement cytoskeletal proteins within cultured cells (Alexander et al., 1991).

These experiments show that at sub-confluence in the SW480/Lamin A cells, endogenous Lamin A expression is greater compared to at confluence and thus Lamin A expression is affected by cellular density in these cells, when there is potential for motility and an absence of cell-cell contacts.

The levels of GFP-Lamin A were not considered along with the levels of endogenous lamin A in these experiments. Changes in the expression of endogenous Lamin A in sub-confluent and confluent cells transfected with GFP-Lamin A has been previously shown to be significant (Foster, 2011- e thesis, page 57). By not considering the levels of GFP-Lamin A in all of the cultures tested this experiment has limitations on how accurately we can draw the conclusion that the endogenous Lamin A is responsible for the observed changes in Slug and Vimentin expression is SW480/Lamin A cells at low cell densities as the contribution of GFP-Lamin A was not quantified. A more detailed future study should consider how the expression of GFP-Lamin A and endogenous lamin A together in the SW480/Lamin A cells could modulate the expression of the 2 other proteins included in this study- Slug and Vimentin.

The levels of the expression of the proteins Slug and Vimentin in the SW480/Lamin A cells at sub-confluence are also greater compared to the same cells at confluence (Table 9). As Slug and Vimentin are both markers of mesenchymal cells (Kalluri and Weinberg, 2009), this suggests that sub-

confluent SW480/Lamin A cultures show evidence of being more mesenchymal in comparison to their confluent counterparts. Upon reaching confluence, the expression of Lamin A, Vimentin and Slug is reduced in the SW480/Lamin A cells, indicating that the up-regulated expression of the two mesenchymal markers and Lamin A itself in SW480/Lamin A cells is transient and appears to be dependent on cell density.

These changes appear to be a result of the expression of Lamin A in SW480 cells, as the levels of Lamin A, Vimentin and Slug were significantly higher in sub-confluent SW480/Lamin A cells compared to sub-confluent SW480/Control cells, which do not express Lamin A (Table 10), thus indicating a relationship between Lamin A, Vimentin and Slug expression.

Experiments in cell culture environments using MCF10 breast cancer cells have shown that these cells can undergo spontaneous morphologic and phenotypic changes associated with EMT at low cell density. These changes include an up-regulation in the transcription factor Slug and a spindle shaped morphology of cells that disappears as they become confluent (Sarrió et al., 2008). Experiments testing the effect of cell confluency on TGFβ1 induced EMT in cultured tubular epithelial cells show that TGFβ is unable to induce EMT in confluent monolayers of cells. Upon wounding of the cells, growth at sub-confluence or contact disassembly by Ca^{2+} removal this effect was reversed, thus indicating the importance of a low cell density in the induction of EMTs in cultured cells.

Slug is a well-known repressor of the cell-junction protein E-cadherin (Yilmaz and Christofori, 2009) and various studies where Slug has been over-expressed in epithelial cells have shown them to adopt a mesenchymal phenotype (Medici

et al., 2008; Yilmaz, 2009). Connacci-sorrell et al. (2003) have shown an induction of Slug expression in SW480 cells at low cell density by β-Catenin-Tcf complex transcriptional activation or through the ERK signaling pathway. Once the SW480 cells were grown to confluency, they formed an epithelial-like sheet, with β-catenin localized to adherens junctions.

The data here indicate a similar induction in Slug expression in sparse cultures of SW480/Lamin A cells, but in this case the induction of Slug expression appears to have been mediated by the expression of Lamin A instead of modification to either the β-Catenin or ERK signaling pathways.

The SW480 cell line typically expresses E-cadherin and β-catenin and lacks expression of Vimentin (Buck et al., 2007). The greater levels of expression of Vimentin in sparse cultures of SW480/Lamin A cells thus suggests that Lamin A may also be able to induce the expression of Vimentin when cells are unable to form contacts. There is an abundance of information reporting the importance of the Vimentin cytoskeleton in the behavior of motile cells. Studies using knock-out mice have demonstrated a reduced migratory capacity in epithelial cells that do not express Vimentin (Eckles et al., 2000). Within the context of cancer, Vimentin can be used as a biomarker for epithelial cell invasiveness in both prostate and breast cancers (Lang et al., 2002; Gilles et al., 1999). A study looking at Vimentin expression in malignant glioma cells at low density treated with chemotherapeutic agents has also indicated a correlation between high Vimentin expression at low cell density as a marker of invasive cells during chemotherapeutic treatment (Trog et al., 2008). Higher levels of Vimentin in sparse cultures of SW480/Lamin A cells thus suggests they may be more invasive than their control counterparts.

Together these results provide preliminary evidence that Lamin A expression in SW480 cells provides a signaling environment that allows EMT to occur in sparse cultures of SW480/Lamin A cells, demonstrated by a significant increase in the levels of Slug and Vimentin in these cells at low cell density in the presence of increased Lamin A expression.

Judgement of the confluency of cells in culture can lead to variation, thus making the standardization of a 50% confluent cell culture difficult in between experimental replicates. A more detailed future analysis may therefore consider using cellular counting as a method for standardizing confluency, whereby sub-confluent flasks should have a fixed percentage of fewer cells such as 50% of the number of cells of confluent samples. In this experiment eye-based judegments of confluency could have lead to potential experimental error, given that the level of confluency between cell cultures may vary. A sample taken from a sub-confluent cell population generally yielded less protein, due to the presence of fewer cells. This was overcome to an extent by using multiple growth flasks of cells for sub-confluent samples, but it was still difficult to obtain an equal amount of protein in the sub-confluent SW480/Lamin A sample compared to the other samples. The reasons for this are not known but may be due to the fact that the extra Lamin A in sub-confluent cells caused phenotypic and structural changes within these cells that made protein extraction using an SDS buffer more difficult.

Immunoblotting as a quantitative technique also offers several limitations. The level of protein that was extracted from sub-confluent samples of the SW480/Lamin A cells was poor, hence resulting in a faint band in loading control experiments. As the loading control is used as a standard when

determining the relative levels of expression of proteins, the presence of a faint band could be a cause for error, as the analysis using densitometry is dependent on the area of the band and its mean gray value. Therefore, with smaller bands, the small level of background that may be incorporated into the densitometry value will cause a more significant experimental error in comparison to the larger bands. Nonetheless, given that this sample was able to demonstrate a significant level of both Lamin A and Vimentin expression despite the apparently low protein level, evidence is provided in favour of the conclusion drawn from the data that the levels of Lamin A protein and Vimentin protein were higher in the SW480/Lamin A cells compared to the SW480/Control cells.

The quality of both the Vimentin and Lamin A/C antibodies was consistently good. The Slug antibody often resulted in some non-specific binding of the antibody to the membrane, demonstrated by a grey appearance in each lane. This may be due to the fact that it is more difficult to probe for low expression level proteins, such as transcription factors, in comparison to cytoskeletal proteins and nuclear lamina proteins.

Chapter 4: Scratch wound assays results

4.1: Background and experiment aims

Having observed high levels of expression of the mesenchymal-associated proteins Vimentin and Slug in SW480/Lamin A cells at low cells density compared to at high cell density, scratch wounding assays were carried out on SW480/Lamin A cells to observe whether they displayed any mesenchymal properties in a scratch-wound environment. By creating a wound down the centre of a layer of confluent cells, a gap is created in which cells are free to migrate. This assay allows the study of cellular motility and behaviour the form of an *in vitro* study of cancer cell metastasis as the wounding of a layer of confluent cells can roughly mimic a tumour environment, where cells on the edges of a primary tumour may detach and migrate away from the tumour (Cory, 2011; Hulkower and Herber., 2011). Immunofluorescence was used to investigate the transcription factor Slug, the cell junctional protein E-cadherin and the cytoskeletal intermediate filament protein Vimentin. TRITC-conjugated phalloidin, a toxin that binds F-actin was used to observe changes in the actin cytoskeleton.

SW480/Lamin A cells and SW480/Control cells were grown to confluence on glass coverslips before being scratch wounded. Confocal imaging of immunofluorescent staining was used to observe the levels and organisation of EMT proteins in SW480/Lamin A cells compared to controls to determine what effect Lamin A expression has on the expression of classical EMT markers in SW480 cells.

