Role of Flightless I in Cancer Growth and Metastasis

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Noraina Muhamad Zakuan, BSc, Msc

School of Health and Biomedical Sciences

College of Science Engineering and Health

RMIT University

November 2016

Declaration

I certify that except due acknowledgement has been made, the work is that of the author alone; the work has not submitted previously, in whole or in a part, to qualify for any other academic award; the content of the thesis is the results of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and ethics procedures and guidelines have been followed.

Noraina Muhamad Zakuan

Date: 17 November 2016

Acknowledgements

My journey as a PhD student has been an amazing learning experience. This would not have been possible without a number of people who were there for me to share both the challenges and rewards of my scientific endeavours. This acknowledgement is devoted to all those wonderful people who were a part of this journey and who contributed in their own way to make this thesis possible.

First and foremost, I would like to express sincere gratitude and appreciation to my supervisor, Associate Professor Ian Darby, for offering me a wonderful opportunity to do my doctoral research in his laboratory. His understanding, support and personal guidance have been crucial to my PhD journey. One simply could not wish for a better or more supportive supervisor. Thank you!

I would like to express my deepest thanks and gratitude to my co-supervisor, Dr Dodie Pouniotis, for her endless help, generous advice and assistance, which added clarity to my research. It has been a privilege and a pleasure to work with her.

I would also like to take this opportunity to thank the staff and colleagues in the Cancer and Tissue Repair Laboratory at RMIT University; it has been a great pleasure working with each of you. To Lynda, thank you for your understanding, support and assistance in many ways throughout this rollercoaster journey. I would also like to thank the Ministry of Higher Education Malaysia and the University Putra Malaysia for awarding me a scholarship to pursue my doctoral study.

Professional accredited editor Mary-Jo O'Rourke AE provided proofreading services according to the university-endorsed national 'Guidelines for editing research theses'.

This thesis is dedicated to my parents, who have always had faith and belief in me. Thank you both for giving me the strength and ultimate support to chase my dreams. I would also like to express my heartfelt thanks to the rest of my family and in particular my sisters, Norazrin, Noraisyah, Norasma and Noramira, for their moral support and encouragement.

Finally, my wonderful husband, Kamarul Zaidi, deserves a special mention because he believed that I could do this. Without your understanding, endless patience, support and encouragement, it would have been impossible for me to start, let alone finish this work. To the apples of my eyes, Adriana and Afiq, thank you for your love, understanding and sacrifice. I owe very much to all of you.

List of Publications

Darby, I. A., **Zakuan, N**., Billet, F. and Desmoulière, A. (2016). The myofibroblast, a key cell in normal and pathological tissue repair. *Cellular and Molecular Life Sciences, 73*(6), 1145–1157.

Zakuan, N., Pounitis, D. S., Cowin, A. J., Kopecki, Z. and Darby, I. A. (2016). Inhibition of Flightless I inhibits cancer cell invasion and reduces tumor growth and spread in a mouse colon cancer model. Manuscript submitted to *Cellular and Molecular Life Sciences.*

Conference Abstracts

Zakuan, N., Pouniotis, D., Kopecki, Z., Cowin, A. and Darby, I. A. (2015). Flightless I expression affects tumour growth. Lorne Cancer Conference, Lorne, Australia. Poster presentation.

Zakuan, N., Pouniotis, D., Kopecki, Z., Cowin, A. and Darby, I. A. (2015). Flightless I expression affects tumour growth. Malaysian Post Graduate Colloquium, Melbourne, Australia. Oral presentation.

Zakuan, N., Pouniotis, D., Jackson, J. E., Kopecki, Z., Cowin, A. and Darby, I. A. (2016). Inhibition of Flightless I reduces tumour growth and spread in a mouse colon cancer model. Australian Society for Medical Research Victorian Student Symposium. Poster presentation.

Table of Contents

List of Abbreviations

α-SMA	Alpha (α) Smooth Muscle Actin
CAFs	Cancer Associated Fibroblasts
ECM	Extracellular Matrix
EMT	Epithelial–Mesenchymal Transition
FA	Focal Adhesion
FliI	Flightless I
IL	Interleukin
LRR	Leucine Rich Repeat
MMPs	Matrix Metalloproteinases
PCNA	Proliferating Cell Nuclear Antigen
SCC	Squamous Cell Carcinoma
TAM	Tumour Associated Macrophage
TNF-α	Tumour Necrosis Factor Alpha (α)
VEGF	Vascular Endothelial Growth Factor

List of Figures

List of Tables

Abstract

The aim of this project was to investigate the role of the Flightless I (FliI) protein in tumour growth and metastasis *in vitro* and *in vivo* using cultured colon cancer cells and a mouse colon cancer model. FliI is a member of the actin-remodelling protein family, which consists of gelsolin, adseverin, capG, villin and supervillin family members, which play important roles in cellular motility, contraction and adhesion. Several studies have investigated the function of FliI and its mechanism of action in both *in vitro* and *in vivo* models of wound healing in mouse skin. These studies showed that knockdown of FliI improved wound healing via increased cell proliferation, epithelial cell migration and enhanced wound closure, while also resulting in reduced scarring and inflammation. In contrast, overexpression of FliI caused impaired wound healing with larger, fewer closed wounds, reduced cellular proliferation and delayed epithelial migration, as well as excessive scarring.

Since there are parallels between the wound healing/scarring process and cancer progression, the aim of this study was to investigate the role of FliI in cancer growth and spread. Recently it has been shown that FliI expression also correlated with increased tumour growth in a mouse model of squamous cell carcinoma, while FliI inhibition using a neutralising antibody reduced tumour growth and invasion in this model. The FliI protein itself has been shown to be both localised intracellularly and also secreted; therefore, we have studied tumour growth both in mice with reduced FliI expression (FliI heterozygous knockout +/− mice) and also in wild type (WT) mice injected with cancer cells that have FliI knockdown using siRNA. *In vitro*, FliI expression levels were found to have significant effects on cell migration, cell proliferation, cell adhesion and invasiveness. Knockdown of FliI increased cell motility and proliferation, and was found to increase cell adhesion via

upregulation of focal adhesion (FA) proteins, while reducing FliI expression greatly inhibited cellular invasion associated with reduced expression of an invadopodia marker. *In vivo*, colon cancer cells showed decreased spread to form fewer lung metastases in mice with reduced FliI expression (FliI heterozygous mice).

This suggests that host FliI expression levels can regulate tumour growth, possibly through an effect on tumour stromal cell behaviour. Interestingly, knockdown of FliI using siRNA also resulted in smaller primary tumours in mice, suggesting FliI expression in cancer cells could influence tumour growth in this model as well. *In vitro*, FliI expression correlated with decreased adhesion and increased invasion, suggesting FliI may play a role in tumour progression via effects on cell adhesion and invasion, potentially via effects on invadopodia formation. These findings suggest that FliI inhibition in both stromal and cancer cells may be a promising target for limiting tumour growth and spread. In addition, administration of a FliI-neutralising antibody (FnAB) showed potential for reducing tumour progression in metastatic models and reducing tumour spheroid invasion *in vitro.* Overall, the results suggest FliI may be a promising target for inhibiting tumour growth and metastasis.

The significance of this study is that FliI has been shown to play a role in mediating cellular functions which are important in tumour progression. FliI expression increased cellular invasion, possibly via an invadopodia mechanism correlated with increased cortactin expression. FliI expression has also been shown to influence tumour growth and spread in murine colon cancer models, while FliI inhibition either in the host or in cancer cells reduced tumour progression and metastasis. Importantly, this study demonstrates that the inhibition of FliI expression intracellularly and within hosts could modify the tumour microenvironment, thus reducing tumour growth. This present study also serves as a

preliminary finding on the administration of FnAB to potentially reduce tumour growth and spread in animal models, which indicates that FliI could be a potential target for cancer therapy.

Chapter 1 Literature Review

1.1 Introduction

Current therapeutics targeting cancer cells are well established but are often insufficient to control tumour growth and cancer spread. Tumours consist, in many cases, of the tumour cells themselves and a connective tissue stroma that surrounds the cancer cells and includes several other cell types, including fibroblasts and inflammatory cells. This has led to the acceptance of co-targeting both the cancer cells and their microenvironment including cells such as stromal fibroblasts and inflammatory cells, but also the extracellular matrix (ECM) and matricellular proteins (Pollard, 2009; G. Wong & Rustgi, 2013) as another approach to cancer therapy.

Growing evidence suggests that interaction between cancer cells and their microenvironment contributes to cancer growth and progression. The plasticity of cancer-associated stroma is one of the main contributors involved in morphological and functional transitions that support the pathogenesis of cancer cells (S.-Y. Sung et al., 2008). The modification of the cytoskeleton may also be important in modifying cancer cell motility, resulting in cancer spread or metastasis. The balance between cell adhesion and cell motility is likely to be a major determinant of cancer cell movement (Detchokul, Williams, Parker, & Frauman, 2013).

It is known that wound healing in the skin and tumour stroma shares several similar cellular and molecular characteristics. Because of this it has been postulated that tumours represent a ‘wound that never heals’ or possibly a wound that over-heals (Dvorak, 1986). The

development and functional characterisation of the microenvironment, such as recruitment of inflammatory cells, fibroblasts, and the presence of extracellular matrix and matrix remodelling proteins, have been highlighted and show the similarity between wound repair mechanisms and cancer. However, there are crucial differences in terms of the ability to differentiate and the invasive growth of malignant tumours and, in addition, the failure of cancer growth to resolve to scar tissue as occurs in wound repair (Schäfer & Werner, 2008).

The parallels between the scarring process and cancer progression and their microenvironments led us to hypothesise that those factors known to regulate wound healing and scarring may potentially play a role in tumour growth and spread. The focus of this project was to determine the role of Flightless I (FliI), which is a negative regulator of wound repair, in cancer growth and metastasis. FliI belongs to the gelsolin family of actin severing and remodelling proteins, which are involved in cell motility, contraction and adhesion, but it may also mediate other protein interactions.

A number of studies have been done to determine the role of FliI as a negative regulator of wound healing using *in vivo* and *in vitro* models of healing (Cowin et al., 2007; Jackson, Kopecki, Adams, & Cowin, 2012; Z. Kopecki & Cowin, 2008). These studies found that under-expressing FliI by generating heterozygous knockout mice improved wound healing, with wounds showing increased cell proliferation and keratinocyte migration. In contrast, FliI-overexpressing mice that had one or two extra FliI transgenes showed significantly impaired wound healing, resulting in larger wounds associated with reduced cellular proliferation and migration and increased scarring.

A better understanding of how the tumour microenvironment affects cancer progression could lead to new targets for the destruction of cancer cells via interruption of the complex

networks that exist between cancer cells, host cells and the surrounding extracellular matrix or the tumour microenvironment. Therefore, FliI might represent one such target where intracellular inhibition or its secreted form via molecular inhibitors or monoclonal antibodies might enable a reduction in tumour growth and spread.

1.2 Colon Cancer

Colorectal cancer (CRC) is a common lethal cancer in Western countries. Approximately one million new cases of colorectal cancer are diagnosed annually and cause half a million deaths, commonly in the elderly (Boyle & Leon, 2002). The risk of developing CRC is influenced by both environmental and genetic factors. A rise in incidence of young adults diagnosed has been reported in the United States of America and Australia linked to an increase in common risk factors such as obesity, alcohol consumption and sedentary lifestyles (Young et al., 2014).

Approximately 25% of patients present with metastases at initial diagnosis and almost 50% of patients with CRC will develop metastases, contributing to the high mortality rates reported for CRC (Van Cutsem, Cervantes, Nordlinger, & Arnold, 2014). Importantly, distant metastasis, which may be asymptomatic until reaching an advanced stage, comprises about 40% of CRC patient deaths due to metastatic complications.

Even though many of the genetic alterations occurring in colon cancer are now known (Fearon & Vogelstein, 1990; Vogelstein et al., 1988), it still remains the second most common cause of cancer mortality and current therapeutic approaches still need to be improved to increase the effectiveness of the treatment.

1.2.1 Epidemiology

Colorectal cancer (CRC) is one of the main causes of cancer incidence and death worldwide, particularly in developed countries such as Europe, the USA and Australia. It was responsible for 215,000 deaths in 2012 in Europe and over 4000 deaths in Australia (Siegel, DeSantis, & Jemal, 2014; Young et al., 2014). In the United States, it was estimated that about 71,830 men and 65,000 women would be diagnosed with colorectal cancer, and 26,270 men and 24,040 women would die from the disease, in 2014. This data represents about 13.2% and 12.7% frequency of CRC among all cancer cases, and 11.6% and 13.0% of all cancer deaths in men and women, respectively (Siegel et al., 2014).

Australia remains one of the countries with the highest rates of CRC incidence. CRC was the second leading cause of cancer incidence and third leading cause of cancer deaths in Australian adults in 2010 (Young et al., 2014). In 2007, 4047 deaths were caused by CRC (2191 males; 1856 females), which comprised 10.1% deaths from invasive cancer, the second highest after lung cancer. The incidence increased in 2008 with 14,225 people diagnosed with this disease (National Bowel Cancer Screening, 2011).

1.2.2 Colon Carcinogenesis

Tumourigenesis is a multistep process which involves genetic alterations that have the ability to transform normal cells into aggressive malignant cells (Hanahan & Weinberg, 2000). It has been shown in rodent cells that the transformation process itself includes several steps involving genetic changes and acquisition of tumourigenic functions (Hahn et al., 1999).

Hanahan and Weinberg hypothesised that there are six hallmarks of cancer which are acquired throughout a multistep process of tumour development. Most cancer cells become

capable of producing their own mitogenic growth signals to maintain an active proliferative state, they become insensitive to anti-growth signals, they can escape from cell death (apoptosis) mechanisms, they cause sustained angiogenesis, they have no limit in replicative potential and, lastly, they have the ability to invade other tissues and organs (metastasise) (Hanahan & Weinberg, 2000).

1.3 Tumour Microenvironment and Cancer Stroma

Tumour stroma or the tumour microenvironment are terms used to describe the mixture of connective tissue and stromal cells including inflammatory cells, fibroblasts and vascular angiogenic cells that surrounds the tumour cells (Hale, Hayden, & Grabsch, 2013). Previously, it was thought that tumour stroma may have acted as a barrier to tumour growth and spread, or was a relatively inert tissue that was generated as a reaction to the presence of tumour cells and was present in and around tumours, particularly epithelial tumours such as lung, colon and breast carcinoma. However, in recent years it has become accepted that this microenvironment can favour cancer progression and metastatic spread due to cross-talk between tumour cells and stromal cells. This involves recruitment of fibroblasts and their transformation into cancer-associated fibroblasts (myofibroblasts), immune cell recruitment, matrix degradation and remodelling, and the expansion of vascular networks or angiogenesis (Junttila & de Sauvage, 2013). This stroma is known to play a role as a key regulator of tumour progression and has also been shown to have an association with the efficacy of drug delivery. Recent findings have shown that tumour stroma–derived biomarkers can also be used as biomarkers to determine responses to chemotherapy and in some cases may be useful as early biomarkers for detection of cancer (Hale et al., 2013).

The role of tumour stroma in carcinogenesis has been reviewed widely in several studies (Bhowmick, Neilson, & Moses, 2004; Quail & Joyce, 2013). Morphological and biochemical changes in stromal fibroblasts in response to cancer growth form a 'reactive stroma'. There are abundant expression and diverse types of extracellular matrix proteins present in reactive stromal cells associated with alteration of fibroblasts to myofibroblast phenotype, including expression of alpha (α) smooth muscle actin (α-SMA), splice variant forms of fibronectin, actin bundles, paladin and transforming growth factor-beta (TGF-β1) (Dakhova et al., 2009). There is also a direct interaction between reactive stroma and cancer cells in promoting cancer development, and the presence of reactive stroma around and within a tumour in many cases correlates with poor prognosis for the patient (Tsujino et al., 2007). It has also been suggested that tumour progression is controlled by the microenvironment of the tumour, not only by the cancer cells themselves (Bissell & Radisky, 2001).

There are two sequences of events occurring in tumour and stromal cells that involve a series of changes which lead to tumour progression. Firstly, transformation of epithelial cells to become mesenchymal or stromal cells in the stromata can result in paracrine secretion of growth factors and cytokines that regulate tumour cells (Polyak, Haviv, & Campbell, 2009). The tumour stroma also consists of several types of cells including blood vessels that provide sustained angiogenesis and thus nutrient supply to the tumour cells (Lohela, Bry, Tammela, & Alitalo, 2009), and secretion of cytokines and chemokines by immune cells is believed to both promote and, under some conditions, suppress tumour progression (Mueller-Huebenthal et al., 2009). Secondly, an activated form of fibroblasts, myofibroblasts or cancer-associated fibroblasts, are also a key controller between stromal and cancer cells in activating paracrine signalling pathways (Lorusso & Rüegg, 2008). These myofibroblasts

secrete more extracellular matrix than normal quiescent connective tissue fibroblasts, are contractile and can contribute both paracrine growth factors, but also contribute to matrix stiffening and enhanced mechanical signalling in the tumour microenvironment. The major role of stress fibres is to maintain a balance between contraction and adhesion. This balance results in stable actin bundles, which maintain a constant length under tension, especially in ventral stress fibres attached to the extracellular matrix on both sides (Tojkander, Gateva, & Lappalainen, 2012). The multistep process of tumour progression suggested in (Hanahan & Weinberg, 2000) has been revisited recently and the importance of the tumour microenvironment in regulating cancer growth and spread has been acknowledged (Hanahan & Weinberg, 2011).

In the tumourigenic niche, cancer cells may modify the behaviour of fibroblasts, macrophages and NK cells so that they acquire tumour-promoting properties. These cells have been termed cancer-associated fibroblasts (CAFs) and tumour-associated macrophages (TAMs). Inhibition of NK cell cytotoxicity is also frequently observed. TAMs are strongly associated with tumour progression, whereas CAFs may potentially initiate tumour development, modulate immune responses and promote cancer metastasis (Öhlund, Elyada, & Tuveson, 2014). **Figure 1.1** indicates changes acquired by stromal cells, such as CAFs and TAMs, that can influence tumour cells either directly or indirectly via release of several cytokines that lead to promotion of angiogenesis and thus provide a microenvironment that favours tumour growth.

Evolution of multiple stromal cells and extracellular matrix during tumour progression creates modifications in cellular organisation and phenotypic characteristics, thereby allowing cancer cells to dissociate and disseminate to the metastatic sites (Hanahan &

Weinberg, 2011). Cellular interactions between cancer cells and stromal cells in prostate cancer in a 3D co-culture system and in mice *in vivo* studies showed the importance of the stroma and tumour microenvironment in cancer progression (Josson, Matsuoka, Chung, Zhau, & Wang, 2010).