4.2: E-cadherin repression
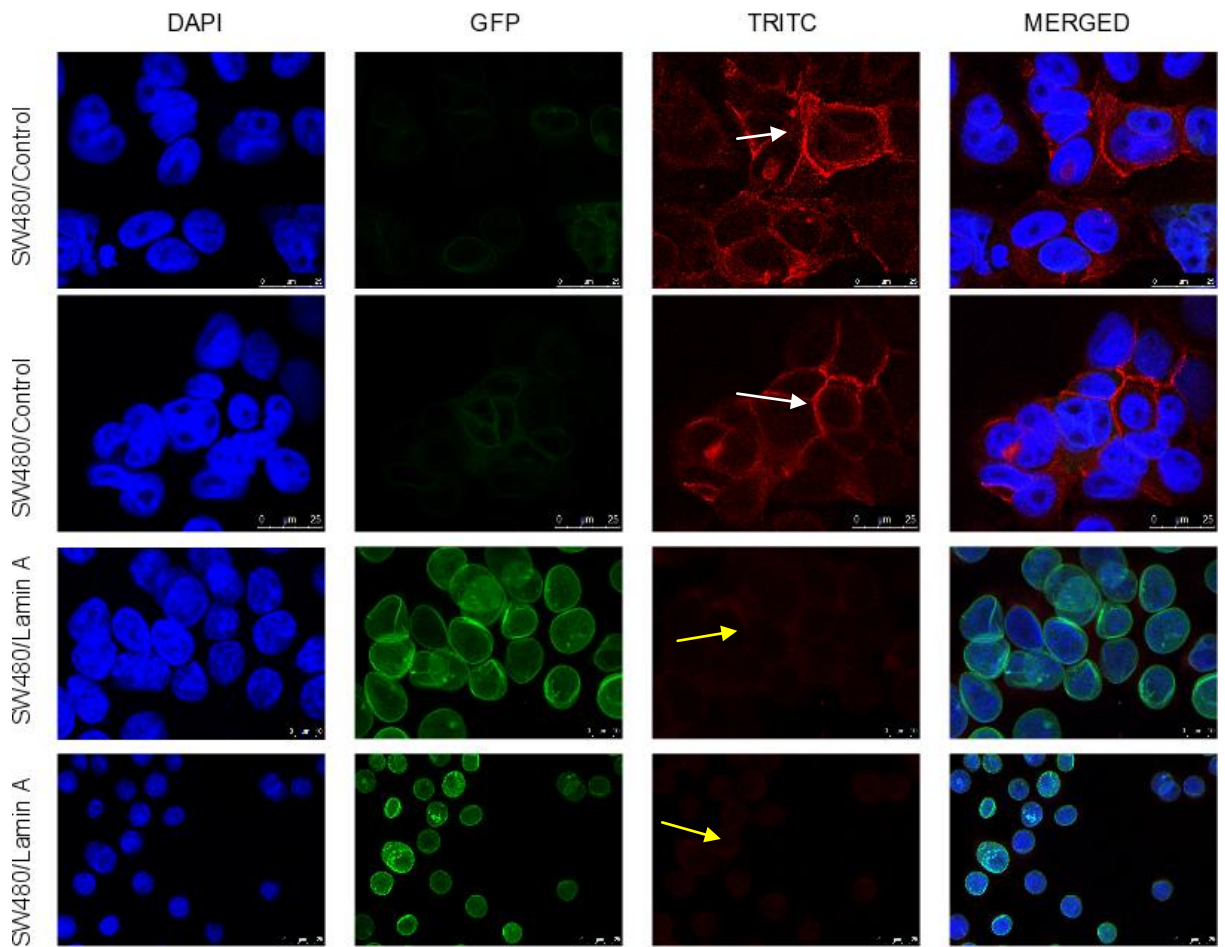
Figure 14: SW480/Control (rows 1 and 2) and SW480/Lamin A (rows 3 and 4) cell cultures that have been stained with an E-cadherin primary antibody and a TRITC conjugated secondary antibody. Rows 1 and 3 represent confluent cultures and rows 2 and 4 represent the edges of scratch wounds. White arrows indicate the presence of cell junctions in SW480/Control cultures. Yellow arrows indicate the absence of cell junctions in SW480/Lamin A cultures- even in areas of high cell density.

The cell adhesion molecule E-cadherin, which forms complexes at epithelial cell junctions, is primarily responsible for the maintenance of epithelial tissues. Repression of the E-cadherin gene promoter is a pre-requisite for cancer cell dissemination and EMT (Mahmut and Yilmaz, 2009). Dense cultures of SW480 cells have been reported to have high level of E-cadherin expression at adherens junctions and to resemble an intact epithelium (Conacci-Sorrell et al., 2003). The induction of Slug expression through β-catenin signaling has been shown to induce the repression of E-cadherin and dissolution of cell-cell contacts at the adherens junctions of SW480 cells and thus cell density dependent relationship between Slug expression and E-cadherin repression in SW480 cells has been previously been established (Conacci- Sorrell et al., 2003).

Confluent cultures of both SW480/Lamin A cells and SW480/ Control cells were fixed following scratch wounding and stained with an E-cadherin antibody conjugated to TRITC. The results were visualized by confocal microscopy. As seen in Figure 14, the SW480/Lamin A cells are clearly distinguishable from the SW480/Control cells due to the peri-nuclear presence of GFP-Lamin A, shown in the GFP channel. The SW480/Control cells had diffuse, lower levels of GFP in this experiment that were not as easy to detect. The SW480/Lamin A cells showed background expression levels of E-cadherin (rows 3 and 4). This occurred both in areas of high density and also in cells at the edges of the scratch wounds. Cell junctions were largely absent from SW480/Lamin A cells regardless of their confluency.

 In contrast, the SW480/Control cells are able to form junctions in areas of cell confluence at the centre and edges of scratch wounds (Figure 14). This data suggests that expression of Lamin A in SW480 cells may repress the expression

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of E-cadherin and leads to loss of cell adherens junctions, shown by a lack of expression of E-cadherin in confluent SW480/Lamin A cells (yellow arrows- Figure 14).

4.3: Slug expression
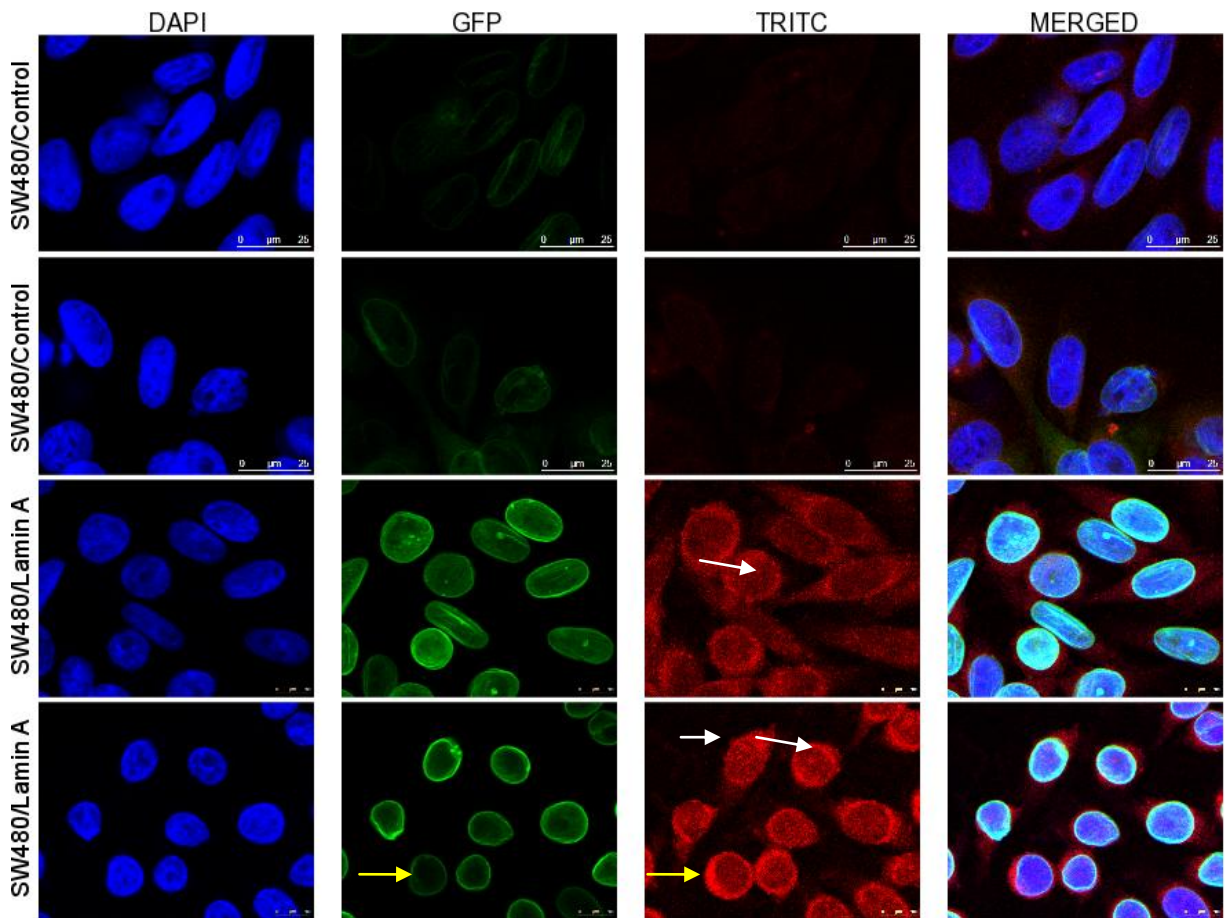
Figure 15: SW480/Control (rows 1 and 2) and SW480/Lamin A cells (rows 3 and 4) that have been stained with a Slug primary and a TRITC conjugated secondary antibody. Rows 1 and 3 represent confluent cultures and rows 2 and 4 represent the edges of scratch wounds. GFP-Lamin A expression is represented in column 2- GFP. White arrows indicate increased nuclear Slug in SW480/Lamin A cells in both confluent cells (row 3) and in particular, at the edges of scratch wounds (row 4).

Slug is a Zinc-finger transcription factor that binds the E-cadherin promoter within the nucleus, repressing its transcription (Harjra et al, 2002), and is an important inducer of EMT (Hoshino et al., 2011. As shown in Figure 15, Slug expression was mostly absent in the SW480/Control cells at both the edges of the scratch wounds and at the centre of the epithelial sheet.

In contrast there was extensive expression of Slug (both cytoplasmic and nuclear) in the SW480/Lamin A cells, which was most prominent in cells at the edges of the scratch wounds (row 4). The expression of nuclear Slug increased in the SW480/Lamin A cells at the edges of scratch wounds (white arrows-row 4), where nuclear "speckles" of Slug protein were present in the nuclei of the SW480/Lamin A cells. The most consistent cellular staining occurred in the peri-nuclear and nuclear regions of the SW480/Lamin A cells at both the centre and edges of the scratch wounds (shown by white arrows in rows 3 and 4- Figure 15). The increased expression of Slug in the SW480/Lamin A cells did not appear to be solely an effect of the levels of GFP-Lamin A in these cells. This is shown by the fact that cells with lower levels of GFP-Lamin A (yellow arrow, GFP channel- row 4, Figure 15), still had high levels of Slug (yellow arrow, TRITC channel- row 4, Figure 15). As previously mentioned, transfection of the SW480 colorectal cancer cells with the GFP-Lamin A also significantly increased the expression of endogenous Lamin A (Foster, 2011- PhD thesis) and therefore the observed increase in Slug in the SW480/Lamin A cells may also be due to the increase in endogenous Lamin A in these cells.