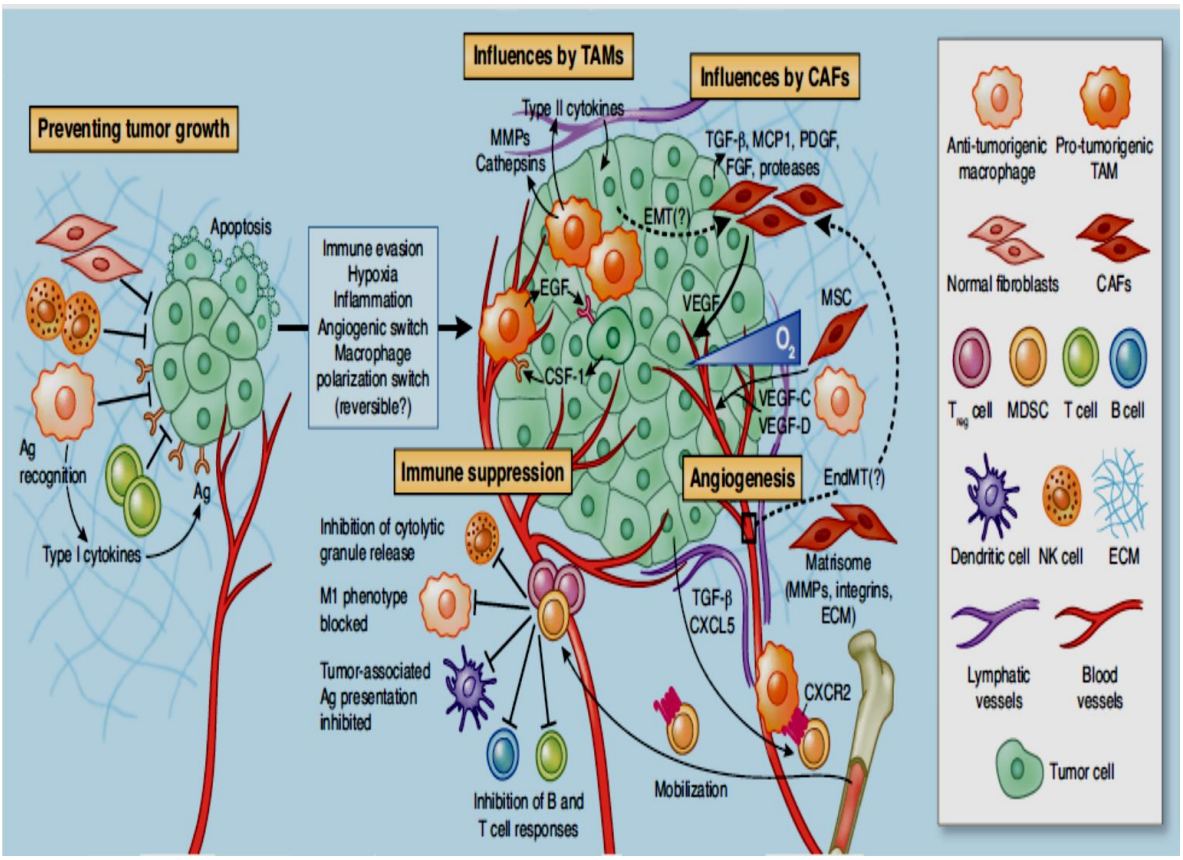


Figure 1.1: Tumour microenvironment support by various types of stromal cells. In the local microenvironment, fibroblasts, anti-tumourigenic macrophages and immune cells such as NK- and T- cells mediate apoptosis to suppress tumour growth. These stromal cells then acquire changes influenced by tumour cells to become CAFs and TAMs, either directly or induced by release of several cytokines, leading to promotion of angiogenesis and escape from immune surveillance, thus favouring tumour growth (Quail & Joyce, 2013).

1.4 Cancer Stroma and Wound Healing

Several cellular and molecular mechanisms are common to wound healing and in cancer microenvironments. It was postulated that “tumours are wounds that do not heal” (Dvorak, 1986) or tumours are wounds that over-healed or fail to resolve (Haddow, 1972) and this idea has been revisited more recently where the tumour microenvironment has been shown to resemble a site of chronic wound healing (Arwert, Hoste, & Watt, 2012; Barcellos-Hoff, Lyden, & Wang, 2013).

It has been suggested that the composition of tumour stroma is very similar to the chronic wound healing involving the formation of granulation tissue which contains differentiated myofibroblasts, inflammatory cells and growing blood vessels (Rybinski, Franco-Barraza, & Cukierman, 2014). However, in contrast to wound healing, the process in cancer tissue is not self-limiting, which results in uncontrolled cellular proliferation, leading to invasion and metastasis. It has been proposed previously that tumours trigger a latent wound-healing program; however, they do so in a prolonged manner, particularly in epithelial cell tumours and/or carcinomas (Schäfer & Werner, 2008).

Besides the similarities between tumors and wounded tissue, the process of wound healing also shows several common characteristics with chronic fibrosis, including similar cell types (inflammatory and activated fibroblasts), and soluble and matrix elements that drive wound healing (including regeneration) via distinct signalling pathways. It has also been shown that chronic fibrosis and tumor progression (Rybinski et al., 2014) have similar characteristics. In fact, prolonged healing processes resulting from chronic injury can result in chronic fibrosis and eventually tumourigenesis (Schäfer & Werner, 2008; Wynn & Ramalingam, 2012). Both tumours and their stroma and chronic fibrosis share several characteristics, as both tissues

are characterised by the presence of hyperproliferating cells (Y. Liu, 2011; Noble, Barkauskas, & Jiang, 2012), while stromal elements consist of abundant myofibroblasts responsible for altered and excessive ECM deposition (Cirri & Chiarugi, 2011). However, mutations and epigenetic changes have also been observed in cancer cells which are absent in fibrotic tissue. Chronic fibrosis has been shown to influence affected tissues leading to development of cancer (Bobba, Holly, Loy, & Perry, 2011; Duell et al., 2012) and also to enhanced tumour progression, which is commonly linked with poor prognosis (Gupta et al., 2011).

The normal wound-healing process involves three phases, which include clotting and inflammation, proliferation (growth of granulation tissue) and tissue remodelling. On injury, a platelet plug temporarily seals the wound and provides a rich source of growth factors. Subsequently, growth factors and cytokines produced by the inflammatory cells and stromal cells stimulate resident cells to form new tissue (Broughton 2nd, Janis, & Attinger, 2006; Lazarus et al., 1994). This phase involves cell migration and hyperproliferation of keratinocytes, which eventually leads the formation of a reconstituted epidermis. Wound contraction occurs due to differentiation of fibroblasts into contractile myofibroblasts, which involves transient expression of α-SMA (I. Darby, Skalli, & Gabbiani, 1990). The new tissue formed at the wound site is called granulation tissue. Finally, this granulation tissue will lay the foundation for mature scar tissue formation, which is characterised by the loss of cells and by matrix remodelling (Schäfer & Werner, 2008).

Velnar et al. 2009 expanded the phases to suggest five major underlying mechanisms of action throughout the healing process, which are inflammatory mediators and growth factors, interactions between cells and extracellular matrix in mediating cell proliferation,

migration and differentiation, re-epithelialisation and angiogenesis associated events, wound contraction and then remodelling (Velnar, Bailey, & Smrkolj, 2009). However, this process does not resolve in cancer, which then may result in tumourigenesis (Schäfer & Werner, 2008). Recently, a WHFC triad has been proposed, which is an interconnection between chronic wound healing, fibrosis and cancer (**Figure 1.2**) (Rybinski et al., 2014). Four major components that are common between wound repair, fibrosis and tumour development are described in this triad, specifically, cell proliferation, epithelial–mesenchymal transition (EMT), myofibroblast differentiation and ECM repair.

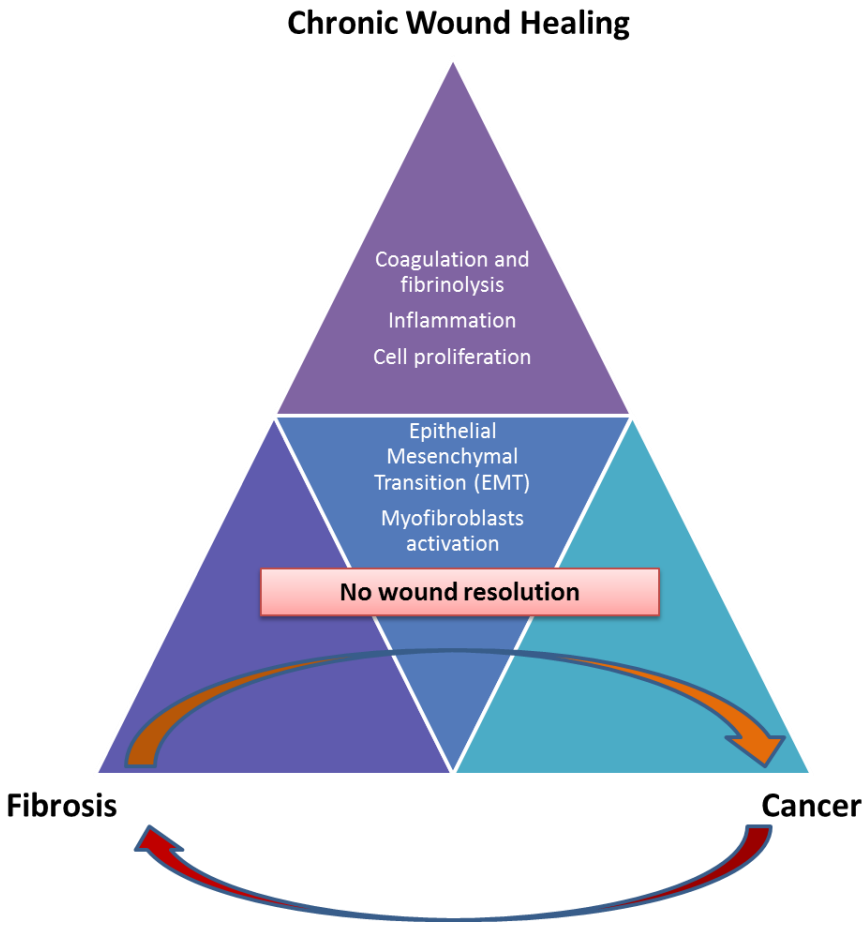


Figure 1.2: The wound healing, chronic fibrosis and cancer (WHFC) triad. Model illustrates the WHFC triad commonalities with wound healing. The triad indicates mechanisms in wound healing that facilitate a cycle between fibrosis and cancer without wound resolution. Adapted from (Rybinski et al., 2014).

1.4.1 Similar characteristics between wounds, chronic fibrosis and tumour stromata

Both wound and tumour stroma show parallels at the cellular and molecular levels (Schäfer & Werner, 2008). There are substantial links showing that the molecules involved in inflammation and wound healing are also involved in cancer (Frances Balkwill, Charles, & Mantovani, 2005). It has also been shown that patterns of gene-expression in stimulated, serum-treated fibroblasts strongly resemble the genes expressed in human carcinomas (Chang et al., 2004). Wound-healing factors have also been found to stimulate the proliferative capacity of tumour cells and increase tumour-forming capacity in primary head and neck cancer cells (Lindgren, Kjellén, Wennerberg, & Ekblad, 2016). In fact, tumour cells that express genes which are similar to those in early wounds commonly show a poor prognosis, favouring tumour progression and leading to metastasis and potentially to death (Chang et al., 2004; Gupta et al., 2011).

The most common hallmarks in wound healing and tumour progression is the high/ increased density of myofibroblastic cells within the tumour microenvironment and EMT that occurs in epithelial cells resulting in differentiation and transformation (Cirri & Chiarugi, 2011). Myofibroblast differentiation, also known as the activation of fibroblasts, is the process whereby cells acquire a myofibroblastic phenotype, which occurs in components of the stroma such as resident fibroblasts, pericytes, endothelial cells and recruited bone marrow–derived mesenchymal cells (Eyden, Banerjee, Shenjere, & Fisher, 2009).

As mentioned in the previous section, the large populations of myofibroblasts in the tumour stroma are referred to as CAFs and pro-fibrotic myofibroblasts are cells that can drive tumour progression and chronic fibrosis (Cirri & Chiarugi, 2011; Pietras & Östman, 2010). Like myofibroblasts, CAFs are believed to promote tumourigenesis and carcinogenesis

(Hanahan & Weinberg, 2011; Orimo & Weinberg, 2006). They are thought to come from the same origin as myofibroblasts and are a heterogenous population of cells that can alter the tumour microenvironment, leading to neoplastic changes (Öhlund et al., 2014). Oncogenic signalling by neoplastic cells could mimic the normal wound-healing process by releasing fibroblast-activating factors, resulting in recruitment and differentiation of the fibroblasts similar to that found in wounds. Injured epithelial cells and activated macrophages are also shown to secrete these factors (G. Yang et al., 2006).

In normal stroma, fibroblasts are in an inactive, quiescent state and functionally secrete few components of extracellular matrix such as fibronection and collagen, and only in small amounts (Brouty-Boye, 2005). In damaged tissues involving tissue remodelling processes such as wound healing and fibrosis, fibroblast cells become activated into myofibroblasts, which are characterised by the expression of α-SMA, and there are the presence of microfilament bundles (contractile stress fibres) and splice variant expression of fibronectin (Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). Myofibroblasts also secrete more extracellular matrix proteins that contribute to matrix stiffening and were originally described in the granulation tissues of healing wounds (Gabbiani, Ryan, & Majno, 1971). In cancer, these myofibroblasts which have escaped apoptosis, are believed to promote carcinogenesis (Eyden et al., 2009). It has been shown that physical matrix remodelling by myofibroblasts promotes the invasion of cells in squamous cell carcinoma (SCC) (Gaggioli et al., 2007). Increased staining for myofibroblasts in the tumour stroma also strongly correlates with poor prognosis in lung, pancreatic and colorectal tumours (Erkan et al., 2008; Maeshima et al., 2002; Tsujino et al., 2007).

An increase in ECM stiffness has also been shown in both fibrotic and tumour-associated ECM compared to normal stroma (Goetz et al., 2011). ECM changes, such as fibrin and fibronectin crosslinking as well as abundant presence of inflammatory cells in the stroma during cancer growth and progression, have been confirmed in numerous studies ((Coussens & Werb, 2002) Schafer et al. 2008; (Solinas, Germano, Mantovani, & Allavena, 2009). In fact, high deposition of interstitial fibronectin has been shown in liver and pancreatic carcinoma, with aggressive progression of tumours observed (Bachem et al., 2005). Extra domains A and B (EDA and EDB) of fibronectin has also been associated with increased tumour growth, angiogenesis and lymphangiogenesis (Sun et al., 2013; Xiang et al., 2012). In fact, EDA has also found to promote myofibroblast activation and fibrosis, with the presence of TGF-β and EDA fibronectin being necessary for fibroblast differentiation to the myofibroblast phenotype (Hinz, 2010).

On the other hand, ECM tension is increased during the wound-healing process due to collagen deposition, where collagen I is often found in fibrotic tissue, while abundant Collagen I and IV were also commonly observed in activated myofibroblasts (Hinz, 2010). The physiological and pathological roles of collagen in wound healing, fibrosis and tumour–stroma interaction have been recently reviewed (Gullberg, Kletsas, & Pihlajaniemi, 2016). Fibrillar collagens are produced by fibroblasts during wound healing, often altering the profile of collagen types present in the tissue (Schäfer & Werner, 2008) and this has been shown to contribute to tumour growth and metastasis in a breast cancer xenograft models (Xiong, Deng, Zhu, Rychahou, & Xu, 2014). Collagen deposition in the tumour tissue may also lay down tracks for metastatic tumour cells to move along by rearrangement of the collagen linkages (Provenzano, Eliceiri, & Keely, 2009).

The predominant inflammatory cells that play a role in wound healing and tumour biology are macrophages. Macrophages are associated with the development of tumour-support tissues by synthesising growth and angiogenic factors, remodelling extracellular matrix and removing apoptotic cells (Condeelis & Pollard, 2006). Therefore, during wound healing macrophages are crucial in mediating tissue repair (Coussens & Werb, 2002). Macrophages can be divided into two phenotypes depending on the cytokines and growth factors that they release and the surface markers that they express. M1 macrophages is predominantly present in inflamed non-cancerous tissue, while in tumours tumour-derived cytokines induce the polarisation of macrophages into an alternate M2 phenotype. It has been suggested that M2 macrophages induce angiogenesis and thus ultimately promote tumourigenesis, while also enhancing cancer cell motility and invasion (Allavena, Sica, Garlanda, & Mantovani, 2008; Condeelis & Pollard, 2006).

Angiogenesis is a fundamental process for both wounds and malignant tumours. It is defined as the development of blood vessels from an existing vasculature and it is necessary in tissue repair to restore blood supply to growing tissues (Crowther, Brown, Bishop, & Lewis, 2001). Generally, vascular networks develop via migration, proliferation and differentiation of vascular endothelial cells (Carmeliet, 2000). In wound healing, angiogenesis functions to restore vascular perfusion in damaged tissues and to assist recruitment of inflammatory cells such as neutrophils and macrophages to the site (M. E. Wong, Hollinger, & Pinero, 1996). In tumours, the ‘stressed’ microenvironment due to hypoxia and high levels of lactate, as a by-product of glycolysis, stimulates tumour cells to secrete pro-angiogenic cytokines and enzymes that establish a local vascular supply in the tumour, allowing the tumour to progress. Hyperpermeability of wound and tumour blood

vessels (capillaries) in the presence of histamine and vascular endothelial growth factor (which was originally called vascular permeability factor) also allows the continuous release of plasma proteins and the deposition of a fibrin and fibronectin matrix in the stroma. This deposition is an acute condition during normal wound repair; however, it is chronic during tumour growth (Schäfer & Werner, 2008).

All of these features are commonly present in wound healing, fibrotic tissue and cancer stroma; however, most of these changes are resolved once the wound-healing process is complete. For example, in wound granulation tissue, myofibroblasts are lost by apoptosis as the wound heals, and scar tissue becomes less cellular and has no transformed fibroblasts remaining (Desmoulière, Redard, Darby, & Gabbiani, 1995). Myofibroblasts and EMT cells were actively involved in regenerative healing wounds, fibrosis and tumour-associated desmoplasia; however, these cells are eventually lost in normal wound repair by the natural elimination process of the myofibroblastic cells via apoptosis (Rybinski et al., 2014).

The cellular and molecular parallels between chronic wound healing and tumour progression are summarised in **Table 1.1**.

Table 1.1: Similar cellular characteristics between chronic wound healing and tumour progression

Similarities between chronic wound healing and tumour progression
• Presence of inflammatory cells including macrophages, neutrophils, mast cells and lymphocytes • Formation of new vasculature (angiogenesis) • Differentiation of fibroblasts into myofibroblasts, creating cancer-associated fibroblasts (CAFs) in tumour stroma (which become reactive stroma) • Fibronectin and collagen deposition, which contribute to ECM stiffness • Transformation of epithelial cells via epithelial–mesenchymal transition (EMT) • Release of matrix metalloproteinases, MMPs, which mediate ECM remodelling

1.5 Local Microenvironment

In cancer, stroma can be classified as local or distant and, in the case of distant, as the metastatic microenvironment. Local microenvironments are heterogeneous, consisting of vascular endothelial cells, smooth muscle cells, inflammatory cells, and soluble and insoluble extracellular matrix proteins. These cells create a complex cancer stroma in the presence of macrophages, platelets, dendritic cells, T and B lymphocytes, and activated stroma and then support local cancer growth, progression and distant metastasis (Josson et al., 2010). Therefore, the microenvironment surrounding the tumour mass can be characterised as containing proliferating tumour cells with local components including stromal cells, newly developed blood vessels and inflammatory cell infiltrate associated with constant tissue remodelling. Soluble factors secreted by cancer and inflammatory cells such as hepatocyte growth factor (HGF) (Ren et al., 2005), epidermal growth factor (EGF) (Chiang, Chou, Hsu, Huang, & Shen, 2008; Mendelsohn & Baselga, 2000), PDGF (Donnem et al., 2008), VEGF (Ai, Lu, Huang, Chen, & Zhang, 2008) and TGF-β (Kitisin et al., 2007; Massagué,

2008), induce an activated form of stromal fibroblast or myofibroblast via multiple signalling pathways and these have a potent growth-promoting effect on various types of cancer, leading to tumour angiogenesis, invasion and metastasis.

1.6 Metastatic Microenvironment

Cancer cells have the ability to invade and metastasise to distant sites (Hanahan & Weinberg, 2011). Soluble and matrix factors secreted by both cancer cells and cells in tumour microenvironment may alter cancer cell adhesion, proliferation and survival (Folkman, 2002) and assist in metastasis to other organs such as the lung, liver, brain and kidney. There are important events that occur during metastasis, which include dissociation from the primary tumour, extracellular matrix remodelling by enzymes that can degrade matrix proteins, cell migration and proliferation and angiogenesis within the tumour stroma (Wells, 2006).

Circulating tumour cells (CTCs) undergo several changes to become able to colonise distant organs, which include infiltration of distant tissue, the ability to escape from immune defence, adaptation to supportive niches, survival of tumour-initiating seeding and eventually breaking out to overtake the host tissue (Massagué & Obenauf, 2016). As mentioned previously, an early stage of the metastatic cascade involves the invasion and migration of cancer cells into tissues and the circulatory system, which is mainly controlled by cytoskeletal rearrangements of cancer cells (Hall, 2009), changes to adhesive interactions between cells and the secretion of extracellular matrix–degrading metalloproteinases (MMPs) allowing destruction of collagen fibres of the ECM (Quail & Joyce, 2013).

Cancer cells that survive after the infiltrating step are known as disseminated tumour cells (DTCs) and later become established in supportive niches (Braun et al., 2005). DTCs are

believed to survive in the circulatory system as single cells or clusters of cells coated with platelets which then enter a latent state as single cells or micrometastases (Massagué & Obenauf, 2016). Finally, cancer cells in supportive niches reinitiate outgrowth and replace the local tissue microenvironment.

Therapeutic strategies have been developed to target different cells in the tumour microenvironment, including targeting angiogenesis, inflammation, CAFs and several growth factors present, including VEGF, PDGF and HGF, as well as targeting other non-cellular components such as the ECM (Joyce, 2005; Sounni & Noel, 2013). The targeting of tumour stromata may be crucial since targeting cancer cells alone may be less effective because of their capacity to adapt to therapy and due to increasing understanding of the importance of the microenvironment (Pietras & Östman, 2010; Quail & Joyce, 2013). Additionally, tumour stroma–derived biomarkers are now known to be useful in determining the tumour response to chemotherapy, as well as having potential impacts on drug delivery and efficacy (Hale et al., 2013).

Figure 1.3 shows the therapeutic strategies that are available to target different types of cells in the tumour microenvironment which actively contribute to cancer development and progression.

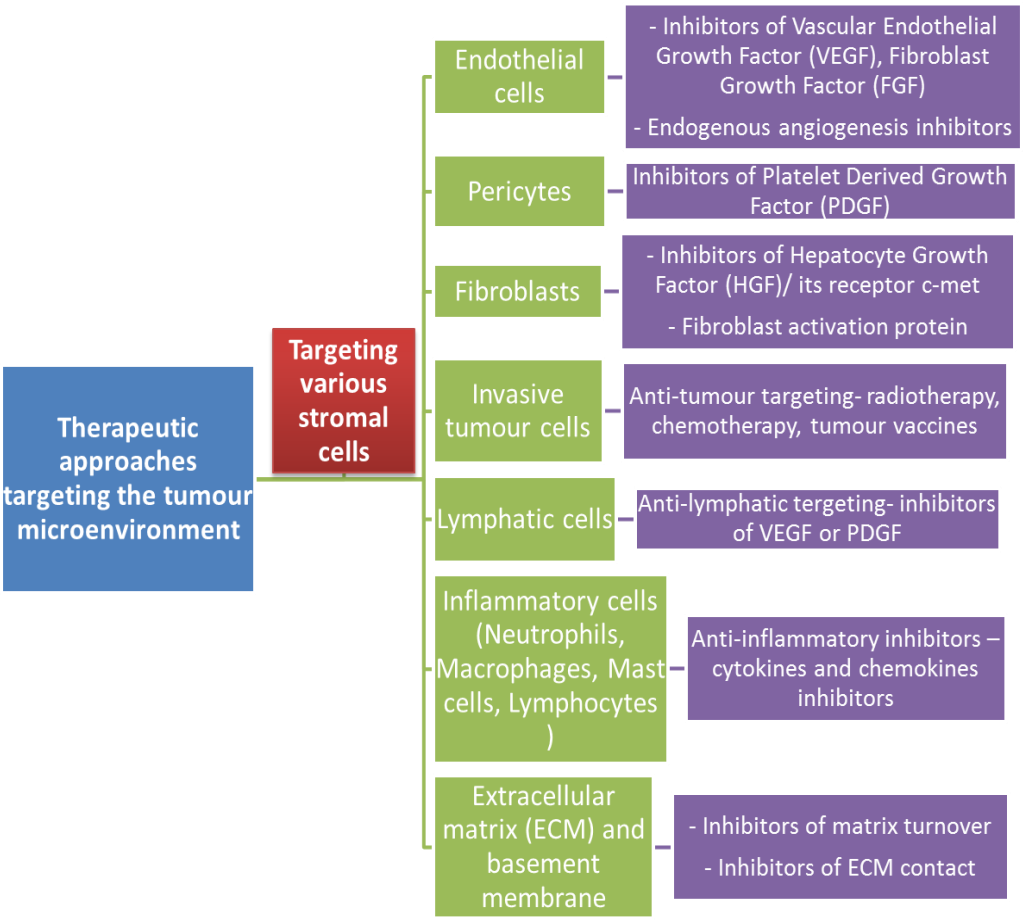


Figure 1.3: Therapeutic approaches targeting the tumour microenvironment. There are several possible therapeutic approaches to target stromal cells including inflammatory, lymphatic and vascular angiogenic cells in the tumour microenvironment. Adapted from (Joyce, 2005).

1.6.1 Cancer Associated Fibroblasts (CAFs) in metastatic microenvironments

Fibroblasts located within the extracellular matrix (ECM) are involved in tissue remodelling and repair, and the ECM itself acts as a reservoir of important cytokines and growth factors that can induce cell differentiation and migration (Öhlund et al., 2014). As described earlier, on injury myofibroblasts are found in various inflamed and fibrotic tissues, and in reactive tumour stromata (Orimo & Weinberg, 2007). The large population of myofibroblasts in the tumor stroma, CAFs and fibrotic myofibroblasts, have the capacity to drive tumor

progression and chronic fibrosis (Rybinski et al., 2014). They are also found in pseudotumours and close to neoplastic cells within the tumour (Eyden et al., 2009). CAFs are defined as fibroblastic, non-epithelial, non-inflammatory, non-vascular and non-neoplastic cells within a tumour (Kalluri & Zeisberg, 2006; Orimo & Weinberg, 2007). They may arise through EMT, which can potentially differentiate them from neoplastic cells. However, the heterogeneous populations and different origins of CAFs make it difficult to define a specific molecular profile of CAFs.

CAFs have been reported to augment cancer development by secreting growth factors and cytokines which are crucial in the tumour microenvironment. Studies have demonstrated that levels of the cytokines CCL7 and CXCL12 are increased in invasive oral squamous cell and breast carcinomas, respectively, expressed by stromal fibroblasts (Jung et al., 2010; Orimo et al., 2005). Importantly, CAFs have the ability to remodel the ECM such that changes in ECM constituents can influence the stiffness of the tissue, which can also favour tumour growth by increasing mechanical signalling (Erler & Weaver, 2009). In fact, CAFs can also mediate matrix degradation by the production of matrix metalloproteinases (MMPs) and contribute to tumour vascularisation (Quail & Joyce, 2013). They can thus facilitate tumour invasion and the escape of tumour cells and spread to secondary sites (Zigrino et al., 2009). Activation of myofibroblasts to cancer-associated (myo) fibroblasts within the stroma is commonly correlated with poor prognosis and cancer metastasis (Castelino & Varga, 2014; Pickup, Mouw, & Weaver, 2014). Recently, more evidence has shown that myofibroblasts and CAFs play a role in many types of cancer and targeting CAFs would thus be beneficial in regulating fibrosis, scarring and cancer (for review see (I. A. Darby, Zakuan, Billet, & Desmoulière, 2016)).

Myofibroblasts in the tumour stroma can influence tumour progression and establishment of the metastatic niche through the ECM molecules that they secrete (Barcellos-Hoff et al., 2013). It has been shown that myofibroblasts secrete ECM molecules which can mechanically influence signalling and cell adhesion, such as the matricellular protein periostin, which can affect tumour growth and establishment of a metastatic niche (I. A. Darby & Hewitson, 2016). ECM rigidity and stiffness can also regulate the action and the activation of TGF-β signaling including TGF-β-induced apoptosis and TGF-β-induced EMT (Leight, Wozniak, Chen, Lynch, & Chen, 2012). In addition, matrix stiffness itself has been shown to promote breast cancer progression via mechanoreciprocal induction of Rho-dependent cell contractility (Levental et al., 2009).

1.6.2 Epithelial–mesenchymal transition (EMT) in metastatic microenvironment

EMT and its converse form, MET (mesenchymal–epithelial transition), are essential phases in many physiological processes which have been shown to contribute to scar formation and fibrosis in response to injury (Stone et al., 2016). At tumour sites, the reverse process of MET, which involves the conversion of mesenchymal cells into epithelial derivatives, also occurs (J. M. Lee, Dedhar, Kalluri, & Thompson, 2006). As EMT progresses, epithelial cells acquire mesenchymal markers and capabilities to evolve towards a mesenchymal phenotype, which involves the regulation of several proteins including cadherins (N-cadherin, E-cadherin), α-SMA, vimentin, integrins, fibronectin and MMPs (Huang, Guilford, & Thiery, 2012).

EMT is crucial in tumour initiation and subsequent invasion of tumour cells into surrounding stroma which is a vital event for cancer metastasis (Grünert, Jechlinger, & Beug, 2003). This transition involves distinct morphological change whereby the tumour cells switch from a

well-differentiated epithelial phenotype to a more invasive mesenchymal phenotype. Excessive cell proliferation and angiogenesis are hallmarks of primary epithelial tumour growth (Hanahan & Weinberg, 2000) in which EMT-derived migratory cancer cells have been shown to establish at distant sites, thus facilitating metastatic dissemination (Kalluri & Zeisberg, 2006). EMT has been shown to facilitate metastasis by promoting ECM proteolysis and enhancing malignant cell motility, thus conferring the ability to invade (Tiwari, Gheldof, Tatari, & Christofori, 2012). In addition, EMT has been found to mediate the metastatic stage by colonisation and subsequent MET transition (Kalluri & Weinberg, 2009). Furthermore, in the EMT process cancer cells change their adhesive, migratory and invasive properties, which involves the reorganisation of the actin cytoskeleton and formation of membrane protrusions required for invasive cells (Yilmaz & Christofori, 2009). It has been also shown that increased EMT in cancer commonly correlates with late stages of tumour progression (Thiery, 2003).

Recently, increased matrix stiffness in human breast tumours has been shown to induce EMT and consequently promote tumour invasion and metastasis (Wei et al., 2015). In addition, morphological features of EMT have been shown to resemble many cellular changes in matrix stiffness that lead to tumour cell dissemination (J. Yang & Weinberg, 2008).

Carcinoma cells can acquire a mesenchymal phenotype and express mesenchymal markers, as demonstrated in both *in vivo* and *in vitro* studies (J. Yang & Weinberg, 2008). EMT-derived migratory cancer cells typically establish secondary colonies via a colonisation process at distant sites that later resemble the primary tumour as the cells revert to their carcinoma phenotype (Massagué & Obenauf, 2016). EMT promotes cell entry into tissue

during extravasation and acts as a facilitator of metastatic dissemination, as metastasising cancer cells are found to shed their mesenchymal phenotype during secondary tumour formation via MET and enter the metastatic microenvironment after extravasation (Kalluri & Weinberg, 2009).

These considerations indicate that induction of an EMT is likely to be a centrally important mechanism for the progression of carcinomas to a metastatic stage and implicate MET during the subsequent colonisation process, as described in **Figure 1.4**.

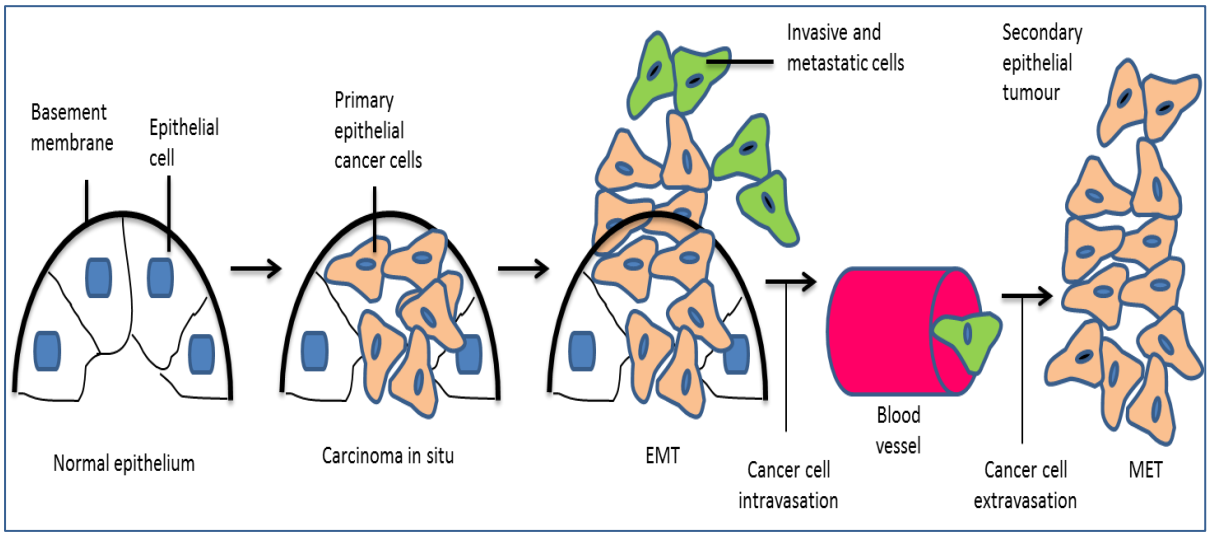


Figure 1.4: EMT in cancer progression. Contribution of EMT to cancer progression involves several stages from normal epithelium to invasive carcinoma. Epithelial cells lose their polarity and detach from the basement membrane in the invasive carcinoma stage. The composition of the basement membrane also changes, altering cell–ECM interactions and activating signalling networks. EMT and an angiogenic switch facilitate the malignant phase of tumour growth, enabling cancer cells to enter the circulation and exit the bloodstream, then undergo MET and thus revert to an epithelial phenotype. Adapted from (Kalluri & Weinberg, 2009).

1.7 Cancer and Inflammation

The relationship between inflammation and cancer has been extensively reviewed. Inflammation is well known as a critical component in the initiation and progression of cancer. Many types of cancer are known to arise from sites of infection (Blaser, Chyou, & Nomura, 1995; Shacter & Weitzman, 2002) and sites of chronic inflammation (Coussens & Werb, 2002). For example, colon cancer commonly occurs in individuals with inflammatory bowel diseases such as Crohn's disease or chronic ulcerative colitis (Coussens & Werb, 2002).

Improving understanding of physiological and pathological changes in wound healing and infection is important in developing understanding of the role of inflammation in relation to cancer development. Various chemical signals are initiated to regulate the healing process on tissue injury, including activation and recruitment of leukocytes such as neutrophils, eosinophils and monocytes to the sites of injury (Velnar et al., 2009). Monocytes which then differentiate into macrophages are the major source of cytokines and growth factors (Osuský, Malik, & Ryan, 1997). ECM remodelling, mediated by secreted proteins such as transforming growth factor α and β1 (TGF-α and TGF-β1), platelet-derived growth factor (PDGF) and platelet-derived angiogenesis factor, causes proliferation of fibroblasts and endothelial cells, which attempt to reconstruct the normal microenvironment (Buckley et al., 2001). However, persistent secretion of cytokines and chemokines such as tumour necrosis factor-α (TNF-α) and TGF-β1 at inflammatory sites may develop into chronic conditions whereby dysregulation initiates neoplastic progression (Coussens & Werb, 2002). In chronic inflammatory diseases, TNF-α was originally shown to have toxic effects at higher levels; however, animal studies clearly showed that TNF-α can exert tumour-promoting

effects (Moore et al., 1999). In fact, activation of a TNF-α signalling pathway mediated by malignant and inflammatory cells also contributes to promotion of cell survival, angiogenesis and invasion (Charles et al., 2009).

The presence of inflammatory cells and inflammatory mediators is a prominent hallmark in tumour tissues, and numerous studies have demonstrated their important roles in cancer development and progression (Candido & Hagemann, 2013; de Visser, Eichten, & Coussens, 2006; Philip, Rowley, & Schreiber, 2004). There are obvious similar characteristics in tissue remodelling and angiogenesis between tumour tissues, chronic inflammatory responses and tissue repair (Mantovani, Allavena, Sica, & Balkwill, 2008). 'Smouldering' inflammation (Frances Balkwill et al., 2005) can also be seen in the tumour without a firm causal relationship to inflammation, for example in breast tumours. In addition, epidemiological studies have revealed that chronic inflammation correlates to cancer development, with colon cancer being one of the typical types of cancer showing a relationship to chronic inflammation as mentioned above (de Visser et al., 2006).

The link between inflammation and cancer can be ascribed to two pathways (**Figure 1.5**), which are, firstly, an extrinsic pathway caused by inflammation that may initiate or promote cancer-promoting conditions and, secondly, an intrinsic pathway activated by genetic events that cause inflammation and neoplasia such as activation of oncogenes (Figure 1.2) (Candido & Hagemann, 2013; Mantovani et al., 2008). Activation of oncogenes such as *myc, ras* and *ret* or inactivation of tumour suppressors such as *p53* and *pVHL* induce initiated cells to produce inflammatory cytokines (Borrello et al., 2005; Soucek et al., 2007).