4.4: Filamentous Actin expression

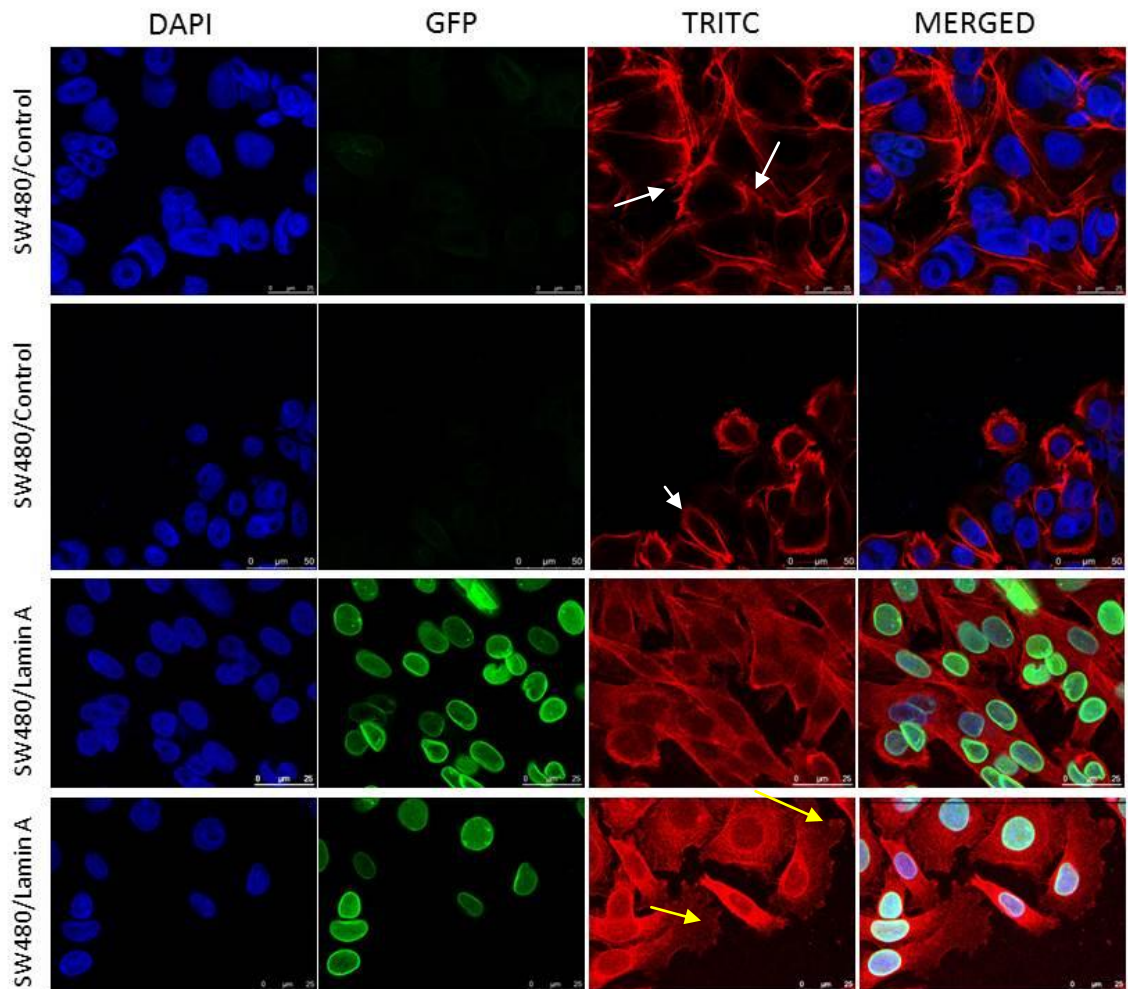Figure 16: SW480/Control (rows 1 and 2) and SW480Lamin A cells (rows 3 and 4cells stained with TRITC-phalloidin which stains filamentous actin. Rows 1 and 3 represent confluent cultures and rows 2 and 4 represent the edges of scratch wounds. White arrows indicate cortical actin bundles typical of epithelial cells in SW480/Control cultures. Yellow arrows indicate lamellipodium formation in SW480/Lamin A cells.

Re-organization of the actin cytoskeleton is an important part of the EMT process as it is a major determinant of cell shape and polarity (Yilmaz and Christofori., 2009). To determine whether there were changes in the actin cytoskeletons of scratch wounded SW480/Lamin A cells, cells were stained with a TRITC-conjugated actin filament binding toxin phalloidin.

SW480/Lamin A cells had a different cellular shape compared to SW480/Control cells. The SW480/Control cells had a "cobblestone-like" appearance in the epithelial sheet but were more rounded at the wound edge. In contrast the SW480/Lamin A cells were elongated and spindle-like in confluent cells at the centre of the culture and at the wound edge. In SW480/Control cells, actin expression was concentrated at the cytoplasmic edges of the cells, and was organized into thin cortical bundles at the cell boundaries which is consistent with its organization into epithelial sheets (Haynes et al., 2011 ;Doherty and McMahon, 2008). The SW480/Control cells at the edges of the scratch wounds did not vary drastically from the cells in the centre of the epithelial sheet except for some evidence of filipodia formation (shown by white arrow in Figure 16-row 2).

In SW480/Lamin A cells phalloidin staining was not concentrated at cytoplasmic edges but was more diffuse through the cytoplasm with some evidence of perinuclear staining. This altered staining is consistent with increasing motility as increased tethering of the Actin cytoskeleton to the nucleus through the LINC complex is important in cellular movement (Chambliss et al., 2013). At the edges of the scratch wounds, the formation of lamellipodia and spreading of the cell membrane was also observed in SW480/Lamin A cells (Yellow arrows).

4.5: Vimentin expression
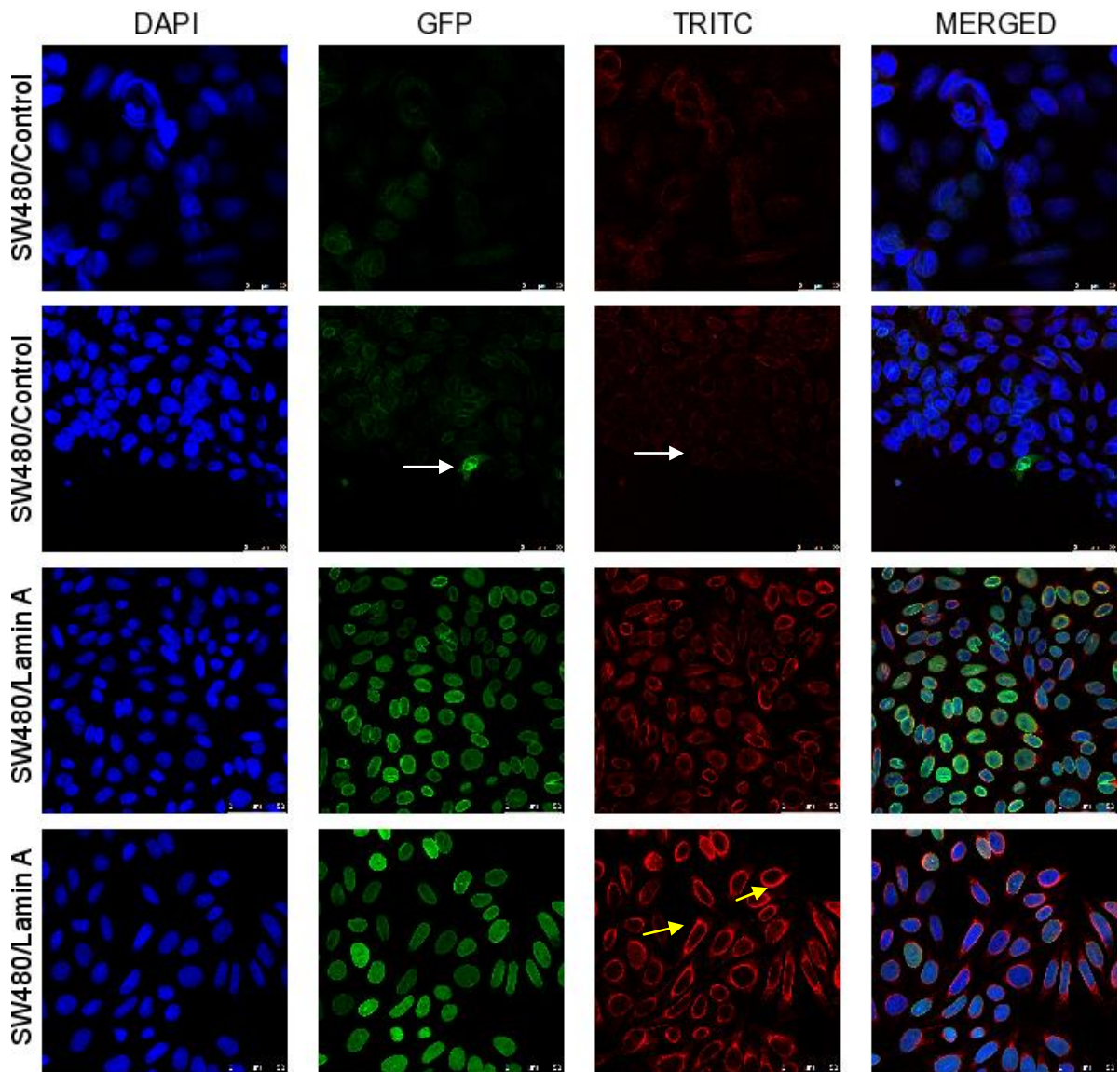
Figure 17: SW480/Control(rows 1 and 2) and SW480Lamin A cells (rows 3 and 4) that have been stained with a TRITC-conjugated Vimentin antibody.- Rows 1 and 3 represent confluent cultures and rows 2 and 4 represent the edges of scratch wounds. White arrow shows background levels of Vimentin staining at the edge of the scratch wound in SW480/Control cultures. Interestingly one of the SW480/Control cultures expressed high levels of GFP (White arrow- row 2, GFP channel) Yellow arrows indicate significant peri-nuclear Vimentin expression.in SW480/Lamin A cells.