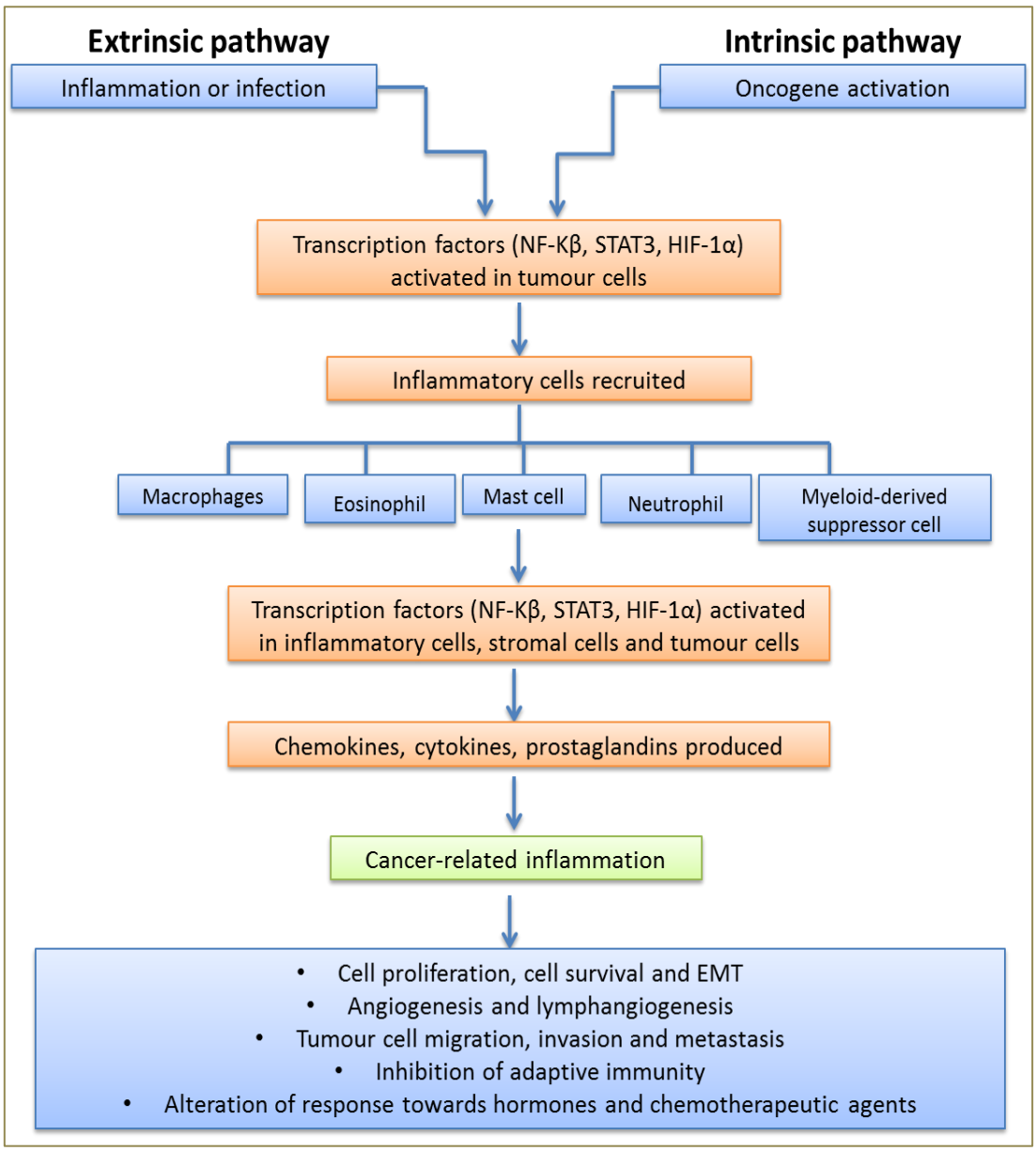


Figure 1.5: Pathways that connect inflammation and cancer. Inflammation and cancer are connected by both intrinsic and extrinsic pathways. The intrinsic pathway involves genetic mutations that cause neoplasia, which in turn activates production of inflammatory mediators, generating an inflammatory microenvironment in tumours. In contrast, recruitment of inflammatory cells at the primary site occurs via the extrinsic pathway. Both of these pathways converge, thus activating various transcription factors and producing inflammatory mediators including cytokines and chemokines. Adapted from (Mantovani et al., 2008).

Inflammatory cells can potentially direct malignant transformation processes as they are potent producers of reactive oxygen species (ROS) and reactive nitrogen species, which can directly damage DNA and also alter proteins that regulate DNA repair, act in cell-cycle checkpoint controls or are involved in regulation of apoptosis (Allavena et al., 2008).

Inflammatory mediators such as cytokines, chemokines, arachidonic acid, and free radicals and cellular effectors are important components of the local microenvironment of tumours (Candido and Hagemann 2013). Release of these potent soluble mediators during immune responses has been found to regulate cell migration, proliferation, angiogenesis, remodelling of ECM and effects on metabolism (Tlsty & Coussens, 2006). Complex interactions between the cell types and these mediators in this microenvironment favour tumour growth and angiogenesis, leading to cancer progression and metastasis (Fran Balkwill, 2005).

1.7.1 Macrophage functions in cancer

Inflammatory responses in the tumour microenvironment involve various types of immune cells, and macrophages are key players (Libby, Ridker, & Maseri, 2002). Macrophages are important in the innate and adaptive immune responses towards pathogens and are also critical mediators in the inflammatory response. Macrophages derived from the bone marrow enter the blood circulation as immature monocytes before undergoing differentiation into resident macrophages in the tissue (Siveen & Kuttan, 2009). Macrophages execute a multitude of functions which are essential for tissue remodelling (D. O. Adams & Hamilton, 1984), inflammation and immunity, including phagocytosis, cytotoxicity (Nathan & Hibbs Jr, 1991) and the secretion of various cytokines, growth factors, lysozyme and complement components (Ross & Auger, 2002).

In order to respond to injury or infection, monocytes and endothelial cells are upregulated or activated by E- and P-selectin to induce production of cytokines and chemokines such as IL- 1β, TNF-α and interferon gamma (IFN-γ) which trigger recruitment of other immune cells (Fran Balkwill, 2004; Fox, Turner, Gatter, & Harris, 1995). Macrophages are involved in the regulation of angiogenesis in both normal and diseased tissues (Leek, Harris, & Lewis, 1994). This observation is based on early studies indicating the presence of wound macrophages in wound fluid associated with angiogenic activity (Greenburg & Hunt, 1978). The importance of macrophages in wound angiogenesis has been shown in mouse models comparing aged and young mice in which macrophages in aged mice produced less VEGF than in younger mice, resulting in reduced angiogenesis and delayed wound healing in older mice (Crowther et al., 2001). Unless activated, macrophages are not tumouricidal for tumour cells, while activation can stimulate anti-tumour functions via direct and indirect cytotoxicity towards tumour cells (Siveen & Kuttan, 2009).

Macrophages can, however, contribute to tumour growth and progression. In some cancers macrophages represent up to 50% of the tumour mass (Solinas et al., 2009). It has also been shown that macrophages may comprise up to 80% of the tumour mass in breast carcinoma. In these cases macrophages may actually behave as a tumour promoter. Macrophages serve as a source for many pro-angiogenic factors, including vascular endothelial factor (VEGF) as mentioned above, tumour necrosis factor-alpha (TNF-alpha), granulocyte macrophage colony-stimulating factor (GM-CSF), Interleukin 1 (IL-1) and Interleukin 6 (IL-6) (Lin et al., 2006), contributing further to tumour growth. Macrophages infiltrate into many tumour types. The quantity of infiltrated macrophages then correlates with poor prognosis in many types of cancers such as breast, cervix, bladder and brain cancers (Bingle, Brown, & Lewis,

2002). The association of macrophages in human and murine tumours called TAMs has been further reviewed in many cancers where their numbers correlate significantly with tumour angiogenesis, such as in pulmonary adenocarcinoma (Takanami, Takeuchi, & Kodaira, 1999), endometrial carcinoma (Salvesen & Akslen, 1999) and malignant melanoma (Torisu et al., 2000). Recently, macrophage infiltration during wound healing was found to promote skin carcinogenesis by stimulation of epidermal cell proliferation (Weber et al., 2016).

1.7.1.1 Macrophage polarisation

Macrophages have a pleotropic biological function and show different phenotypes based on the specific environmental stimuli that they are exposed to (Frances Balkwill et al., 2005). **Figure 1.6** shows a schematic diagram of macrophage polarisation. The M1 phenotype or M1 macrophages are classical activated macrophages that have the ability to kill microorganisms and tumour cells and to produce high levels of immunostimulatory cytokines. In contrast, in the presence of tumour-derived anti-inflammatory molecules such as IL-4, IL-10, IL-13 and transforming growth factor β1, macrophages show an M2 phenotype. These cells are adapted to cell scavenging, promoting angiogenesis and repairing and remodelling damaged tissues (Mantovani, Sozzani, Locati, Allavena, & Sica, 2002). In addition, angiogenesis and ECM breakdown stimulated by these cells may promote tumourigenesis (Allavena et al., 2008). Production of MMPs by macrophages at the invading front of a tumour may also enhance cancer-cell motility and invasion (Condeelis & Pollard, 2006).

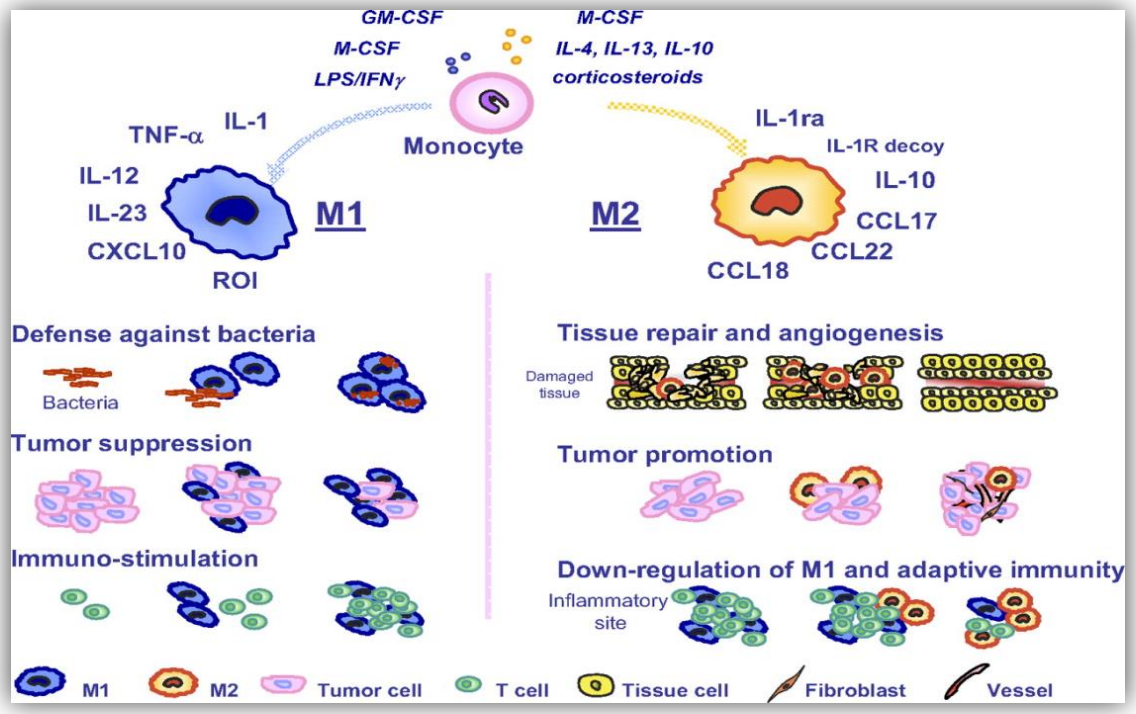


Figure 1.6: Polarisation of macrophage function. Macrophages can be polarised based on their environmental stimuli. They are primarily of the M1 phenotype in normal inflamed tissue. Increased levels of reactive oxygen species (ROS) and inflammatory cytokines provide potent antimicrobial, immunostimulatory and tumour cytotoxic effects on the cells. In tumours, tumour-derived cytokines induce differentiation of macrophages into the M2 phenotype, which favours tumour growth (Solinas et al., 2009).

1.7.1.2 Tumour-associated macrophages (TAM)

Macrophages are known to exhibit either pro- or anti-inflammatory responses depending on the stage of disease and the type of signals they are exposed to in the microenvironment. In the tumour microenvironment TAMs, which resemble M2 macrophages (Sica, Schioppa, Mantovani, & Allavena, 2006), have diverse functions and have the ability to affect neoplastic tissues, which includes promotion of angiogenesis and vascularisation, stroma formation and ECM dissolution, and enhancement or in some cases inhibition of tumour cell growth (Mantovani, Bottazzi, Colotta, Sozzani, & Ruco, 1992).

Both primary lesions and metastases of solid tumours are infiltrated by enormous numbers of tumour-associated leukocytes. The heterogeneous populations of these cells consist of a variable subset of T-cells (helper, suppressor and cytotoxic), B-cells, natural killer (NK) cells and macrophages (Bingle et al., 2002).

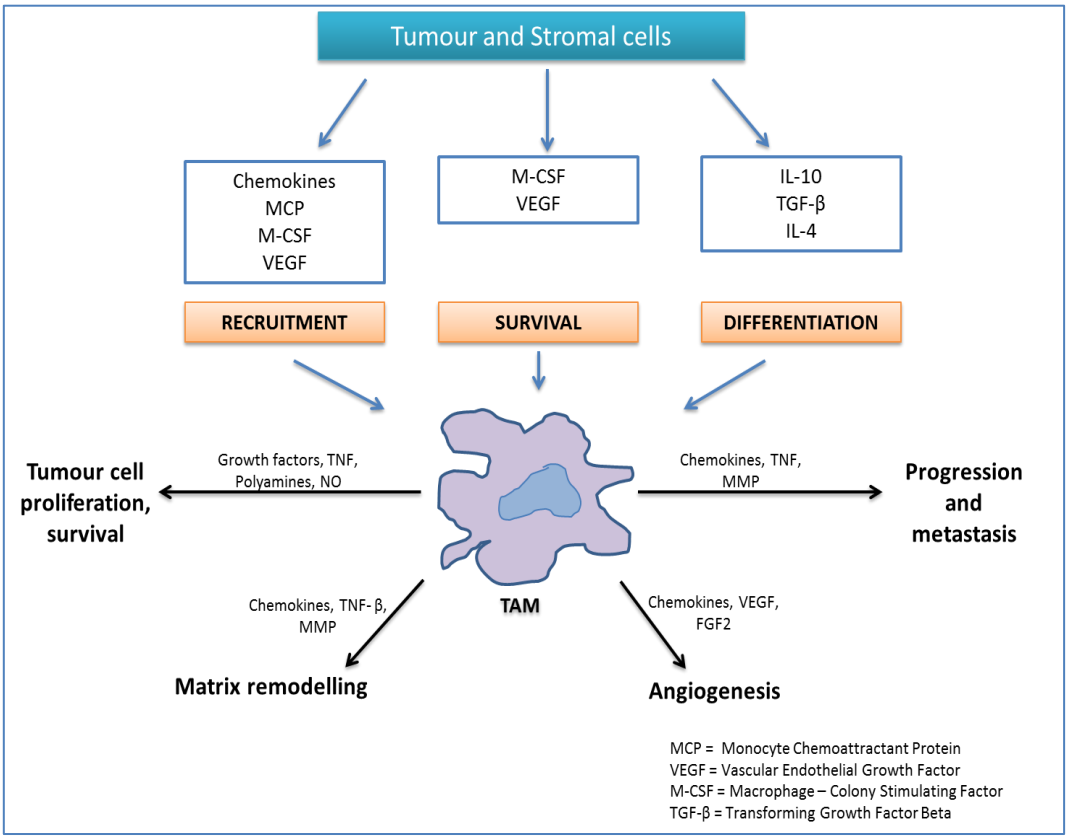


Figure 1.7: Tumour-associated macrophages in cancer progression. TAMs play a role in cancer progression, where they influence several important cellular functions including proliferation, matrix remodelling and angiogenesis (Siveen & Kuttan, 2009).

TAMs have been associated with several stages of tumour progression, as well as tumour proliferation, angiogenesis and tissue remodelling, metastasis and immune suppression, as shown in **Figure 1.7**. TAMs also promote cancer metastasis via all of these mechanisms, including enhancement of cell proliferation, migration and invasion. Several studies have demonstrated that TAMs induced tumour cell invasion via a paracrine signalling loop with the involvement of tumour-derived colony stimulating factor 1 (CSF-1) and macrophage-

derived epidermal growth factor (EGF) in glioma and breast cancers (Coniglio et al., 2012; Goswami et al., 2005). Interactions within the tumour microenvironment enable ECM breakdown and promote cell motility (Condeelis & Pollard, 2006). Modulation of the microenvironment by TAMs has been shown to increase neovascularisation and stroma formation (Crowther et al., 2001).

1.8 Cytokines in Inflammation and Cancer

Cytokines are major mediators of communication between cells in the inflammatory tumour microenvironment (Fran Balkwill, 2004). Neoplastic cells commonly over-express pro-inflammatory cytokines, such as migratory inhibitory factor (MIF) (Verjans et al., 2009; Wilson et al., 2005), TNF-α (Charles et al., 2009; Maniati et al., 2011), IL-6 (Lesina et al., 2011), IL-17 (Charles et al., 2009; Numasaki et al., 2003; L. Wang et al., 2009), IL-12, IL-23, IL-10 and TGF-β (Candido & Hagemann, 2013), and these have been shown in various types of cancers in both experimental animal models and human cancers. Furthermore, growth factors released by tumour cells such as granulocyte macrophage colony-stimulating factor (GM-CSF), platelet-derived growth factor (PDGF), VEGF, TGF-α and other monokines lead to the activation of macrophages, which then have the capability to influence angiogenic processes (Ueno et al., 2000).

These anti-inflammatory cytokines provide a link between inflammation and cancer. Inflammatory cytokines which are produced by stromal and cancer cells may functionally promote or potentially suppress tumours (Mueller-Huebenthal et al., 2009). It has been clearly shown that both TNF-α and IL-17 can act as tumour suppressors at the beginning of tumour development, while later they are seen to promote tumour growth (Mumm & Oft, 2008; Murugaiyan & Saha, 2009). Apart from angiogenesis to help maintain cell survival,

TNF-α has been shown to suppress the function of T-cells and associated macrophages, which then impairs immune surveillance (Kiessling et al., 1999). Stimulation of genotoxic ROS and RNS by TNF-α also may help to initiate tumour growth (Charles et al., 2009).

In addition, both IL-6 and IL-10 are highly expressed in inflammatory bowel disease (IBD) and colitis-associated colon cancer (CAC) (Mudter et al., 2008), and in diffuse large B-cell lymphoma (Lech-Maranda et al., 2006), respectively. IL-6 is commonly shown to target important genes which regulate cell cycle progression and suppress apoptosis; therefore, it has been suggested as promoting cell survival and tumour development (Bommert, Bargou, & Stühmer, 2006).

1.9 Tumour Growth and Proliferation

Tumour growth is determined by three main factors, which are regulation of the cell cycle, the amount of cell proliferation and the amount of cell loss (Quinn & Wright, 1990). The cell cycle consists of four phases: Gap1 (G1), S, Gap2 (G2) and mitosis (Figure 1), where DNA synthesis takes place in the S phase, followed by mitosis. The G1 and G2 phases allow cell preparation before entering DNA synthesis and mitosis, respectively. However, most of the cells in normal tissue are in a quiescent state (G0), having a range of normal metabolic activities outside the cell cycle (Tachibana, Gonzalez, & Coleman, 2005).

Normal tissues have the ability to control the cell cycle process by regulating growth signals, while in tumour cells, acquired damage to cell cycle regulatory genes such as *p53* and retinoblastoma protein (*Rb)* (Staunton & Gaffney, 1998), and deregulation of these signals, can result in uncontrolled cell growth and inappropriate cell survival. Since tumour cells are heterogeneous and diverse, they can acquire the ability to proliferate beyond the growth

restrictions in normal tissue, which then results in an uncontrolled growth rate in tumours (Evan & Vousden, 2001).

Numerous studies have shown that higher tumour proliferation rates, as assessed by various established techniques, commonly correlate with higher risk of metastasis and increased number of malignancies, as seen for example in breast and lung cancer patients (Kawai et al., 1994; Rudas et al., 1994). Inhibiting cell proliferation could delay tumourigenesis and limit cancer spread, currently widely applied using various drugs for treatment and intervention (Mabuchi et al., 2007; So, Guthrie, Chambers, Moussa, & Carroll, 1996; Tsuchiya et al., 2011).

Proliferating cell nuclear antigen (PCNA) is one of the proliferation markers that has a role in normal DNA replication and repair, chromatin remodelling and cell cycle regulation (Stoimenov & Helleday, 2009; Strzalka & Ziemienowicz, 2011). Based on that fact, PCNA has been found to be an effective marker for cell proliferation and has been very useful to determine tumour status, including histological staging, prognosis and cancer relapse (Mayer et al., 1993; S.-C. Wang, 2014). A study showed that serosal invasion and metastasis to lymph nodes were correlated with a high PCNA labelling index in gastric adenocarcinoma (Elpek, Gelen, Aksoy, Karpuzoğlu, & Keles, 2000). Furthermore, high levels of expression of PCNA have also been shown in more aggressive oral tumours (Tsuji et al., 1995). Additionally, survival time was found to be inversely related to the percentage of PCNA-positive cells in primary tumours and lymph node metastases (Mayer et al., 1993).

Even though PCNA is widely used as a tumour proliferation marker, researchers are still concerned with sometimes conflicting results in the correlation between PCNA expression and cancer prognosis. Evidence shows that in several types of cancer, PCNA was highly

expressed in tumours which are associated with poor survival rates (Ebina, Steinberg, Mulshine, & Linnoila, 1994; L.-F. Wang et al., 2006). However, there were variations in the distribution and intensity of PCNA staining in cancer tissues (Ebina et al., 1994). In addition, a few studies have claimed that expression of PCNA and Ki-67 were not correlated with cancer prognosis and survival rates (Bukholm, Bukholm, Holm, & Nesland, 2003; Grossi et al., 2003; Haerslev, Jacobsen, & Zedeler, 1996), even though expression may be associated with larger tumour size and poor tumour cell differentiation (Haerslev et al., 1996).

1.10 Cell Motility and Adhesion

Cell motility is one of the characteristics of invasive tumours which facilitate migration into adjacent tissues or transmigrate through basement membranes and ECMs. Invasive tumour cells have frequently been demonstrated to show dysregulated cell motility in response to extracellular signals from growth factors and cytokines. Previous findings suggest that this growth factor receptor–mediated motility is one of the most common aberrations in tumour cells leading to increased invasiveness and may represent an altered cellular behaviour during migration (Wells, 1999).

After cellular differentiation, most cells are non-motile (with the exception of wounded keratinocytes and sprouting capillaries in wounded tissue) due to attachments, via membrane proteins such as integrins, to the ECM scaffold and these attachments limit locomotion (Peter Friedl & Wolf, 2003). In contrast, during invasion reorganisation of the cytoskeleton occurs via actin protrusions, producing lamellipodia and invadopodia that help the cell to invade across basement membranes. Additionally, EMT may occur and this has been widely described in cancer cell dissemination (Grünert et al., 2003). The changes of the cytoskeleton result in changes of the cell shape that equip the cell for migration and

extravasation (Wells, 2006). This may be accompanied by a loss of lateral attachments to other cells, which is a hallmark of EMT and is characterised by a loss of cadherin expression.

Cell movement involves the extension of lamellipodia at the leading edge of the migrating cells which allow protrusion towards the motogenic signal, attachment, contraction and detachment at the rear of the cells (Ridley et al., 2003). In addition there are filopodia, described as membrane projections that consist of gathered actin filaments to form tight bundles and have the ability to respond to cell migration signals (Mattila & Lappalainen, 2008). The biophysical processes involved in cytoskeletal reorganisation are described in **Figure 1.8**. Limitation of cell motility and tumour invasion could be achieved by inhibiting one of these processes (Kassis, Lauffenburger, Turner, & Wells, 2001). Chemokinesis or chemotaxis towards released growth factors and cytokines can influence cell movement, and these are directly related to tumour progression (Wells, 2006). Targeting tumour cell motility will be crucial for future therapeutic approaches to enhance effectiveness in limiting tumour progression and in preventing metastasis to secondary sites.

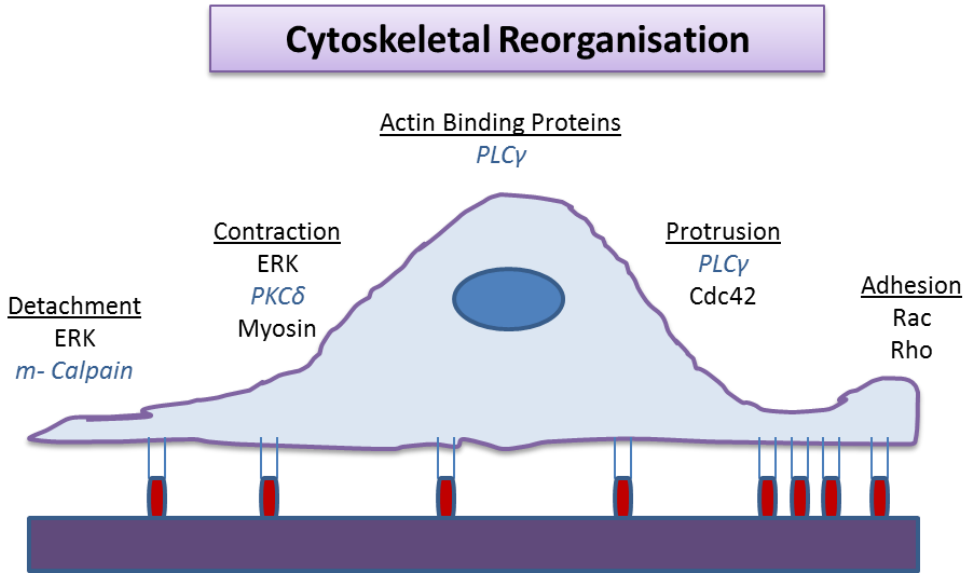


Figure 1.8: Cytoskeleton reorganisation in migration. The process involves protrusion of lamellopodia, anterior adhesion, cellular contraction and posterior detachment of the cell. Figure indicates the integrating molecules for each process; those involved in adhesion and growth factor–driven motility (**black**) and those activated during the enhanced movement due to growth factor receptor activation (**blue italics**). Abbreviations: ERK, extracellular signal-regulated kinase; PKCδ, protein kinase Cd; PLCγ, phospholipase Cγ. Adapted from (Wells, Grahovac, Wheeler, Ma, & Lauffenburger, 2013).

Adhesiveness of the cells may also influence the invasion process. Cell adhesion is very closely related to important cellular processes such as cell proliferation, migration and motility, differentiation and death (Thiery, 2003). Cell adhesion molecules such as cadherins, integrins and immunoglobulin-like cell adhesion molecules (Ig-CAMs) were found to directly mediate signal transduction pathways which are involved in diseases as well as cancer development (Cavallaro & Christofori, 2004). Adhesion signalling also frequently enhances signalling from growth factors and acts in a synergistic fashion. Cellular movement and cell-matrix binding can be controlled by complexes of integrins, adaptor proteins and the actin cytoskeleton termed the cytoplasmic cell-adhesion complex (CCC) which is involved in potential cell signalling pathways such as RTK and Wnt signalling (Hynes, 2002). Moreover, adhesion sites also influence the maintenance of an adhesive interaction between cells and

the basement membrane via specialised junctions including tight junctions, adherens junctions, gap junctions, desmosomes and hemidesmosomes (Thiery, 2003).

The ability of malignant cancer cells to dissociate from the primary tumour site and metastasise to other distant organs indicates changes in the adhesive properties of the tumour cell to basement membrane components that thus facilitate tumour progression (Cavallaro & Christofori, 2004). Invasive cells are less adhesive and become highly mobile, with additionally higher mitotic activity compared to normal cells. They are also increasingly metabolically active and have the ability to secrete proteolytic enzymes that can change their motility (Turner & Sherratt, 2002) by degrading adjacent ECM molecules.

1.10.1 Focal adhesion proteins

Cell spreading and migration are driven by focal complexes in which focal adhesion (FA) proteins adhere to the ECM (Zaidel-Bar, Milo, Kam, & Geiger, 2007). FAs are specialised cellular domains that provide networking junction between the cell cytoskeleton and the ECM (Burridge, Fath, Kelly, Nuckolls, & Turner, 1988). The formation of lamellipodia and the reorganisation of cell adhesion proteins enable the attachment and detachment of actin filaments throughout the cycle (Mohammad et al., 2012; Vicente-Manzanares, Choi, & Horwitz, 2009).

In most adhesion sites, extracellular domains of the integrin receptors mediate the binding to the ECM, which then either directly mediates or strengthens the mechanical linkage between the ECM and the cytoskeleton, or participates in adhesion-mediated signalling (Zamir & Geiger, 2001). FAs commonly located at the periphery of the cell possess specific FA proteins such as paxillin, vinculin and β-subunits of integrins, such as αvβ3 integrin, which are then associated outside the cell with matrix molecules such as fibronectin fibrils

(Zaidel-Bar, Ballestrem, Kam, & Geiger, 2003). Tyrosine phosphorylation of FA via Rho signalling pathways allows the assembly and turnover via those FA proteins, mediated by activation of focal adhesion kinase (FAK) (Panetti, 2002). Focal complexes (FXs) are temporary structures that assemble under the lamellipodium in a hierarchical manner, being activated by Rho A and resulting in force-dependent transformation into FAs (Zaidel-Bar et al., 2003). These structures are then associated with actin- and myosin-containing stress fibres (Zamir & Geiger, 2001).

Figure 1.9 (adapted from (Sandbo & Dulin, 2011)) shows the connection signalling between matrix stiffness, contraction of FA and formation of stress fibre during actin cytoskeletal regulation of myofibroblasts. Involvement of actin cytoskeleton in this bidirectional signalling was shown to increase ECM organisation, FA turnover and contraction, resulting in the regulation of transcriptional proteins, which are vital processes in wound repair and fibrogenesis (Sandbo & Dulin, 2011).

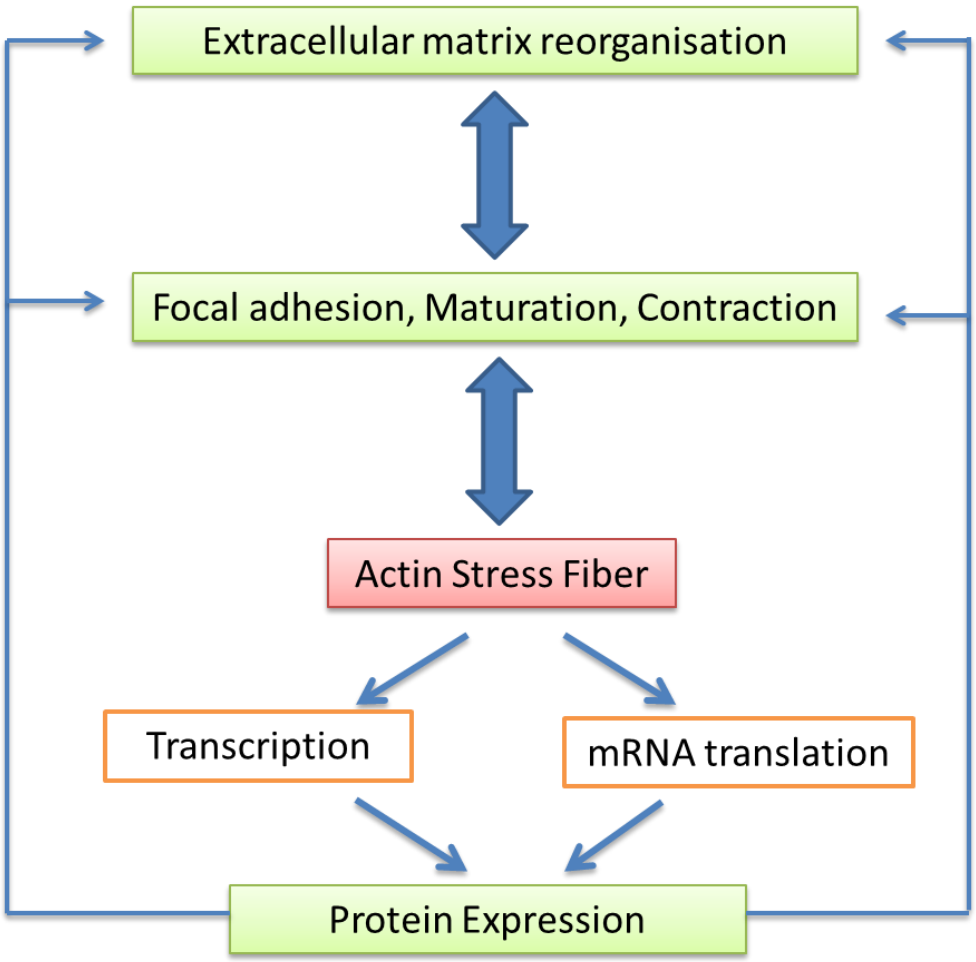


Figure 1.9: The role of the actin cytoskeleton in modulating myofibroblast functions. The actin cytoskeleton regulates several mechanical functions during myofibroblast differentiation (focal adhesion formation, contraction and matrix remodelling), which controls the transcription and translation of several genes simultaneously that are involved in these same mechanical functions. The actin stress fibre amplifies the signals leading to myofibroblast differentiation and contributes to matrix stiffness, focal adhesion formation and contractility of stress fibre formation. Adapted from (Sandbo & Dulin, 2011).

Cell adhesions, connecting cells to their surrounding stroma, have been shown to influence tumour malignancy via their role in regulating the rigidity and reorganisation of the ECM, thus regulating tumour cell survival and favouring metastasis. Activation of both growth factor pathways and FA proteins such as paxillin, Hic-5 and leupaxin has been shown to correlate with poor prognosis and aggressive cancera (Jianxin, Deakin, & Turner, 2009; Short et al., 2007). Cortactin, paxillin and PKCm have been shown to form a complex associated with invadopodial membranes that enables the cell to extend into the matrix, which produces cellular protrusions via matrix degradation and an invasive phenotype in invasive breast cancer cells MDA-MB-231 (Bowden, Barth, Thomas, Glazer, & Mueller, 1999).

1.10.1.1 Paxillin

Paxillin is characterised as a 68-kDa FA protein involved in cell adhesion assembly and turnover via tyrosine phosphorylation, and has been identified as a novel binding partner for the FA and actin-binding protein vinculin (279). The paxillin family comprises three proteins: paxillin, hydrogen peroxide inducible clone-5 (Hic-5) and leupaxin, which functions as a molecular scaffold protein. Paxillin comprises multiple discrete protein-binding modules, including numerous tyrosines as well as Ser/Thr phosphorylation sites. Many of these phosphorylated forms have been detected in tumour samples and/or cancer cell lines, and are expressed in a variety of invasive/metastatic cancers, including but not limited to breast, lung and prostate tumours (Deakin, Pignatelli, & Turner, 2012). Paxillin family expression in human cancers varies, as shown in **Table 1.2** (based on mRNA expression in tumour samples compared to normal tissue).

Table 1.2: Variability of paxillin expression in human cancers. Variability of paxillin expression in human cancers as indicated by a number of studies which determined expression in tumour tissues compared to normal tissue. Adapted from (Deakin et al., 2012).

Cancer Type	Paxillin		Hi-5		Leupaxin	
	Increased	Decreased	Increased	Decreased	Increased	Decreased
Bladder cancer				3		
Brain and CNS cancer	5		4	1		1
Breast cancer	1	6	2	3	3	1
Cervical cancer	2	1		1	1	
Colorectal cancer		3	4	5		4
Oesophageal cancer	5		1			
Leukaemia		10	2	2	1	1
Ovarian cancer	6					
Pancreatic cancer	1		2			
Prostate cancer				4	1	1

Paxillin phosphorylation has been shown to increase lamellopodia protrusions, while non-phosphorylated paxillin is crucial for fibrillar adhesion formation (Zaidel-Bar et al., 2007). Paxillin phosphorylation was also shown to promote invadopodia dynamics by expansion of an invadopodia ring which allows invadopodia disassembly (Badowski et al., 2008). However, there is contradictory evidence on the role of paxillin phosphorylation in remodelling integrin adhesion and cell migration that leads to membrane protrusion (Zaidel-Bar et al., 2007); phosphorylation was shown to increase migration in some cell types

(Iwasaki et al., 2002), whereas it was reported to inhibit cell migration in another study (Yano et al., 2000). The dynamic adhesion process may depend on the proportions of phosphorylated and non-phosphorylated paxillin. It has been demonstrated that overexpression of a phosphomimetic paxillin mutant enhances lamellipodial projection associated with formation of FA complexes (Zaidel-Bar et al., 2007). Recently, increased levels of paxillin have been shown to reduce cell migration and invasion from the primary tumour to metastatic sites due to the inability to disassemble of FAs (Mowers, Sharifi, & Macleod, 2016).