Expression of Vimentin was largely absent in SW480/Control cells (rows 1 and 2 Figure 17). In contrast the SW480/Lamin A cells displayed uniform expression of Vimentin in both the cells at the confluent centre and edges of scratch wounds. Increased Vimentin expression was more dramatic at the edge of the scratch wounds (Yellow arrows in Figure 17). In these cells Vimentin appeared to be organized into cytoskeletal filaments which were dense in peri-nuclear areas before radiating out along the cell axis. The SW480/Lamin A cells showed varying levels of GFP-Lamin A (rows 3 and 4- GFP channel) but the increases in Vimentin staining compared to the SW480/Control cells were mostly consistent across the cellular populations shown in the fields imaged (rows 3 and 4 –TRITC channel)

4.6: Chapter discussion

Using confocal microscopy SW480/Lamin A and SW480/Control cultures have been visualized at the edges and centres of scratch wounds with the aim of observing the expression of classical EMT markers.

The cell junction protein E-cadherin is tethered to actin filaments by a series of proteins including β-Catenin. As E-Cadherin maintains the integrity of an epithelium through its connections via adherens junctions, the dissolution of cell-cell contacts through repression of E-Cadherin expression also leads to wide-spread changes in configuration of the actin cytoskeleton that allow it to direct cell motility (Yilmaz and Christofori, 2009). Willis et al (2008) have previously shown that SW480/Lamin A cells have down-regulated E-cadherin expression using RT-PCR and this was visualized at the level of protein expression in SW480/Lamin A scratch wound assays.

Previously microarray analysis on SW480/Lamin A cells has shown an increase in the level of Slug mRNA in comparison to SW480/Control cells (Foster 2011, PhD thesis). As mRNA data suggesting an up-regulation in gene expression does not always necessarily lead to a linear increase in functional protein expression, due to potential mRNA degradation or poor protein folding (You and Yin, 2000), these immunofluorescent experiments demonstrate an important increase in Slug at the protein level in SW480/Lamin A cells. The zinc finger transcription factor Slug behaves as a repressor of E-cadherin expression by binding to the E-cadherin gene promoter, ultimately causing Histone modifications such as hyper-methylation. The result is epigenetic silencing of the gene (Bolós et al., 2002). In the absence of E-cadherin, β-Catenin is released into the cytoplasm, where it can traverse into the nucleus and activate the Wnt signalling pathway by interaction with members of the Tcf/Lef family of transcription factors. This causes changes in expression of a large number of genes involved in cellular adhesion, migration, invasion and morphogenesis (Wong and Pignatelli., 2002). For these reasons Slug is a well-known inducer of the EMT process, which has been demonstrated in a number of different cancer cell lines, including oral (Joseph et al., 2009), pancreatic (Zhang et al., 2011) colon (Camp et al., 2011) and breast (Vuoriluoto et al., 2011). Slug is also as a marker of poor patient prognosis in many human cancers (Peinado et al., 2007). Slug expression was up-regulated in SW480/Lamin A cultures, particularly in nuclear regions. This is a second indicator of a more aggressively motile phenotype in SW480/Lamin A cells and due to the location of the Slug staining, suggests that Slug may be acting on the E-cadherin promoter region in SW480/Lamin A cells to repress E-cadherin protein expression. Future work should look to identify whether Slug is acting alone to repress E-cadherin, or whether other commonly identified repressors, such as Snail, ZEB1 and Twist (Kalluri and Weinberg, 2009) could

also be responsible for the observed dissolution of cellular junctions in SW480/Lamin A cells that is a crucial part of the EMT process.

Previously Willis et al (2008) found an up-regulation in expression of the actin-bundling protein T-Plastin, which is a protein commonly associated with invasive and metastatic cells. Foster et al (2011) subsequently demonstrated an increase in the levels of filamentous actin in SW480/Lamin A cells. Here, filamentous actin, which is associated with cells of greater metastatic potential (Nowak et al., 2002), was visualized in SW480/Lamin A cells in these immunofluorescent experiments. The cells showed significant changes in the arrangement of the actin cytoskeleton compared to SW480/Control cells that indicated a more motile phenotype, including lamellipodia formation at the edges of scratch wounds. The formation of a lamellipodium is a pre-requisite for cell movement across a 2D planar surface (Yamaguchi and Condeelis., 2007) so the observed formation of these structures at the edges of scratch wounds suggests that these cells were moving into the area of free space created. Change in cellular shape from a regular, "cobblestone" epithelial- type shape to a mesenchymal-like fibroblastic shape were also observed which were similar to those recorded by other groups using TGFβ–induced EMT on epithelial cells (Miettinen et al., 1994; Haynes et al., 2011; Kalluri and Weinberg, 2009). Very elongated cells also displayed the formation of thick stress-fibres, ventral to the cell surface, which ran along the cell axis. These fibres could contribute to cellular contraction in order to allow motility, given that contractile stress-fibre formation is also a pre-requisite for cell movement (Xu et al., 2009; Haynes et al., 2011).

Foster (2011) has previously demonstrated that SW480/Lamin A cells express more Vimentin using proteomic analysis. An increase in Vimentin expression was visualised for the first time here in the SW480/Lamin A cells using immunofluorescence. The type III intermediate filament is up-regulated in most cancers and like Slug, can be a marker of poor patient prognosis (Satelli and Li, 2011). Increasingly, multi-functional roles for Vimentin in cellular adhesion, migration and signaling are being uncovered (Ivaska et al., 2007;Satelli and Li, 2011). Vimentin is an important signaling mediator in the EMT process (Ivaska, 2011), has been shown to co-localize with Lamin A (Mergui et al., 2010). Recently the ability of Vimentin to functionally contribute to the EMT process was shown by Mendez et al., (2010), whereby transfection of epithelial cells with Vimentin caused an increase in mesenchymal morphologic and motile characteristics in the cells.

The relationship between Vimentin expression and Slug expression has been studied in breast cancer where Vuoriluoto et al (2011), showed that ectopic expression of the EMT-inducing transcription factor Slug induces Vimentin expression and migration in pre-malignant epithelial cells. Vimentin expression was necessary for Slug-induced migration and the motile characteristic demonstrated by the cells. Using immunofluorescence, this group reported up-regulation of both Slug and Vimentin at the wound edges of scratch wounding assays when the cells expressed Slug. The results also indicated important changes in the shapes of the epithelial cells that were transfected with Slug, whereby a more fibroblastic morphology was observed and the migratory capacity of the cells was increased. Finally, gene expression profiling carried out by the group has indicated a positive correlation between Vimentin expression and genes that contribute to invasiveness. The scratch wounding assays

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performed here on colorectal cancer epithelial cells show strikingly similar results in terms of both an induction of Slug and Vimentin expression contributing to a more mesenchymal cellular phenotype, with observations of cells demonstrating the most invasive phenotypes at the edges of scratch wounds. This suggests that the two proteins may be working together in a larger signalling network involving the Lamin A protein and other potential signalling partners.

Together the above results indicate a significant change in the SW480 cells from a less invasive, less motile epithelial cell type (SW480/Control) to a more motile, more invasive mesenchymal cell phenotype (SW480/Lamin A) as a result of stable transfection of Lamin A . There was clear variation in the levels of GFP-Lamin A in the SW480 cells used in these experiments, where some SW480/Lamin A cells showed significantly more Lamin A signal in the GFP channel. As cells with less GFP-Lamin A still showed significant mesenchymal properties such as increased Vimentin and Slug expression, this suggests the changes observed could partly be due to the increased levels of endogenous Lamin A that have been observed previously in the SW480/Lamin A cells by western blot in this thesis (Chapter 3) and from previous work (Foster, 2011-PhD thesis). The induction of Slug and Vimentin expression, dissolution of cell-cell contacts through E-cadherin repression and re-arrangement of the cytoskeleton, which are all present in SW480/Lamin A cells and not in SW480/Control cells gives substantial evidence of the occurrence of an EMT in the SW480/Lamin A cells. It thus appears that high levels of Lamin A expression in SW480 cells provides a suitable signaling environment for EMT to occur. Lui et. al (2013), have found that the de-regulated expression of the three proteins Vimentin, E-Cadherin and Slug correlated with poor clinical outcome in patients

with breast cancer. As the data shows here that expression of these three proteins is also increased in scratch-wounded SW480/Lamin A cells, further confirmation is given towards the significance of the Lamin A protein as a risk biomarker in colorectal cancer (Willis et al., 2008), as it contributes to the production of a more aggressive cancer cell phenotype.

In a scratch-wounding assay cells are cultured in a 2 dimensional environment. In vivo, cells are in a 3-dimensional environment and thus the assay has several limitations in terms of mimicking the EMT process in live tissue, as important properties that contribute to the EMT process such as cell adhesion and cell-cell signaling may be altered (Muthuswamy, 2011). Future studies may therefore consider observing Lamin A expressing SW480 cells in artificial 3-dimensional cultures to observe their cell shape, levels of expression of Slug, Vimentin and E-cadherin and arrangement of the cytoskeleton in an environment that more closely mimics a real adenocarcinoma to determine whether SW480 cells are still able to display the mesenchymal characteristics that have been observed in these 2-Dimensional experiments.