1.10.1.2 Vinculin

Vinculin is a 116 kDa actin-binding protein which localises to integrin-mediated cell–matrix interactions in the FA complex and cadherin-mediated cell–cell junctions (Ziegler, Liddington, & Critchley, 2006). It has been shown to bind with talin to the actin cytoskeleton of the cytoplasmic domain of β-integrin, and is required for integrin activation and FA stabilisation (Nayal, Webb, & Horwitz, 2004).

Vinculin acts as a key regulator of FAs and regulates cell migration depending on the availability of paxillin in the system (DeMali, 2004; Zaidel-Bar et al., 2007). Depletion of vinculin reduced cell adhesion to different substrates of the ECM and increased cell migration (Saunders et al., 2006). Expression of vinculin also is correlated with local protrusion of the cell membrane and lamellopodia formation (Humphries et al., 2007).

Similarly, overexpression of vinculin reduces cell migration, while downregulation of vinculin enhances cell motility, supporting findings that vinculin knockout cells are less adherent but more motile and associated with fewer and smaller FAs, also showing elevated expression of both FAK and paxillin (Ziegler et al., 2006). Induction of cell motility associated with lower

vinculin expression is thought to enhance tumour metastasis (Carisey & Ballestrem, 2011). Tyrosine phosphorylation of vinculin has been confirmed to be involved in the regulation of cell spreading (Z. Zhang et al., 2004). Vinculin has also been claimed to act as a tumour suppressor, as it has been shown to be involved in apoptosis-signalling pathways, leading to increased cell survival (Subauste et al., 2004; Ziegler et al., 2006).

1.11 Tumour Cell Migration

Cell migration can be defined as the ability of cells to migrate and change their positions within tissue or organs, a non-destructive process (Kramer et al., 2013) and crucial for tissue repair and immune function (Ridley et al., 2003). In contrast, invasion involves penetration through tissue barriers, which allows malignant cells to pass through the basement membrane and infiltrate into interstitial tissues. Therefore, in order to invade, tumour cells require migration, adhesion, proteolysis and remodelling of the ECM (Peter Friedl & Wolf, 2010). A cell cannot invade without migration, but can move without invasion (Kramer et al., 2013). Disruption of this motility behaviour contributes to pathological conditions such as atherosclerosis (Heldin & Westermark, 1999), as well as tumour progression and metastasis (Hood & Cheresh, 2002).

The migration cycle consists of cell polarisation and protrusion before the cell can migrate in a certain direction (Ridley et al., 2003). Actin filaments are polarised, commonly via actin polymerisation that drives membrane protrusion. It also involves formation of lamellipodia or filopodia, and adherence to the ECM via transmembrane receptors that are linked inside the cell to the actin cytoskeleton (Welch & Mullins, 2002) via adaptor proteins.

Cells can migrate in several ways, including single cell, amoeboid, chain and collective migration (Peter Friedl & Wolf, 2003). Commonly, the shape and structure of migrating cells

are both changed, allowing cell protrusion at the edge of the cells, followed by cell adherence to the substrate before forward translocation of the cells finally occurs (Lauffenburger & Horwitz, 1996). It has been described that tumour cells collectively migrate via protrusion sheets and strands at the primary site or site of local invasion, and then detachment from the cluster, becoming a 'nest' of cells, which is then responsible for metastasis. The migration mechanism used by tumour cells has been found to be most likely similar to that of normal cell migration, such as that seen in morphogenesis, immune responses and wound repair (P Friedl & Bröcker, 2000).

In addition, extrinsic controls regulated by chemokines and growth factors can also influence cell migration. Epidermal growth factor (EGF) and insulin-like growth factor 1 are known factors that enhance cell migration (Brooks et al., 1997). Moreover, chemokines have been shown to promote cell metastasis by increased cell migration via CXCR4-signalling pathways (Wells et al., 2013).

1.12 Tumour Invasion and Metastasis

Most cancer morbidity and mortality occurs on spread of the tumour from an original localised tumour site. This is termed tumour dissemination and is responsible for about 90% of cancer deaths. Therefore, key molecular controls that enable this process must be identified in order to prevent metastatic spreading. Understanding the mechanisms of inhibition of migration, invasion and metastasis will likely lead to the development of novel therapeutic approaches.

Tumour dissemination involves invasion and the metastasis process. Multistage processes are involved in metastasis, including infiltration of cells through ECM, migration of cells through blood and lymphatic vessels – termed invasion – and establishment of malignant

cells at secondary sites (Kramer et al., 2013). Adaptation by aggressive cancer cells to particular tissue microenvironments creates favourable surroundings which allow tumour cells to survive and exploit new environments. In order to spread to distant sites, cancer cells need to survive in the circulation prior to intravasation; this leads to 'cell seeding' at the target organ, successful parenchymal extravasation and persistent cell proliferation at the distant site (Joyce & Pollard, 2009).

Different cellular characteristics of the dissemination process are observed in both invasion and metastasis, as listed in **Table 1.3**. At the primary sites, tumour cells need to separate from the primary mass, reorganise and penetrate the basement membrane barrier so as to migrate through it and survive among neighbouring cells (Wells et al., 2013). Sustained angiogenesis and stable adhesion are crucial at this stage to provide support to the tumour cells, since without angiogenesis growth is limited (Kienast et al., 2010). Metastatic seeding then occurs by extravasation of surviving cancer cells at the distant sites and establishment of distinct secondary or metastatic tumours (Klein, 2008).

While much is known about cellular contractility and traction forces of cells including cell adhesion, migration and actin remodelling (Lemmon, Chen, & Romer, 2009) in 2D, force generation in more physiologically-relevant 3D environments has not been well characterised. There are differences in cell–matrix adhesions and cytoskeletal organization (Rhee, Jiang, Ho, & Grinnell, 2007) between 2D and 3D microenvironments which can alter cell morphology, proliferation and migration (Fraley et al., 2010). In contrast, study on the role of the cytoskeleton in 2D and 3D was showed no difference in modulating cell force (Kraning-Rush, Carey, Califano, Smith, & Reinhart-King, 2011). As for the advantage of 3D microenvironment, it has been shown that multiple modes of 3D migration, including

lamellipodial, lobopodial and amoeboid and collective migration were involved that influence the mode of cell motility and migration (Peter Friedl, Sahai, Weiss, & Yamada, 2012).

In vivo, tumour cells move through three dimensional matrices, meaning two-dimensional models *in vitro* have limitations compared to tumour invasion and metastasis in animal models (Wells, Chao, Grahovac, Wu, & Lauffenburger, 2011). Transcellular contractility/cell-substratum adhesion was found to be dependent on the 3D environment, including changes in actin polymerisation which allow cell protrusion and also potentially allow differences in matrix stiffness (Wells et al., 2013). In fact, ECM stiffness, density and fibre orientation have been shown to modulate cell adhesion and reorganisation of the actin cytoskeleton (Peter Friedl & Wolf, 2010).

Table 1.3: Properties needed for invasion and metastasis

Property acquired	Invasion	Metastasis
Dissociate from tumour mass	Partial	Full
Reorganize/remodel matrix	At invasion site	At metastasis site
Migrate	Major	Limited to major
Recognize endothelial cells	N/A	Limited to major
Proliferate within ectopic stroma	TBD	Major
N/A – not necessary, TBD – to be determined		

Adapted from (Wells, 2006).

1.12.1 Invadopodia mechanisms

In order for tumour cells to disseminate from the primary tumour sites, they must degrade the surrounding ECM–basement membrane barrier to escape into surrounding tissues, and subsequently metastasise and be sustained at the distant sites. These protrusion membrane structures are called lamellipodia and are usually associated with dynamic membrane ruffle formation, characterised by the co-localisation of many proteins that are found in FAs and lamellipodia, as well as membrane-trafficking proteins and proteases (Buday & Downward, 2007; Yamaguchi, Wyckoff, & Condeelis, 2005).

Invadopodia and podosomes are highly dynamic and specialised adhesive structures associated with finger-like protrusions that can degrade ECM in order to invade into and across tissue boundaries (Yamaguchi et al., 2005). There are three stages of invasive cancer cells needed to cross the ECM: perforation of the ECM by invadopodia formation, followed by invadopodia elongation and maturation, then finally the cells' entry into tumour stromata and establishment in a new microenvironment (**Figure 1.10**). In addition, the elongation of invadopodia is commonly dependent on filopodial actin formation associated with establishment of microtubule and vimentin filament networks for further growth (Schoumacher, Goldman, Louvard, & Vignjevic, 2010).

These structures are crucial in tumour cell invasion, as they cause focal disruption of the dense meshwork of ECM barriers through localised activity of membrane binding, and release of soluble MMPs, which are responsible for degradation of constituents of the ECM and thus able to promote tumour cell dissemination (Curran & Murray, 2000). Degradation of the ECM that is present in the basement membrane and tumour stroma is essential for local invasion and formation of metastatic sites by malignant cancer cells (Kessenbrock,

Plaks, & Werb, 2010) and this capacity is useful in determining the invasiveness of the cancer cells (Artym, Yamada, & Mueller, 2009). MMPs have also been shown to interact with cell-matrix adhesion molecules such as integrins to promote cell migration and matrix degradation, and EMT has been shown to further enhance these processes by increasing the expression of MMP-2 and MMP-9 (Bourboulia & Stetler-Stevenson, 2010).

In many ways invadopodia are similar to lamellipodia, having branched actin cytoskeleton into membrane protrusions that are present at the leading edge of migratory or invasive cells. Lamellipodia instead could be thought of as an initiating event for invadopodia formation (Baldassarre et al., 2006). However, their structures differ in the formation of FA complexes and FAK, which are found only in podosomes but not in invadopodia (Bowden et al., 2006). In addition, significant differences in the key regulatory proteins, such as Nck1 and Mena, was observed in tumor cell invadopodia compared to pedosomes (Sibony-Benyamini & Gil-Henn, 2012).

Recently, invadopodia were shown to be a major contributor to and hallmark of tumour cells that undergo systemic dissemination and metastasis due to oncogenic mutations in tumour cells, driving tumour initiation in the tumour microenvironment (Eddy, Weidmann, Sharma, & Condeelis, 2017). In addition, a range of signals from tumour microenvironments, such as ECM stiffness, mitogenic and angiogenic growth factors, cell contact-mediated signalling and hypoxia, have all been shown to influence invadopodia functions *in vivo* (Eddy et al., 2017).

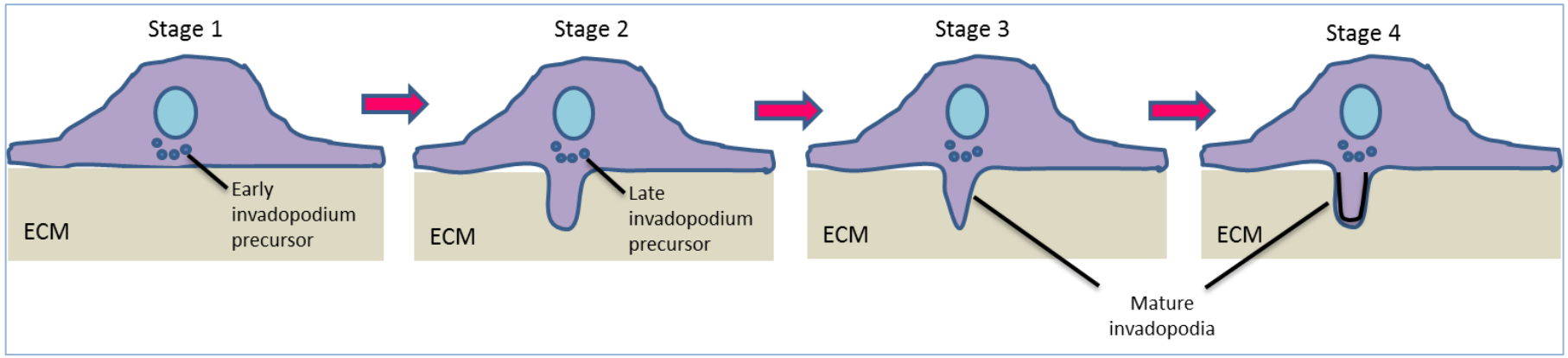


Figure 1.10: Stages of invadopodium maturation. Stage 1 or early precursor stage: invadopodia initially form as non-degradative precursors that consist of a core structure containing actin, cortactin, cofilin, N-WASp, Tks5 and other proteins. Stage 2 or late precursor stage involves activation of kinases, recruitment of β1 integrin and talin, and Tks5 anchors the precursor. Stages 3–4, which are the maturation of invadopodium stage: Stage 3 involves activation of actin polymerisation by stimulation of the NHE-1-cofilin pathway, and actin polymerisation drives invadopodial elongation and stabilisation. In Stage 4, microtubule and intermediate filament recruitment facilitates further elongation of the protrusion, and matrix proteases are recruited to degrade the ECM. Adapted from (Beaty & Condeelis, 2014).

1.12.2 Cortactin as invadopodia marker

Cortactin is an actin-binding protein that was originally identified as a substrate for the protein kinase Src (Roncucci, Stamp, Medline, Cullen, & Robert Bruce, 1991). It contains an N-terminal acidic region (NTA) that binds to and activates the Arp2/3 complex. The major function of cortactin is to regulate the actin cytoskeleton through promotion and stabilisation of the Arp2/3-induced branch-chained actin networks, suggesting that cortactin facilitates actin network formation (Weaver, 2008).

Cortactin is distributed in the cytoplasm and enriched in the front of the extending lamellipodia at the adhering side of cultured cancer cells (L.-H. Zhang et al., 2006). In invadopodia, cortactin is involved in the activation and stabilisation of the branched actin assembly to form an Arp2/3 complex, which allows cellular protrusion (Clark & Weaver, 2008) by projection of lamellipodia (Yamaguchi & Condeelis, 2007) and pedosomes (Tehrani, Faccio, Chandrasekar, Ross, & Cooper, 2006). Dynamic regulation of pedosomes has been shown to result in differentiation into invadopodia, which are specialised actin-rich protrusions in metastatic tumour cells and in most transformed cells, where they play crucial roles in ECM degradation and invasion (Artym, Matsumoto, Mueller, & Yamada, 2011).

In addition, cortactin has been suggested to act directly to promote actin assembly at invadopodia puncta (Yamaguchi & Condeelis, 2007). Importantly, the plasma membrane delivery of MT1-MMP, which can activate the proteases MMP-2 and MMP-9, is correlated with the level of expression of cortactin (Clark, Whigham, Yarbrough, & Weaver, 2007). The role of cortactin in vesicular trafficking and dynamic branched actin assembly is thought to be a potential mechanism to mediate ECM degradation associated with invadopodia by

releasing proteases (Clark & Weaver, 2008). The secretion of MMPs has been found to be correlated closely with cortactin expression levels.

In humans, the amplification of segment 11q13 on chromosome 11 – a region that includes the CTTN gene – and the associated overexpression of cortactin have been linked to many cancers, including head and neck squamous cell carcinomas (HNSCC), oral SCC, lung SCC, gliosarcoma, breast cancer, colorectal cancer and melanoma (Akervall et al., 1995; Cai et al., 2010; Campbell et al., 1996; Chen et al., 2010; Faoro et al., 2010; Schuuring et al., 1993; Schuuring et al., 1992; van Damme et al., 1997; Xu et al., 2010; Yamada et al., 2010).

Cortactin is commonly overexpressed in several human cancers, including breast and squamous cell carcinomas of the head and neck (Buday & Downward, 2007). It has been well documented that cortactin is overexpressed in several human cancers, including hepatocellular carcinoma, where the expression of cortactin correlated with metastatic level (Chuma et al., 2004) and increased phosphorylation of cortactin has been shown to induce metastasis in a mouse cancer model (Y. Li et al., 2001).

Overexpression of cortactin has also been linked to invasive cancers, including melanoma, colorectal cancer and glioblastoma, making cortactin an important biomarker for invasive cancers (Hirakawa et al., 2009; Kirkbride et al., 2011; Rothschild et al., 2006; Weaver, 2008; Xu et al., 2010).

Since cortactin is an important regulator in cell motility and invasion, it is frequently used as an invadopodia marker, based on its localisation to sites of focal ECM degradation (Weaver, 2008).

1.13 Apoptosis and Necrosis

Apoptosis, or programmed cell death, is defined as a genetically encoded cell-death program which can be differentiated from necrosis, or accidental cell death, based on distinct morphological and biochemical characteristics (Vermes, Haanen, & Reutelingsperger, 2000). Thus, necrosis occurs due to biological changes in the cell's environment (Nanji & Hiller-Sturmhöfel, 1997). Apoptosis involves an elimination process of unwanted, damaged, transformed or infected cells, and plays a fundamental role in the maintenance of tissue homeostasis in the adult organism, while dysregulation of apoptosis results in pathological conditions including cancer, autoimmune and neurodegenerative diseases (Ola, Nawaz, & Ahsan, 2011). Morphological signs of apoptosis are characterised by cellular shrinkage, membrane blebbing, nuclear condensation and fragmentation, and it can be distinguished from necrosis by these unique events (Vermes et al., 2000).

It has been shown that aggressive tumours commonly show obvious signs of apoptosis, but this may be offset by proliferation, since there are significant linear correlations between apoptotic and cell division (mitotic) indices in many tumours types (Staunton & Gaffney, 1998). A high level of proliferating cells correlates with aggressive tumours in various types of cancer; however, many tumours grow relatively slowly due to a high rate of cell loss, which involves apoptosis (Arends, McGregor, & Wyllie, 1994). Therefore, in certain tumour types increased levels of both apoptosis and mitotic activity may reflect aggressive tumour growth.

Excessive apoptosis can also lead to immune deficiency (Saito et al., 1999) depending on the cell type undergoing apoptosis. Since resistance to apoptosis is a hallmark of some cancer

types, it is important to understand the mechanisms underlying apoptosis and how it might impact on tumour growth.

1.13.1 Molecular mechanisms of apoptosis

The sequence of events of apoptosis are well characterised (Rich, Watson, & Wyllie, 1999). Apoptosis starts when cells undergo nuclear and cytoplasmic condensation, with blebbing of the plasma membrane and finally creation of apoptotic bodies containing portions of nucleus and solid organelles (**Figure 1.11**). Phagocytes then ingest and degrade these apoptotic bodies, and apoptosis typically does not therefore induce inflammation and scarring (Jacobson, Weil, & Raff, 1997). In contrast, necrosis is an accidental mode of cell death stimulated by several conditions such as hyperthermia, metabolic poisons, toxic stimuli and cell trauma. In some pathological conditions, a combination of necrosis and apoptosis may be present, and hypoxic and poorly perfused areas in tumours often contain necrotic cells (Fadeel & Orrenius, 2005).

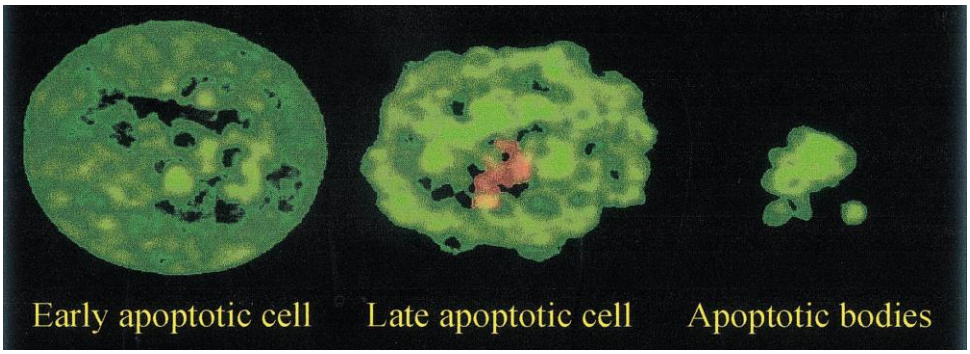


Figure 1.11: The various phases of apoptosis. These images are visualised via confocal laser scanning microscopy of cells undergoing early and late apoptosis with production of apoptotic bodies (Vermes et al., 2000).

The coordinated activation of subsets of enzymes called caspases and subsequent cleavage of specific cellular proteins lead finally to nuclear DNA fragmentation and consequently induce cell death (Hengartner, 2000).

Apoptosis may be initiated via the mitochondrial or intrinsic pathway. The outer mitochondrial membrane has pores formed in it during apoptosis which allow the release of the pro-apoptotic factor cytochrome c, which leads to the activation of initiator caspases such as caspase-3, caspase-6 and caspase-7 in the cytosol. Subsequently, effector caspases are activated by pro-caspase-9, which is proteolytically processed via apoptosome activation (Bremer, van Dam, Kroesen, de Leij, & Helfrich, 2006). This pathway is regulated by the B-cell leukemia/lymphoma 2 (BCL-2) family of pro- and anti-apoptotic proteins (Breckenridge & Xue, 2004), which may either block pore formation in the mitochondrial membrane or promote pore formation.

The second main pathway for apoptosis is the death receptor–mediated or extrinsic pathway. This pathway plays a maintenance role in tissue homeostasis. Association of the death-inducing signalling complex (DISC) leads to activation of initiator and effector caspases and subsequently results in apoptotic cell death (Bremer et al., 2006). Hyperactivation of proliferating signals, for example involving RAS and MYC oncoproteins, which would result in corresponding increases of cancer cell proliferation and thus tumour growth, has also been shown to be able to induce cell senescence and/or apoptosis (Collado & Serrano, 2010).

Many cancers are characterised by inactivating mutations in pro-apoptotic proteins, mainly in the tumour suppressor protein p53, which is very important in the activation of the mitochondrial pathway of apoptosis (O'Brate & Giannakakou, 2003). Tumour cells generally lose p53 function through either mutation or silencing, whereby the normal function of the protein in suppressing transformed cell proliferation and inducing cell cycle arrest and apoptosis is lost (Woods & Vousden, 2001). Anti-apoptotic proteins such as Bcl-2 and Bcl-x_L

are also often found to be overexpressed in several tumour types, which then leads to resistance to apoptosis (Soengas et al., 2001). Conversely, activation of several downstream pro-apoptotic pathways by growth-deregulating oncoproteins such as Myc seems to promote apoptosis. Myc is found to have effects on mitochondria that can trigger cytochrome release and caspase-9 activation (Evan & Vousden, 2001).

1.14 Flightless I

Flightless I (FliI) is a member of the gelsolin family of proteins, which regulates actin polymerisation and depolymerisation, and consists of gelsolin, adseverin, capG, villin, advillin and supervillin (**Figure 1.12**). These proteins are involved in cell motility, contraction and adhesion, and have been hypothesised to mediate other protein interactions (Cowin et al., 2007).

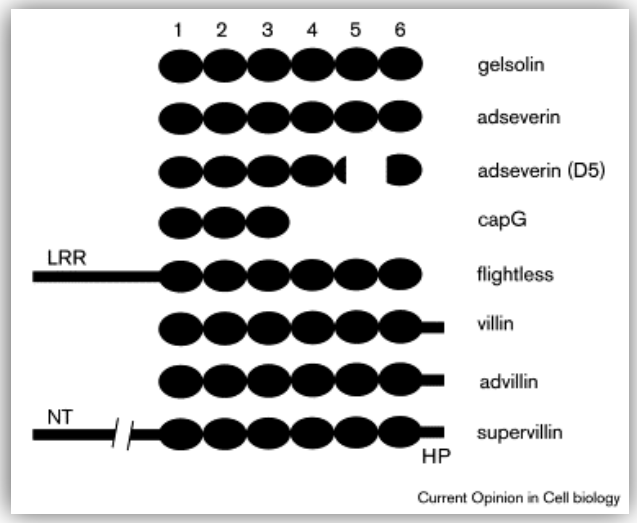


Figure 1.12: Family members of gelsolin protein. The core domain, which is repeated six times, is shown: leucine-rich repeat (LRR), headpiece (HP), nuclear translocation (NT) domain. Adapted from (Kwiatkowski, 1999).

Originally, the FliI protein was identified as a gene mutation in the fruit fly, *Drosophila melanogaster*, where mutations in this gene cause irregular actin organisation and defects in the flight muscles (H. Campbell et al., 1993), hence causing the flightless phenotype. The human homologue of the FliI gene was discovered four years later (H. D. Campbell et al., 1997). Drosophila FliI and the human homolog FliI has shown to contain the classic 6-fold gelsolin segmental repeats and an amino-terminal extension of 16 tandem leucine-rich repeats (LRR) (Y.-T. Liu & Yin, 1998).

FliI is a cytoskeletal-remodelling protein crucial in early stages of development. It has been shown that homozygous knockout of FliI in mice is embryonically lethal with gastrulation and cellularisation defects (H. D. Campbell et al., 2002), but heterozygous knockout mice survive and express lower levels of the FliI protein. The flightless and cellularisation phenotypes suggest that FliI is required for actin organisation as defective cellularisation of the syncytial blastoderm is associated with a disorganised cortical actin cytoskeleton during myogenesis and embryogenesis (Straub, Stella, & Leptin, 1996). The human flightless I (FLI) locus has been mapped to a region deleted in the Smith-Magenis syndrome, which is associated with a spectrum of developmental and behavioral abnormalities (H. D. Campbell et al., 1997).

The protein structure is characterised by leucine-rich repeat (LRR) domains, a protein-interacting domain, and six gelsolin-like segmental repeats in the C-terminal tail, as illustrated in **Figure 1.13** (Z. Kopecki & Cowin, 2008). The FliI gene encodes a protein with 1256 amino acids and an estimated molecular weight of 143,672 Da which is characterised by the fusion of these LRR domains and gelsolin-like domains in the subdivision 19F on the X chromosome (Davy, Ball, Matthaei, Campbell, & Crouch, 2000). In cells, FliI is localised at the

periphery, where it co-localises with the GTP-binding proteins ras, cdc42 and rhoA, which are important regulators of cytoskeletal reorganisation (Ben-Ze'ev, 1997). In this respect, FliI may stabilise the cytoskeleton and thereby inhibit turnover of adhesions, as it likely interacts with adaptor proteins on the cytosolic side of the FA, as shown by its co-immunoprecipitation with paxillin.

The difference between FliI and other family members is that FliI contains 6 repeat gelsolin domains and 11 LRR domains which are not present in the other gelsolin members. As a multifunctional protein, FliI distribution was expected and found to be present in multiple cellular compartments, including the nucleus, cytosol and lysosomes (Z. Kopecki & Cowin, 2008; Lei et al., 2012), as well as being secreted from the cell.

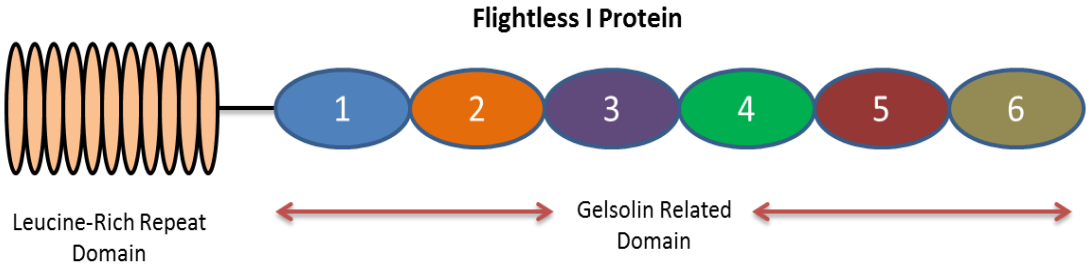


Figure 1.13: Schematic diagram of FliI structure. The FliI protein contains gelsolin domains and unique leucine-rich repeat (LRR) domains. Adapted from (Z. Kopecki & Cowin, 2008).

FliI also has the ability to act as a transcription factor. FliI acts as a co-activator of GRIPI and CARM1, and has been shown to bind with oestrogen and thyroid hormone receptors. In addition, FliI is involved in regulating hormone-stimulated gene expression through binding of its gelsolin region to the oestrogen receptor (Y.-H. Lee, Campbell, & Stallcup, 2004).

The actions of FliI defined to date relate to intracellular FliI; however, the protein has been shown to be secreted via endosomes and is measurable in serum (Davy, Campbell, Fountain,

de Jong, & Crouch, 2001). Studies done by Cowin, Adams et al. 2007 also confirm that FliI is secreted. Recent findings suggest that FliI might not be secreted through the classical pathway (Lei et al., 2012), which involves trafficking proteins from the endoplasmic reticulum to the cell surface via the Golgi complex (Bonifacino & Glick, 2004). The non-classical pathway of secretion involves protein transport to the cell surface via late endosomes/lysosomes and it has been shown that FliI may be secreted via this pathway (Lei et al., 2012).

1.14.1 Role of Flightless I in wound healing

Generation of mice that overexpress or underexpress FliI has enabled studies on FliI function during skin wound repair. FliI-overexpressing mice (FliI $^{Tg/Tg}$), which have two extra copies of human FliI, have impaired healing, with larger, less-closed wounds and reduced cell proliferation, and delayed epithelial migration, which also resulted in excessive scar formation (Cowin et al., 2007). This could be due to the ability of FliI to inhibit turnover of cell adhesions and thus affect cell migration. Changes in cell adhesion and migration could also possibly occur due to effects on the linkage between the actin cytoskeleton and ECM mediated by FliI, since FliI has also been suggested to interact with linker proteins found close to FAs (Zlatko Kopecki, Ruth Arkell, Barry C Powell, & Allison J Cowin, 2009). In addition, FliI was observed to impair hemidesmosome formation and regulate adhesion in keratinocytes and fibroblast cells in wound healing (Zlatko Kopecki et al., 2009). It has been shown that cell adhesion was improved in heterozygous FliI$^{+/-}$ fibroblasts, while significantly impaired in transgenic FliI$^{Tg/-}$ keratinocytes in scratch wound assay (Cowin et al., 2007; Zlatko Kopecki et al., 2009).

In contrast, FliI heterozygous knockout mice ($FliI^{+/-}$), which have reduced expression of FliI, showed improved wound healing, with increased epithelial migration and enhanced wound closure (Cowin et al., 2007). Cells typically found in wounds including fibroblasts and keratinocytes, show different rates of cell migration depending on their level of FliI expression, with those expressing less FliI migrating faster in two-dimensional models of scratch wound healing *in vitro*.

1.14.2 Role of Flightless I in inflammation

In recent years, a number of studies have suggested that FliI may have an important role in dampening immune response interaction with Toll-like receptors (Dai et al., 2009; Lei et al., 2012; J. Li, Yin, & Yuan, 2008; T. Wang et al., 2006). FliI contains LRR domains which are 50% similar to LRR domains of the immune-related receptor and toll-like receptor 4 (TLR4) (Lei et al., 2012). Excessive activation of TLR receptors leads to increased and prolonged inflammatory responses and can induce tissue damage, which can then impair the wound-healing process, as well as potentially inducing tumour formation in certain chronic environments. Inhibition of TLR-signalling pathways and reduction of cytokine secretion have been shown as possibly due to the interaction of FliI with nucleoredoxin and the TLR adaptor protein MyD88 in macrophages. In addition, a reduction in endogenous FliI enhanced the activation of NF-KB and its downstream cytokine production (Dai et al., 2009; T. Wang et al., 2006).

FliI also binds to pro-inflammatory caspase 1, acting as a pseudosubstrate of caspase-1 and caspase 11, which would then reduce caspase-1–mediated interleukin 1β maturation and secretion (J. Li et al., 2008). Recently, FliI has also been shown to mediate caspase-1 inhibition by the interaction of the leucine-repeat FliI-interacting protein 2 (LRRFIP2), which

negatively regulates NLRP3 inflammasome activation. This is supported by evidence that knockdown of FliI significantly promotes NLRP3 inflammasome activation, whereby interaction between FliI and caspase-1 facilitates an inhibitory effect of FliI on caspase-1 activation (J. Jin et al., 2013). Thus, several studies have shown possible mechanisms for FliI to play a role in dampening inflammation. Therefore, further analysis of this negative regulator of wound repair in prolonged conditions of inflammation, which have been shown to be one of the possible causes of tumour initiation, is warranted.

Apart from the intracellular functions of FliI, it has been shown that FliI secreted by macrophages may also have effects on inflammatory responses or innate immunity. Recent data showed that macrophages secrete FliI via the late endosome/lysosome pathway regulated by Rab7 and Stx11, so the secreted form of FliI might then be upregulated on wounding (Lei et al., 2012). It is therefore possible that FliI exerts effects either via intracellular mechanisms, such as regulation of adhesion and migration or activation of inflammatory pathways, or via a possible role of the secreted form of FliI on inflammatory cells such as macrophages.

1.14.3 Role of Flightless I in cell adhesion and migration

Cell migration can be defined as the ability of cells to migrate and change their positions within tissue or organs, a non-destructive process (Kramer et al., 2013) which is crucial for tissue repair and immune functions (Ridley et al., 2003). FliI associates with β-actin, so FliI can contribute to the actin remodelling and polymerisation processes, and regulate cell adhesion to matrix proteins that are associated with changes in cell arrangement and motility. During the adhesion process, protein complexes bind to the ECM, then trigger a linkage signal between integrin receptors and the actin cytoskeleton to form an active FA

site (Hehlgans, Haase, & Cordes, 2007). It is further suggested that FliI mediates cell migration via localisation and maturation of FA proteins (Mohammad et al., 2012). FliI stabilises FAs and its overexpression leads to a decrease in the hemidesmosome component CD151, laminin-332 and altered expression of laminin integrin receptors α3β1 and α6β4 (Z. Kopecki, R. Arkell, B. C. Powell, & A. J. Cowin, 2009).

In addition, FliI has been shown to co-localise with talin, paxillin and vinculin, which are also important in regulating cell adhesion (Mohammad et al., 2012). It has been shown that cell adhesion was increased in heterozygous $FliI^{+/-}$ while significantly impaired in transgenic $FliI^{Tg/-}$ keratinocytes and fibroblasts on different substrates (Zlatko Kopecki et al., 2009). Wound-healing models used in the same study also demonstrated that overexpression of FliI increased FA complexes, leading to actin stabilisation with elevated expression of α-actinin. Reduction of paxillin activity was also shown in FliI-overexpressing fibroblasts via inhibition of paxillin tyrosine phosphorylation signalling pathways mediated by the adhesion molecules Src and p130cas (Z. Kopecki, O'Neill, Arkell, & Cowin, 2011). It has been suggested that paxillin phosphorylation increases the formation of focal complexes associated with lamellipodia formation (Zaidel-Bar et al., 2007), which in turn increases the cell's ability to migrate. Consequently, increased expression of FliI has been shown to impair cellular spreading, with a reduction in active Rac1 and Cdc42, and enhanced formation of fibrillar adhesions, suggesting that the effect of FliI is Rac1-dependent (Z. Kopecki et al., 2011).

1.14.4 Role of Flightless I in cancer invasion and metastasis

Several studies have shown that the expression levels of actin-remodelling proteins are affected during tumour progression, and development of therapies targeting those proteins may thus inhibit cell motility and invasion (Baig et al., 2013; Dong, Asch, Ying, & Asch, 2002;

K. W. Jeong, 2014; A. Van den Abbeele et al., 2007). FliI has been identified as a tumour promoter with transcriptional activity *in vitro* in several types of cancer cell, including colorectal, breast and hepatocellular carcinomas (K. W. Jeong, 2014; L. Wu et al., 2013). FliI has been found to negatively regulate ChREBP functions in cancer cells via the ChREBP transcriptional complex, where ChREBP has been shown to enhance glucose-stimulated proliferation in cancer cells (Lifang Wu et al., 2013).

Knockdown of endogenous FliI in the breast cancer cell line MCF-7 has been shown to significantly inhibit cell proliferation (K. W. Jeong, 2014). It is important to highlight that FliI has been suggested to exert most of its effects at the cellular level, to mediate tumour progression via its ability to influence cell migration, adhesion and proliferation. FliI could also affect proliferation via interactions with calmodulin-dependent protein kinase type II (Seward, Easley, McLeod, Myers, & Tombes, 2008) and inflammation and cytokine production via caspase activation and maturation of IL-1b (J. Li et al., 2008).

A recent study by our collaborator's laboratory showed that FliI-overexpressing mice ($FliI^{Tg/Tg}$) with a chemically induced cutaneous SCC developed more invasive tumours as compared to $FliI^{+/-}$ mice. Additionally, FliI negatively regulated apoptosis due to inhibition of annexin V and caspase-1 expression (Z Kopecki et al., 2015). Intradermal injection of FnAb was shown to be effective in reducing tumour size and SCC progression (Z Kopecki et al., 2015; Z Kopecki et al., 2014). Similarly, *in vitro* findings demonstrated less three-dimensional invasion in MET-1 SCC keratinocytes when FliI expression was reduced, as well as co-localisation of FliI and the invadopodia marker cortactin (Z Kopecki et al., 2015). Invasive cells are generally less adhesive and become highly mobile, with additionally higher mitotic activity compared to normal cells. They are also metabolically active and have an ability to

secrete proteolytic enzymes that can change their motility (Turner & Sherratt, 2002) by degrading adjacent ECM molecules. The effect of FliI on tumour progression and invasion was suggested to be mainly due to its ability to regulate cell adhesion and migration, thus favouring tumour growth. This is in contrast to FliI's suggested role in wound repair, where at least in two-dimensional models FliI appears to negatively regulate motility. The tumour microenvironment may also serve as an important factor in influencing FliI activity and this needs to be further investigated.

Table 1.4 summarises FliI functions at the cellular level, including cytoskeletal reorganisation, transcription, and FliI regulation in wound healing, inflammation, cell adhesion and migration, and cell invasion.