Chapter 5: siRNA knockdown results

5.1: Background and aims

In the previous chapter, SW480/Lamin A cells have demonstrated an up-regulation in Vimentin and Slug expression, repression of E-Cadherin expression and a re-organization of the actin cytoskeleton using immunofluorescence. This gives further evidence that Lamin A is able to induce mesenchymal characteristics in SW480 cells. Lamin A silencing experiments in SW480/Lamin A cells were therefore performed to determine whether the cells express these mesenchymal markers in the absence of Lamin A expression. The siRNA used in this experiment has been custom-produced and optimized by Foster (2011- PhD thesis). The oligonucleotide targeted exons 11 or 12 of the post-transcriptional products of the LMNA gene, thus making it specific for the Lamin A protein and not Lamin C (given that the two proteins are alternatively spliced products of the same gene).

5.2 Confirmation of Lamin A silencing

Silencing of Lamin A protein expression was confirmed using western blotting. Samples for western blotting were prepared from T25 cm^3 cell culture flasks containing cells subjected to siRNA treatment. Immunoblotting was performed using a Jol2 antibody with β-actin used as a loading control at successive time points of 48, 96, 120 and 144 hours. As Figure 18 shows, silencing of endogenous Lamin A was effective from 48h to 144h after initial transfection of cell cultures with the oligonucleotide. This is demonstrated by the faint bands at 75kDa in the "si" samples. The faintest bands were seen between 96 and 120

hours after transfection, which indicates where the knockdown was most effective. The siRNA knockdown was less effective at reducing the expression of GFP-Lamin A, where expression levels appeared to be maintained at the same levels as the control samples across all time points.
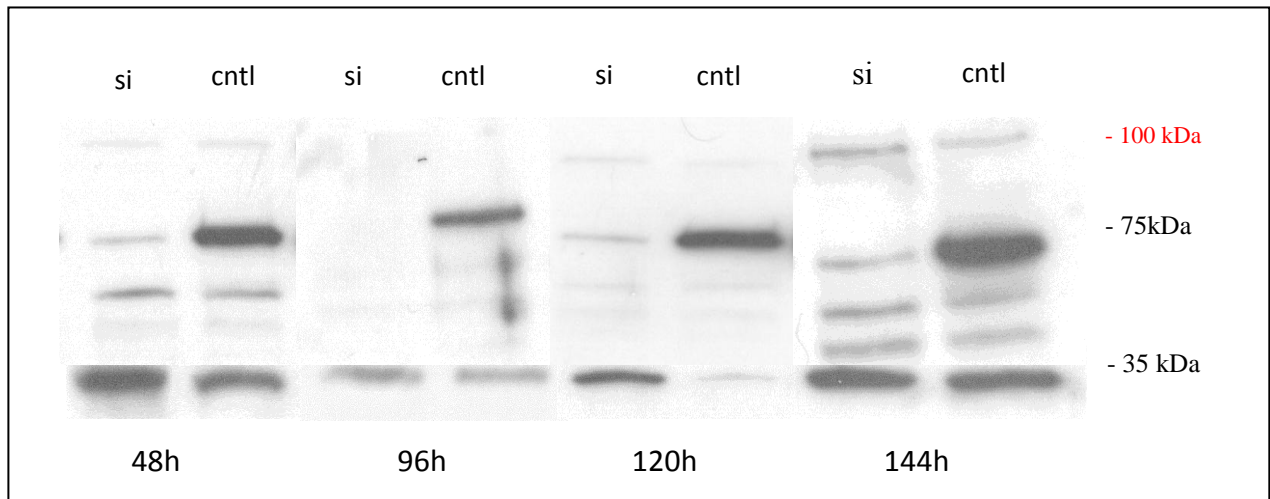

Figure 18: Western blotting of whole cell lysates prepared from SW480/Lamin A cells that have been transfected with siRNA targeting the Lamin A gene (si) and scrambled siRNA (cntl). Bands at 100kDa represent GFP-Lamin A. Bands at 75kDa represent Lamin A and the bands underneath are Lamin C. Bands at 35kDa represent β-actin.

Immunofluorescent experiments were then performed on cells in 6 well plates that had been subjected to 96h to 144h of siRNA transfection. Immunofluorescence microscopy revealed that Lamin A knockdown caused structural changes to the nucleus, shown by the increased presence of shrunken, dysmorphic nuclei. Although not shown consistently in the Western analysis, effective knockdown of GFP-Lamin A may have occurred in some cells where GFP expression was significantly reduced within the region of the nuclear lamina. These cells were targeted for analysis, given that they demonstrated Lamin A repression to some degree. Cells demonstrating successful Lamin A knockdown occurred in small groups within the seeded cultures, suggesting

multiple divisions of cells successfully transfected with the siRNA.

5.3: Vimentin expression
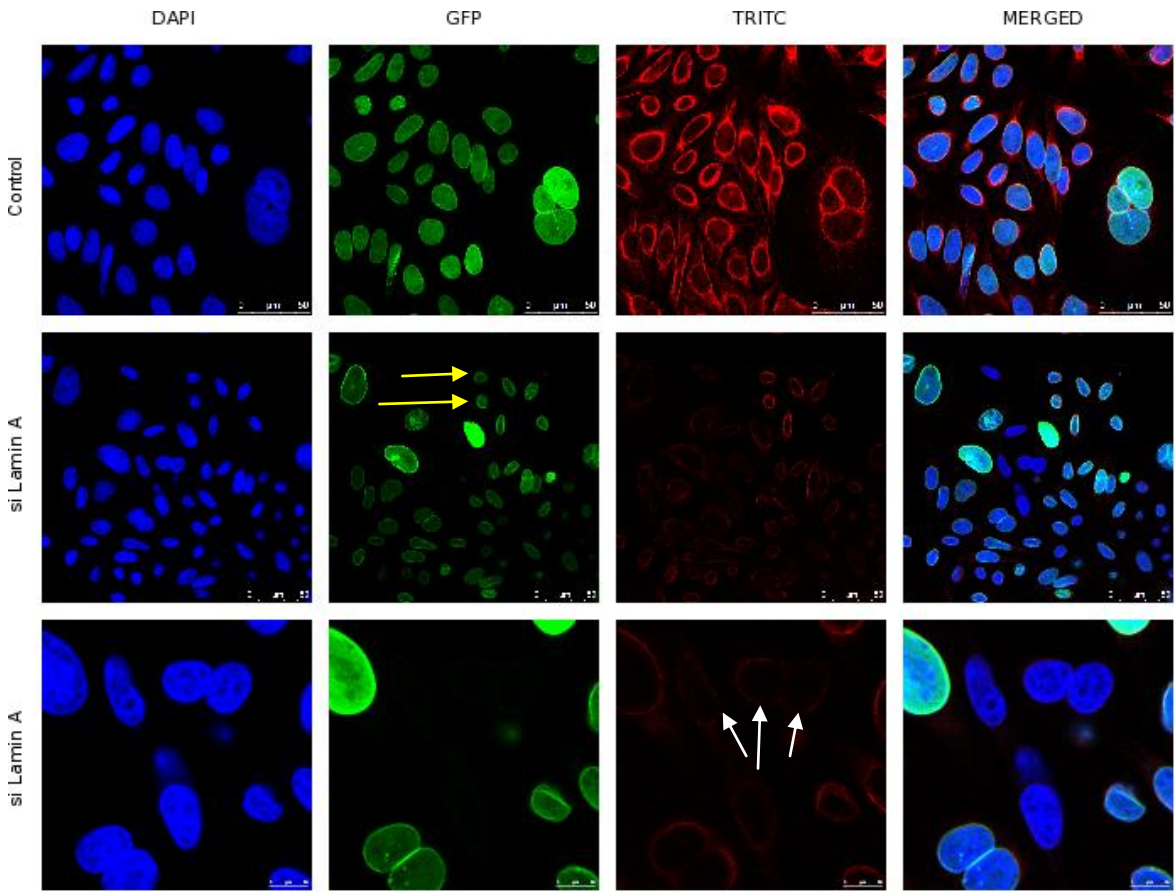
Figure 19: 70% confluent SW480/Lamin A cell cultures that have been subjected to transfection with scrambled siRNA (Control-row 1) and siRNA targeting the transcriptional products of the LMNA gene (si Lamin A- rows 2 and 3). All cells are stained with a Vimentin antibody. White arrows indicate limited Vimentin cytoskeleton formation in si Lamin A cells. Yellow arrows indicate the presence of shrunken and dysmorhic nuclei in si Lamin A cells.

The effect of Lamin A knockdown on the Vimentin cytoskeleton (TRITC channel-Figure 19) was seen most markedly at 144 hours, where Vimentin expression was shown to be greatly reduced. Although western blotting experiments show that Lamin A knockdown is most effective at 120h, the most significant observed reduction in Vimentin cytoskeleton expression seen using

immunofluorescent microscopy was at a later time period. This may be due to the stable nature of intermediate filaments and signalling changes that would have occurred in order for an initial reduction in Lamin A expression to have an effect on the Vimentin cytoskeletal networks present in the cells. In the si Lamin A cells, knockdown of the GFP-Lamin A was variable. Vimentin expression was reduced in all of the cells transfected with the siRNA however, suggesting that the decrease in Vimentin may be partly due to decreases in the endogenous Lamin A seen in the western analysis in Figure 18. Sparse expression was maintained in the peri-nuclear areas of the cells (white arrows). There was a significant reduction in the number of filaments seen in the cytoplasm in comparison to the control cells, where strong staining was observed for Vimentin.

5.4: Filamentous Actin expression

Figure 20: SW480/Lamin A cultures that have been transfected with scrambled siRNA (Control- row 1) and siRNA targeting the Lamin A gene (si Lamin A- rows 2 and 3) for 120 hours. All cells have been stained with TRITC phalloidin. White arrows indicate a change in cell architecture in the si Lamin A cells, where cells have become more rounded, with reduced adherence to the surface substrate.

siRNA knockdown of Lamin A caused significant changes in the organisation of actin cytoskeletons of the SW480/Lamin A cells at 120 hours. Changes in the shape of the cells from a flattened and spindle-like, morphology to a more rounded and cobble-stone shaped morphology was observed in cells that underwent successful lamin A knockdown. The prominent stress fibre formation seen in in the control cultures was not observed in cells that were transfected with the siRNA that targeted Lamin A (si Lamin A cells). si Lamin A cells also demonstrated collapse of the cytoskeleton towards the nucleus and detachment from the surface substrate (white arrows). Control cells maintained a flattened morphology with extensive actin cytoskeletal filaments maintained in the cytoplasm. Overall the effect of transfection of siRNA against the Lamin A gene had variable effects on the actin cytoskeleton, with some cells remaining flattened and spindle-like (yellow arrow) but others demonstrating a more rounded, epithelial cellular architecture. (white arrows).
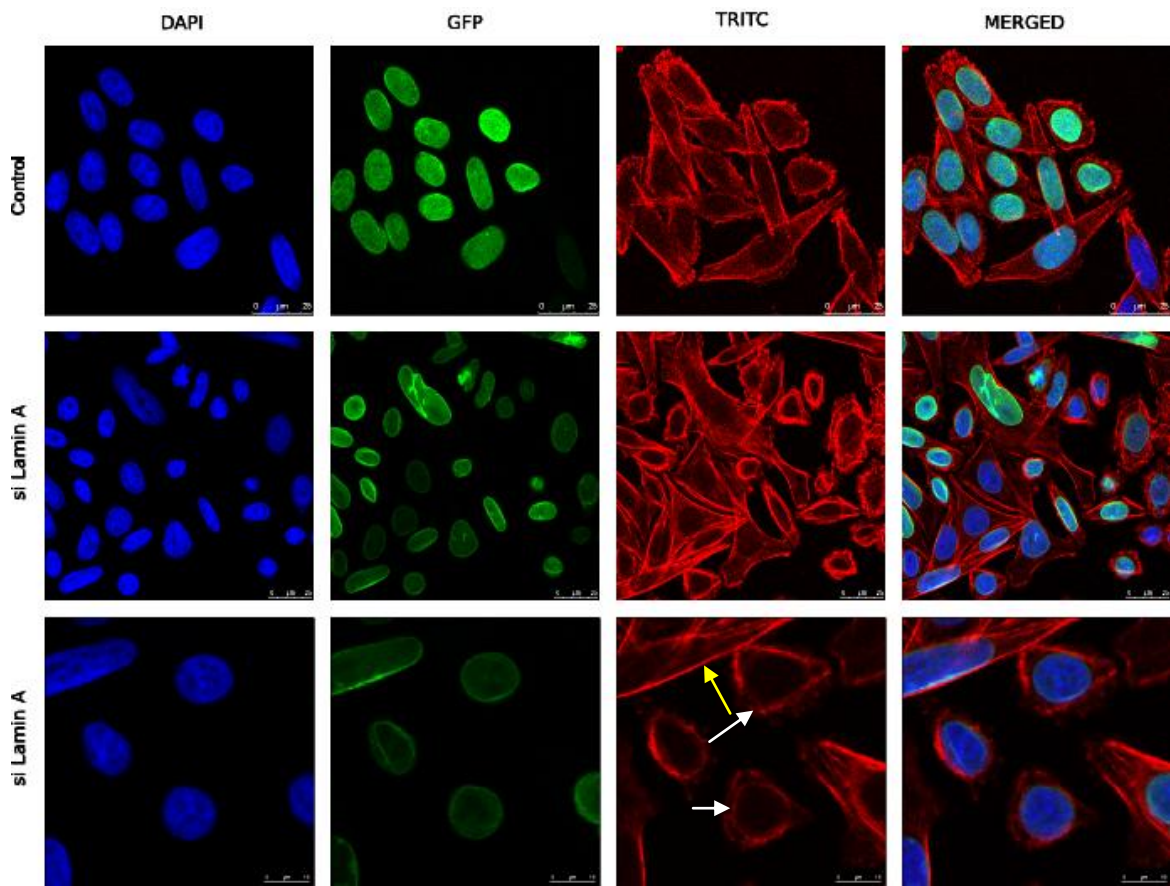
5.5: Slug expression

Figure 21: SW480/Lamin A cultures that have been transfected with scrambled siRNA (Control- row 1) and siRNA targeting the Lamin A gene (si Lamin A- rows 2 and 3) for 120 hours. All cells have been stained with a Slug antibody. Yellow arrows indicate an absence of nuclear Slug in si Lamin A cells that have undergone Lamin A knockdown, shown by an absence of signal in the GFP channel (shown by white arrows).

The expression of Slug was variable in SW480/Lamin A cells that had undergone silencing of Lamin A with most effective knockdown occurring between 120h and 144h. Cells demonstrating the most successful Lamin A knockdown, shown by a marked decrease in the presence of GFP-Lamin A, also demonstrated significantly reduced levels of Slug. This reduction was most prominent in the nucleus, where staining was almost absent (yellow arrows in Figure 3.4). Other

cells, where knockdown had been less effective, maintained some nuclear

expression of Slug In control experiments expression of Slug was uniformly

maintained in the nuclei of the SW480/Lamin A cells, where staining was

observed to occur in small aggregates or "speckles" as previously observed.

5.6 Fibronectin expression

As the previous Chapter 3 has indicated an increase in the levels of expression

the mesenchymal markers Slug and Vimentin in the SW480/Lamin A cells at low

cell density, and Chapter 4 has shown that these markers are increased in the

SW480/Lamin A cells in a scratch wounding environment, a further

confirmation of a mesenchymal phenotype in the SW480/Lamin A cells would

complement these results. Fibronectin is another robust marker of

mesenchymal cells (Kim et al, 2013) and was therefore selected to further
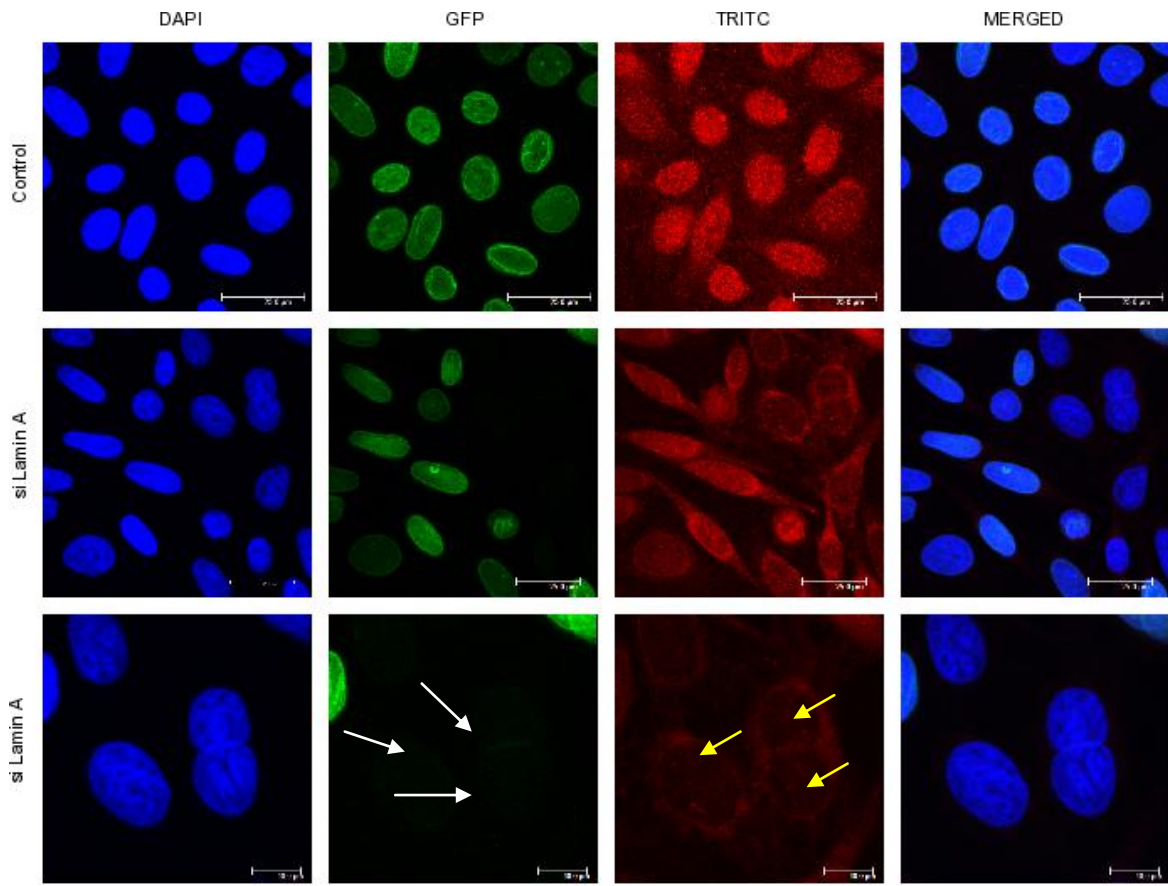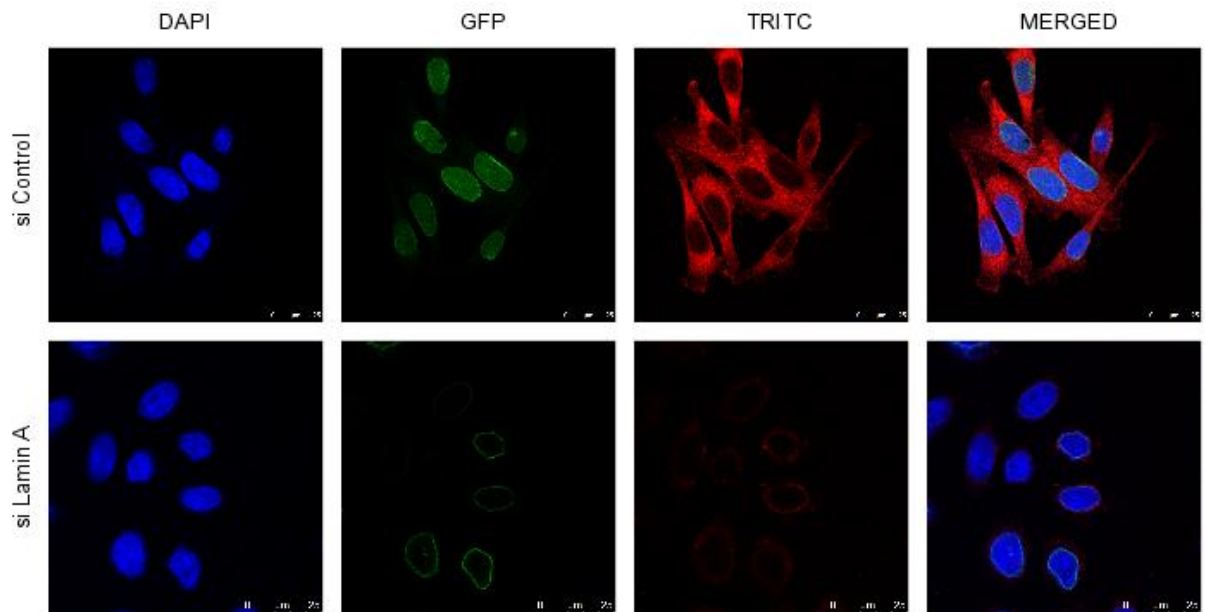
complement previous analyses.

Figure 22: SW480/Lamin A cultures that have been transfected with scrambled siRNA (si Control- row 1) and siRNA targeting the Lamin A gene (si Lamin A- row 2) for 120 hours. All cells have been stained with a fibronectin antibody.

As Figure 22 shows, there was a marked reduction in Fibronectin expression in the si Lamin A cells shown by a reduction in red cytoplasmic staining in the siLamin A cells.

5.7: Chapter Discussion

RNA interference (RNAi) is a method in which transfection of a cell with short double stranded oligonucleotides results in the nuclease-induced destruction of mRNAs of the target gene and as a result, reduced expression of protein(s) encoded for by the gene, which is known as Post transcriptional gene silencing (PTGS) (Bernstein et al., 2000). Here, PTGS of the Lamin A gene was achieved successfully, demonstrated by reduced expression of the Lamin A protein by western blotting analysis.

The aims of these experiments were to consolidate our understanding of the relationships between Lamin A expression and expression of proteins Vimentin, Actin and Slug in relation to cell motility and invasive cellular behaviour.

Lamin A knockdown in the SW480/Lamin A cells caused a decrease in the expression of Vimentin. The role of Vimentin in both cancer and EMT has been discussed in chapter 4, where Vimentin was shown to be up-regulated in SW480/Lamin A cells using immunofluorescence. As silencing of Lamin A has decreased Vimentin expression, this suggests that Lamin A may regulate the expression of Vimentin through a signalling pathway.

The observed decrease in Slug expression in cells that had undergone Lamin A gene silencing also suggests a relationship between Lamin A expression and Slug expression in the SW480/Lamin A cells. Foster (2011, PhD thesis), has also

demonstrated that knockdown of Lamin A in the SW480/Lamin A cells results in a reduction in the expression of Slug mRNA using microarray analysis, but this is the first time that Lamin A silencing has been shown to reduce the expression of Slug at the protein level. Slug is a transcription factor that is a well-known inducer of EMT. It is known to promote cell motility and invasion in a number of different cancer cells (Park et al 2008; Hotz et al., 2007; Uchikado et al., 2005). In SW480/Lamin A cells that had undergone knockdown of Lamin A, Slug expression was almost absent in the nucleus, suggesting it did not behave as a transcriptional repressor of the E-cadherin promoter in the cells that were successfully silenced for Lamin A.

A relationship between Slug expression and Vimentin expression has been deduced in breast cancer (Vuoriluoto et al., 2011) where Vimentin is necessary for Slug-induced, EMT-associated migration. High Vimentin and high Slug expression is also associated with poor patient prognosis in Basal-Like Breast Cancer (Lui et al., 2013. As Slug expression can be induced through TGFβ signalling (Medici et al., 2008) and Lamin A can regulate TGFβ signalling (Van Berlo et al., 2005), future work could look to determine whether TGFβ signalling may be responsible for the Lamin A-dependent up-regulation of Slug and Vimentin expression in SW480/Lamin A cells.

As knockdown will have compromised the regular structure of the nuclear lamina, it thus follows that the interactions Lamin A forms as part of the LINC complex, bridging the nucleus with the cytoskeleton, caused changes to the actin cytoskeleton and overall cell architecture (Meinke et al., 2011). Willis et al (2008) have previously shown using semi-quantitatice RT-PCR that knockdown of Lamin A in SW480/Lamin A cells significantly reduces their motility and the

levels of the actin-bundling protein T-Plastin. Here the impact of Lamin A knockdown on the Actin cytoskeleton was visualized. Knockdown caused the disruption of actin cytoskeleton networks and a reduction in the levels of filamentous actin in SW480/Lamin A cells. The reduction in stress fibre formation, along with changes in architecture and morphology suggests that knockdown in Lamin A in SW480/Lamin A cells causes them to adopt a more epithelial phenotype. Extensive stress fibre formation and spindle-like morphology are typical hallmarks or mesenchymal cells (Haynes et al., 2011). As fewer cells demonstrated these characteristics in SW480/Lamin A cultures that were subjected to siRNA treatment, this suggests that the presence of Lamin A in SW480 cells contributes to a motile organisation of the Actin cytoskeleton, which is reversed upon Lamin A silencing.

The observed reduction in fibronectin expression in the cells that had undergone Lamin A knockdown compared to the SW480/Lamin A control cells further suggests that Lamin A contributes to an mesenchymal phenotype in the SW480/Lamin A cells as this protein is a marker of mesenchymal cells and also of poor patient prognosis in cancer (Sudo et al., 2012). Together, down-regulated Fibronectin, Vimentin and Slug expression, coupled with the conversion of the Actin cytoskeleton to a more epithelial structure in the silenced cells show that in the absence of Lamin A, some of the mesenchymal properties previously observed in SW480/Lamin A cells in Chapter's 3 and 4 are reversed and that silencing of Lamin A in SW480/Lamin A cells reduced their mesenchymal phenotype.

Chapter 6: Discussion

The nuclear envelope (NE) is an important mediator of transcriptional control, cell polarity and migration, genomic stability and cell division. These cellular processes are often aberrant in a cancerous environment (Chow et al., 2012). The Lamin A protein, which is a multi-functional constituent of the nuclear lamina, forms part of the nuclear envelope. This protein has also been found to be aberrantly expressed in a variety of cancers (Foster, 2010). In the case of colorectal cancer, Lamin A has been found to be a risk biomarker. Willis et al (2008) have shown that patients with colorectal cancer tumours positive for Lamin A expression were almost twice as likely to die in comparison to clinicopathologically identical patients whose tumours did not express Lamin A.

To explore why Lamin A expression lead to poor patient prognosis, the SW480 pre-metastatic colorectal cancer cell line was stably transfected with a Lamin A-GFP fusion protein. This significantly increased the motility of SW480 cells. Elucidation of the reasons behind this increase in motility has involved genomic and proteomic studies. Willis et al (2008) have shown that the increase in Lamin A expression in SW480 cells causes a change in the actin cytoskeleton and the adhesive properties of the cells. Further data produced by Foster (2011) showed futher changes in the cytoskeletal fractions of SW480/Lamin A cells that showed an increase in filamentous actin and Vimentin, which are both markers of a mesenchymal cell architecture.

Ingenuity Pathways Analysis (IPA) analysis of microarray data allows us to deduce significant signaling networks by considering the simultaneous up and

down-regulation of several genes within an experiment. Microarray data recorded when SW480 cells were transfected with GFP-Lamin A to give SW480/Lamin A cells has been subjected to IPA analysis. The changes in the levels of the mRNA trascripts of a number of genes, including Slug and Vimentin, gave evidence for the role of Lamin A in a signalling regulatory network contributing to an increased invasive and metastatic phenotype in SW480 cells. This data also showed an increase in the levels of TGFβ mRNA and a down-regulation in E-Cadherin mRNA present in the SW480/Lamin A cells compared to SW480/Control cells transfected with GFP only. Furthermore, observations of the morphologies and behaviour of SW480/Lamin A cells using live cell imaging has indicated that the cells are typically mesenchymal in shape. It was thus speculated that an increase in Lamin A expression in SW480 colorectal cancer cells may make them more amenable to undergo an EMT (Foster 2011, PhD thesis).

The aims of this thesis were therefore to further understand how increased Lamin A expression in SW480 cells changes their cellular phenotype. Initially the effect of cell confluency in relation to expression of Lamin A, Vimentin and Slug was tested. Immunofluorescence was then used to look for changes in expression of EMT proteins in a scratch wound-assay. Finally siRNA knockdown of Lamin A was performed to determine whether this had any effect on the expression of proteins in relation to the EMT process.

Immunoblotting experiments indicated that SW480/Lamin A cells demonstrate a mesenchymal phenotype that is prominent at low cell density, shown by an increase in Vimentin and Slug expression. This effect was Lamin A dependent,

as in the SW480/Lamin A cultures at high cell density, expression of Lamin A, Slug and Vimentin was reduced.

Scratch wounding experiments show SW480 cells lack cell junctions and have high Vimentin and high Slug expression. Along with these mesenchymal cellular properties, the Actin cytoskeletons in SW480/Lamin A cells showed re-arrangements necessary for cell motility.

The contribution of Lamin A to the changes observed in the first 2 experiments, assessed by Lamin A silencing on SW480/Lamin A cells, caused the SW480/Lamin A cells to significantly down-regulate their Vimentin and Slug expression. The Actin cytoskeletons of silenced SW480/Lamin A cells also underwent morphologic changes that suggested a decrease in their motile phenotype and their fibronectin expression, a classical EMT marker, was reduced.

This data has established an important link between Lamin A and Vimentin expression in SW480/Lamin A cells. The two proteins have been found to co-localise in neuroblastoma tissue (Mergui et al., 2011) and furthermore, there has been recent recognition of the multi-functional importance of Vimentin, the type 3 intermediate filament, as both a structural and signalling protein like Lamin A. Vimentin has been identified to have distinct roles in cell adhesion and migration (Ivaska et al, 2007) and in the specific context of cancer, Vimentin behaves as a marker of aggressive breast cancer cell lines (Neve et al., 2006) and of the EMT process (Yilmaz and Christofori, 2009). In the proposed signaling EMT signaling pathway mediated by Lamin A in Figure 23, Vimentin is thus indicated as having effects on both cell motility and cell architecture, due

to the changes expression of this protein induces on both the shape of SW480/Lamin A cells and previous evidence of its roles in adhesion and migratory changes that allow cellular motility. Given the established roles for Vimentin in the context of many cancers, the observed relationship that the protein may share with Lamin A should be further elucidated. Future experiments should look to determine the effect of Vimentin knockdown on Lamin A expression in SW480/Lamin A cells, as this well allow further establishment of a signalling relationship between the two proteins. Vimentin has been shown to regulate ERK signalling, which is an important pathway in development and maintenance of cancers (Ivaska et al., 2007). ERK signalling is also able to induce the expression of Slug in SW480 cells (Connacci-Sorrell et al., 2003), and thus would make an important future signalling pathway to study.

The fact that high levels of Lamin A expression in SW480/Lamin A cells leads to high levels of Slug expression and vice versa could partly explain why colorectal cancer patients with high expression of Lamin A in their tumours can have a poor prognosis (Willis et al., 2008), as the potential expression of Slug in these tumours may contribute to a more motile and invasive phenotype of the colorectal cancer cells through the process of EMT. Slug has also been shown independently to be a marker of poor prognosis in colorectal carcinoma patients (Shioiri et al., 2006), thus demonstrating the implications for Slug expression as a contributor to more aggressive colorectal cancer cells. The up-regulation in Slug protein expression occurred simultaneously with a significant down-regulation in E-cadherin expression, demonstrated by a lack of junctional formation in SW480/Lamin A cells, even in areas of high cell density. E-cadherin repression, along with an increase in expression of an EMT-inducing transcription factor such a Slug is one of the earliest pre-requisites for the

occurrence of EMT and thus shows that the process can be initiated in SW480/Lamin A cells. Vuoriluoto et al (2011) have shown that Slug induces the expression of Vimentin in breast cancer cells leading to a more invasive phenotype and therefore in Figure 23, Slug is indicated as an inducer of Vimentin expression. Slug is also indicated as inducing re-arrangements in the Actin cytoskeleton and repression of E-cadherin in Figure 23, as the transcription factor has been shown to repress E-cadherin expression and re-model the actin cytoskeleton in a number of EMT's in cancer (Zhang et al., 2011; Yilmaz and Christofori, 2009) . These changes, as previously mentioned, allow a change in cellular architecture and an increase in cellular motility (Figure 23).

As the signaling basis behind the contribution of Lamin A to Slug up-regulation is un-known, future experiments should also look to elucidate these. Lamin A has a significant number of signaling partners (Andrés and Gonzàlez, 2009). In prostate cancer Lamin A-induced an increase in prostate cancer cell motility which was identified as a result of signaling through the PI3K/PTEN pathway (Kong et al., 2012). This pathway would therefore make an interesting target of study in SW480 colorectal cancer cells. As TGFβ is an established signaling partner to the Lamin A protein (Van Berlo et al., 2005) and has shown evidence of increased activity in SW480/Lamin A cells (Foster, 2011 PhD Thesis), it has been indicated as the initial effector on the expression of Lamin A and of Slug in the proposed EMT signaling pathway (Figure 23). Future work may look to also determine the levels of TGFβ in SW480/Lamin A cells at low cell density to observe whether signaling is induced in SW480/Lamin A cells and whether it contributes to Slug expression. TGFβ is a well-known tumour promoter and inducer of EMT, and induction of TGFβ signalling induces downstream PI3K and

MAPK signalling (Zavadil and Böttinger, 2005). These pathways are implicated in tumour cell motility and migration. TGFβ signalling is also able to induce expression of Slug in a number of different tissues (De Craene et al., 2005) and therefore forms the primary candidate for future signaling studies (Figure 23).

Together these results confirm speculations made by Foster (2011- PhD thesis) that the presence of Lamin A expression in SW480 colorectal cancer cells induces mesenchymal characteristics in SW480 cells. As Lamin A modulates the expression of a number of signaling pathways, expression of Lamin A is SW480 cells permits a signaling environment that allows EMT to occur. This effect was particularly marked in cells cultured at low cell density and also at the edges of scratch wounds, which are both environments that are permissible of increased cellular motility and migration. As a link is thus established between Lamin A expression and cancer cell metastasis in SW480 colorectal cancer cells, future work should look to further elucidate the molecular signaling mechanisms behind this phenomenon and to explore whether Lamin A contributes to a mesenchymal cell phenotype in other cancer cell lines, such as breast, ovarian and lung. This would allow us to determine the extent to which Lamin A contributes to a more agressive cancer cell phenotype in other kinds of cancer and to further elucidate the role of Lamin A in a variety of cancers.
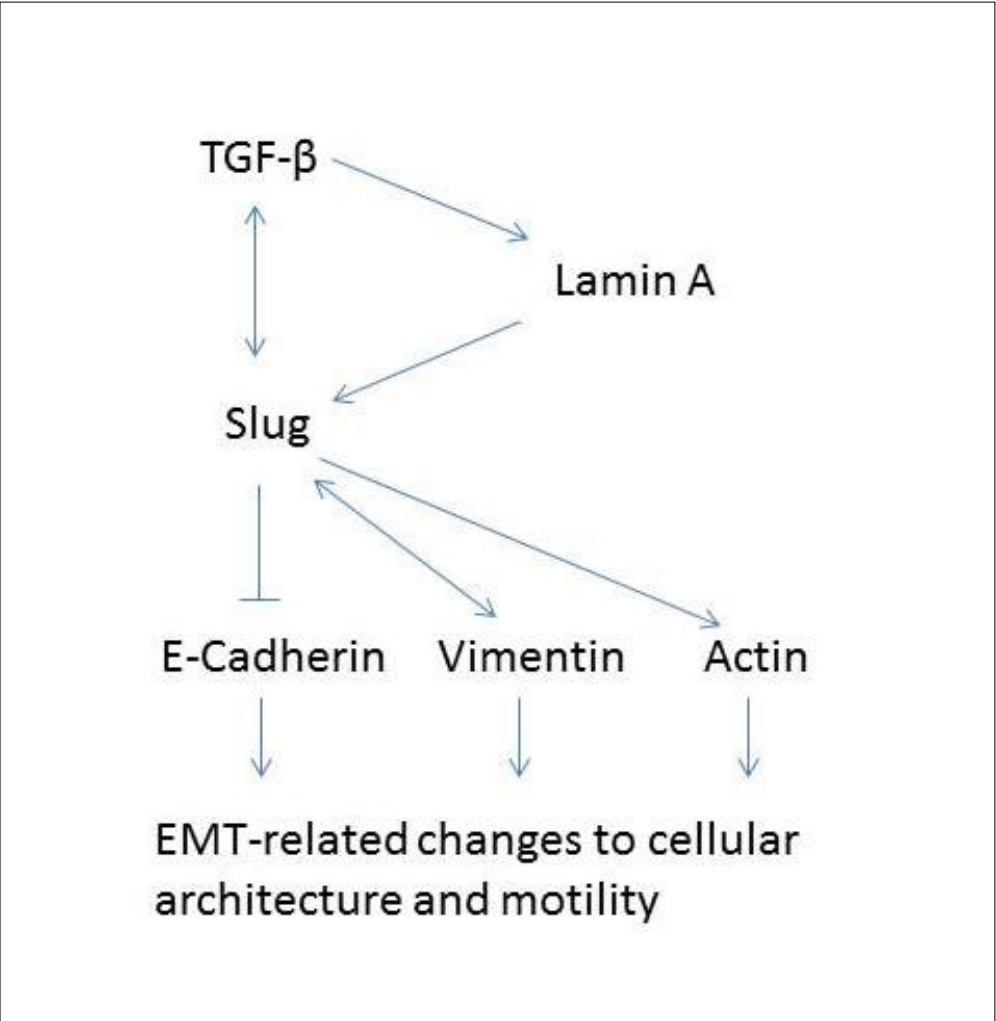
Figure 23: Proposed EMT signalling pathway in SW480 cells that is mediated by Lamin A expression.

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