Table 1.4: Summary of Flightless I cellular functions

Flil Cellular Functions	Mechanism	Associated Molecules	References
Cytoskeletal reorganisation	Inhibit turnover of adhesion by interaction with adaptor proteins integrin, paxillin	Flil co-localised with GTP-binding proteins ras, cdc42 and rhoA	(Ben-Ze'ev, 1997)
Transcription	Bind to oestrogen and thyroid hormone receptors	Co-activator of GRIPI and CARM1	(Y.-H. Lee et al., 2004)
Wound healing	Impaired wound healing, reduced cell migration and proliferation, and increased scar formation	Interacted with linker proteins of focal adhesion proteins (CD151 and laminin-binding integrins α3, β1, α6 and β4) and regulated hemidesmosome formation	(Zlatko Kopecki et al., 2009)
Inflammation	1) Reduced immune response via toll-like receptors 2) Promoted NLRP3 inflammasome 3) Regulated inflammatory pathway by macrophages	1) Activation of NF–KB pathway 2) Interaction with leucine-repeat Flil-interacting protein 2 (LRRFIP2), leading to caspase-1 activation 3) Regulation of Rab7 and Stx11	(Lei et al., 2012), (Dai et al., 2009), (J. Jin et al., 2013)
Cell adhesion and migration	Associates with cell adhesion proteins in cell adhesion, regulating cell arrangement and motility	Co-localised with β-actin, talin, paxillin, vinculin and α-actinin	(Mohammad et al., 2012), (Zlatko Kopecki et al., 2009)
Cell invasion and metastasis	Known as tumour promoter with transcriptional activity *in vitro* and promotes cutaneous squamous cell carcinoma model *in vivo*	1) Affected proliferation via interactions with calmodulin-dependent protein kinase type II via caspase activation 2) Co-localised with invadopodia marker cortactin	(J. Li et al., 2008) (Z Kopecki et al., 2015)

1.15 Hypotheses

The hypotheses in this research study are:

1) Similarities between cellular functions in wound healing, fibrosis and cancer, including cell migration, proliferation, apoptosis and cytokine release, lead to the investigation of the role of FliI in tumour growth and metastasis. We hypothesise that FliI inhibition will reduce tumour growth and establishment of secondary tumours.
2) FliI regulates cell invasion and adhesion associated with adhesion complexes and invadopodia formation, which mediates tumour development and spread to distant sites.
3) FliI expression enhances tumour growth and spread, and therefore FliI inhibition by using FliI knockdown (siRNA in cancer cells) will reduce tumour growth and lung seeding.
4) The therapeutic effects of a FliI-neutralising antibody will reduce lung seeding in an animal cancer model via effects on the tumour microenvironment.

1.15.1 Specific aims of the project

The aims of this research study are:

1) To determine the effects of FliI on cell migration, proliferation and apoptosis, and on the levels of cytokine secretion in CT26 and HT29 (mouse and human colon carcinoma cell lines). Additionally, to examine the effects of knockdown of FliI (siRNA FliI) in CT26 and HT29 cells and administration of exogenous recombinant FliI on these cells.
2) To determine the role of FliI in cell invasion and adhesion using a 3D culture model in CT26 WT and siRNA FliI CT26 colon carcinoma cells. Expression of FA proteins vinculin

and paxillin, and expression of the invadopodia marker cortactin will be further evaluated to elucidate the mechanisms affected by FliI in these cellular processes.

3) To determine the role of FliI in modulating cancer growth, lung seeding and establishment of lung nodules in murine colon-tumour models in wild type and underexpressing FliI mice. Additionally, to examine tumour growth using cancer cells that have FliI expression reduced via siRNA knockdown.
4) To determine the therapeutic effects of a FliI-neutralising antibody (FnAB) in establishment of lung nodules in murine colon-tumour models and tumour spheroid invasion assay *in vitro*.

Chapter 2 Materials and Methods

2.1 Materials and Methods for *in vitro* study

2.1.1 Cell lines

The human colon carcinoma cell line (HT29) (ATCC HTB-38), murine colon carcinoma cell line (CT26 H2k^d) (ATCC CRL-2638), mouse leukaemic monocyte macrophage cell line (RAW 264.7), human embryonic kidney cells line (HEK 293T) and primary mouse fibroblasts were obtained from ATCC and were available in the Cancer and Tissue Repair Laboratory, RMIT University. HT29 and CT26 H2k^d adherent epithelial colon cancer cells were used in this study, as they are compatible (from the same mouse strain) for animal studies.

2.1.2 Mammalian and murine cell cultures

Human colon carcinoma cell line HT29 cells were cultured in 10% fetal bovine serum (FBS) in McCoy's Medium 5A with L-Glutamine (Life Technologies, USA) and penicillin-streptomycin (Life Technologies, USA), and murine colon carcinoma cell line (BALB/c background), CT26 H2k^d (mouse) cells were cultured in 5% FBS in RPMI-1640 with L-Glutamine (Life Technologies, USA), 1% (v/v) HEPES buffer solution (Life Technologies, USA) and 1% (v/v) penicillin-streptomycin. These cells were incubated at 37 °C in a tissue culture incubator with humidified air, supplemented with CO_2 to 5%. Adherent cells were grown in 75 cm^2 and 125 cm^2 vented tissue-culture flasks up to 80% confluence until required for experimental use.

2.1.3 Administration of exogenous recombinant Flightless I (rFliI)

Recombinant mouse protein Flightless I homologue (rFliI) (Cusabio, USA) was used in *in vitro* studies at a final concentration of 1μg/ml. Full-length Flightless I protein is not currently available.

2.1.4 RNA extraction

Total RNA was extracted from WT mice and transfected with HT29 and CT26 using TRIzol reagent (Invitrogen, USA). Contaminating DNA was removed using DNase Turbo (Ambion, USA). cDNA was synthesised from 1 μg RNA using SuperScript III First Strand Synthesis Supermix (Invitrogen, USA).

2.1.5 Real-time quantitative polymerase chain reaction (qPCR)

A 25 μl reaction was set up containing 1 μg cDNA template, 125 ng of each primer and 12.5 μl SYBR Green Supermix (Invitrogen and Roche, USA). A polymerase chain reaction (PCR) was then run using the following program, and reaction parameters and PCR amplification products were generated using the following primers (Invitrogen, USA):

Table 2.1: PCR program and cycle reactions

Cycle/s	Process	HIF1α
1	Pre-incubation (initial denaturation)	95 °C, 5 minutes and 30 seconds
42	Denaturation	95 °C,15 seconds
	Annealing	60 °C, 30 seconds
	Extension	72 °C, 1 minute
1	Final extension	55 °C, 10 seconds

Table 2.2: List of primers sequences for qPCR

Name	Sequence (5'-3')
FliI human forward	CCT CCT ACA GCT AGC AGG TTA TCA AC
FliI human reverse	GCA TGT GCT GGA TAT ATA CCT GGC AG
RPL32 human forward	GGC AGC CAT CTC CTT CTC GGC
RPL32 human reverse	TGC CTC TGG GTT TCC GCC AGT
FliI mouse forward (1)	AAG GGT GGC TAC TTC CCT GA
FliI mouse reverse (1)	GTT GTG GCT CAC AGA CAG GT
FliI mouse forward (2)	GGG TGG CTA CTT CCC TGA GA
FliI mouse reverse (2)	GTG GTC AGA TGG TTG TGG CT
18S mouse forward	AGT TCG CTC ACA CCC GAA AT
18S mouse reverse	AGT GCG TTC GAA GTG TCG AT

Fold changes were calculated by normalising the value to housekeeping gene 18S and using the ΔΔCt formula to compare transfected cells to control samples (WT cells).

2.1.6 Cell lysis and immunoblotting

Treated or non-treated cells, WT and transfected cells, were lysed in a lysis buffer for whole-cell protein isolation on ice for 10 minutes, scraped and the cell suspension collected into a pre-cooled microfuge tube. The cell suspension was then centrifuged at 12000x*g* for 20 minutes to remove cell debris. Cell lysates were mixed with 4X SDS sample buffer, boiled at 95°C for 5 minutes and either used immediately or stored at −20 °C. Proteins were resolved by SDS-PAGE and immunoblotted for the desired protein.

Up to 30 µl of whole-cell lysates were loaded into each well on SDS-PAGE gels covered with 1x running buffer and migrated through the gel using 100 V for approximately 2 hours. After electrophoresis, proteins were transferred from the gel onto a nitrocellulose membrane using a semi-dry transfer system (Invitrogen, USA). For immunodetection, membranes were

then blocked in 5% milk/TBS-Tween for 1 hour at room temperature, with rocking to prevent non-specific antibody binding. Then the membrane was washed in TBS-T and incubated with primary antibodies overnight at 4 °C with agitation by roller.

Following three washes in TBS-Tween for 10 minutes, the blots were incubated with the appropriate horseradish peroxidise-conjugated (HRP) (Dako, Denmark) secondary antibodies for 1 hour at room temperature. After three more washes with TBS-T for 10 minutes, immunoprobed proteins were visualised by Enhanced Chemiluminescent Reagents (GE Healthcare, Australia) using the ChemiDoc XRS System (Biorad, USA). To re-probe the membrane with another primary antibody, membranes were incubated in a stripping buffer twice for 15 minutes, followed by 5 minutes with PBS-Tween at room temperature, then blocked again in 5% milk or BSA in TBS-T for 1 hour and incubated overnight with new primary antibodies at 4 °C.

2.1.7 Quantification of blots

To quantify the protein levels, protein bands exposed onto ECL films were scanned onto a computer and saved as TIF files. The intensity of each protein band was determined using Image J software, and from this the FliI protein expression was calculated and normalised to the WT cells.

2.1.8 Transformation of *E. coli* cells

E. coli cells were transformed with DNA plasmids via a heat shock protocol. Competent *E. coli* cells stored at −80 °C were thawed out slowly on ice prior to the addition of 1–10 μg of plasmid DNA. Bacteria with the added plasmid DNA were incubated on ice for 30 minutes, placed at 42 °C for 40 seconds and then quickly returned to the ice. S.O.C medium (Thermo Fisher Scientific, USA) was added to the transformed bacteria and incubated at 37 °C with

shaking for 1 hour. The bacteria were then plated onto LB-agar plates supplemented with the required antibiotic, kanamycin, and incubated overnight at 37 °C.

2.1.9 Purification of plasmid DNA

A Maxi-prep kit (Invitrogen, USA) was used to isolate and purify plasmid DNA from *E. coli* cells. Transformed bacteria from a single colony were inoculated into L-broth supplemented with the required antibiotic, kanamycin (50 μg/ml), grown in 200 ml of LB broth at 37 °C overnight with shaking. A Maxi-prep kit was used to isolate up to 500 μg from 200 ml of broth. Plasmid DNA was eluted in TE Buffer from Invitrogen columns. Concentration and purity of the plasmid were determined using nanodrop. DNA plasmid was then stored at −20 °C prior to use.

2.1.10 Stable transfection using Lipofectamine 2000

Lipofectamine 2000 transfection reagent (Invitrogen, USA) was used to transfect full-length FliI pEGFP plasmid into HT29 and CT26 cells. Cells were plated the day before transfection to allow them to adhere overnight. Prior to transfection, complete growth medium was replaced with antibiotic-free growth medium. Between 6–8 hours after the transfection mix was added to the cells, the medium was replaced with fresh complete growth medium. Transfected cells were selected using resistance to Geneticin (G418) (Santa Cruz Biotechnology, USA) up to 1 mg/ml. The trypsinised G418-resistant cells were then further analysed to determine the expression of FliI.

2.1.11 siRNA transfection

CT26 and HT29 cells were seeded at $2x10^5$ cells/ml in 2 ml antibiotic-free normal growth medium supplemented with serum, then allowed to adhere for 24 hours to attain 60–80%

confluence. 6 µl mouse or human Flil I siRNA oligonucleotide duplex (at 10 µM), Flil I siRNA mouse (m): sc-35387 and Flil I siRNA human (h): sc-35386 (Santa Cruz Biotechnology, USA) were added to 94 µl of transfection medium (Opti-MEM) (Life Technologies, USA) to make up a total volume of 100 µl. A separate tube was then prepared by adding 6 µl of Lipofectamine 2000 (Invitrogen, USA) to 94 µl of transfection medium (Opti-MEM), then gently mixed with the diluted siRNA and incubated for 45 minutes to allow the formation of transfection complexes.

Subsequently, the complexes were added to the washed cells with PBS containing 1 ml of transfection medium. Cells were further incubated for 5–7 hours at 37 °C in a tissue culture incubator. Medium containing the transfection complexes was aspirated, then replaced with medium containing double the normal concentration of serum and antibiotics. Expression of the knockdown gene was then measured using RT-PCR and immunofluorescence staining. Universal negative control siRNA (Sigma, USA) was used as the transfection control.

2.1.12 Scratch motility assay

WT and knockdown CT26 and HT29 cells (silencing of Flil) were seeded in 6-well culture plates and allowed to create a confluent monolayer of cells. Monolayer cells were scraped in a straight line to create a 'wound' using a yellow (p200) pipet tip. Debris was removed and cells were washed once with 1 ml of growth medium and then replaced with 3 ml of reduced serum culture medium (1% of serum for CT26 cells and 2% of serum for HT29 cells). The scratches were observed and images taken at 0, 24 and 48 hours interval time points using phase contrast microscopy on an inverted microscope (3 images per well in triplicates). The same field of images were photographed in each condition at the same point by marking reference points on the culture plate close to the scratch. Images were

analysed using a Scratch Assay Analyser (MiToBo) in Image J Software. The distance of cell migration (distance of the scratch gap) was determined via measuring the scratch gap of images generated by Image J by subtracting the value from the initial gap (0 hour) in each condition.

2.1.13 Apoptosis assay

Apoptosis assay was carried out using a BD Pharmingen PE Annexin V Apoptosis Detection Kit (BD Bioscience, USA) (Catalogue number: 559763). Cells were seeded at $2x10^5$ cells/ml in 2 ml and were maintained overnight in medium containing a normal percentage of serum. The next day, the culture medium was replaced with a fresh medium and subsequently exogenous recombinant FliI was added to the medium and then further incubated for 6, 24 and 48 hours. The cells were collected by trypsinisation and washed with phosphate buffered saline (PBS). The cells at a concentration of $1x10^6$ cells/ml were resuspended in 1X Binding Buffer. 100 µl of the solution containing $1x10^5$ cells/ml were transferred into microcentrifuge tubes according to conditions. Controls, which were unstained cells and cells stained with either PE Annexin V or 7-AAD, were prepared to set up compensation and quadrants. 5 µl of PE Annexin V and/or 7-AAD were added into the solutions, then incubated for 15 minutes in the dark. 400 µl of 1X Binding Buffer were added into the solutions and then transferred into flow cytometry tubes. The percentages of cell death, early apoptosis and necrosis were analysed using flow cytometry.

Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD ($7AAD^+$, Annexin V^-). Cells that stain positive for PE Annexin V and negative for 7-AAD are undergoing early apoptosis (Annexin V^+, $7AAD^-$) and cells that stain positive for both PE Annexin V and 7-AAD are either in the end stage of

apoptosis, are undergoing necrosis or are already dead (Annexin V^+, $7AAD^+$). The percentages of cells undergoing early apoptosis and necrosis were determined from the flow cytometry dot plots.

2.1.14 Proliferation assay

Indirect intracellular staining was performed to determine cell proliferation using the proliferation marker, proliferating cell nuclear antigen (PCNA) (Dako, Denmark), a mouse monoclonal antibody that detects cells in stages S and G2 of the cell cycle. Cells were fixed with 2.5% paraformaldehyde and permeabilised with 0.5% saponin in PBS for 15 minutes. Cell suspensions were centrifuged at 250xg for 5 minutes and the pellets were washed with PBS and then subsequently resuspended in 0.5% (w/v) bovine serum albumin (BSA) (Sigma, USA) in PBS. Cells were adjusted to a concentration of $1x10^6$ cells/ml and aliquoted into tubes following conditions including negative control. The required concentration of primary antibody for FliI (ab109015) (Abcam, UK) was added to the cells in a buffer containing 0.5% (w/v) BSA/PBS and incubated for 30 minutes at room temperature. Cell suspensions were centrifuged at 250xg for 5 minutes and then the supernatant removed without disturbing the pellet. Fluorochrome-conjugated secondary antibody (Alexa 488) at 1:200 dilution was added to the resuspended pellets and incubated for 30 minutes on ice. The centrifugation step was repeated and the pellets were resuspended in 500 µl FACS fix buffer. Tubes were then stored in the dark for prior to analysis using flow cytometry.

2.1.15 Cell cycle analysis using propidium iodide staining

Cellular DNA content can be quantitatively measured using flow cytometry by measuring the fluorescence signal produced by cells stained with propidium iodide (PI) (Sigma, USA),

which is proportional to the amount of DNA. Four distinct phases could be recognised in the cell population, which were G1, S (DNA synthesis phase), G2 and M phase (mitosis).

Cells were harvested and cell suspensions were prepared in a wash buffer. Cells were washed twice and resuspended at $2x10^6$ cells/ml in PBS. Cell suspensions were centrifuged at 250 g for 5 min and cold (−20 °C) absolute ethanol was added to the pellet to fix the cells for at least 1 hour or preferably overnight. Cell suspensions were then centrifuged at higher speed (1000xg) and washed twice with PBS. Cell pellets were resuspended in 1 ml of PI staining solution (40 µg/ml in PBS), which was added to the cell pellets and vortexed to resuspend. 50 µl of RNase A (10 µg/ml) (New England Biolabs Inc, USA) was added into the mixture and incubated at 37 °C for 20 minutes. Data was acquired from flow cytometry, as stained cells triggered the PI signal detected. For the gating, a secondary gate was placed around the single-cell populations and then represented as histograms. The measurements of cell cycle parameters were analysed using FlowJo software.

2.1.16 3D cell invasion assay

Chemotatic cell invasion assay was performed using pre-coated 24-well plates of BD Matrigel Invasion Chamber (BD Biosciences, USA) with 8 µm pores in the membrane according to the manufacturer's instructions. Each lower chamber of the transwell was filled with 750 µl culture medium containing 5% FBS as a chemoattractant. Prior to addition of the cell suspension, the layer of Matrigel matrix was rehydrated with a culture medium without serum for 2 hours in a tissue culture incubator. Cell suspensions in the presence or absence of exogenous recombinant FliI were prepared and seeded at a concentration of $5x10^4$ (500 µl) in serum-free culture medium into each transwell. Subsequently, transwells were transferred to the chamber containing the chemoattractant and incubated for 22 hours in a

humidified tissue culture incubator, at 37 °C with 5% CO_2 atmosphere. Cells remaining in the upper surface of the membrane were completely removed by gentle swabbing. Cells on the lower surface of the membrane were fixed and stained with cold absolute methanol and 1% Toluidine blue, respectively. The percentages of cell invasion and the invasion index were determined based on the equations below.

(A) Determine the percentage of invasion:

$$\% \text{ Invasion} = \frac{\text{Mean number of cells invading through the Matrigel insert membrane}}{\text{Mean number of cells migrating through the control insert membrane}} \times 100$$

(B) Determine the invasion index:

$$\frac{\text{\% of invasion test cells}}{\text{\% of invasion control cells}}$$

2.1.17 Cell adhesion assay

Poly-L-Lysine (Sigma, USA) and laminin-coating substrates were used to coat 96-well plates at 37 °C for 1 hour. The coated wells were blocked with medium containing 2% BSA and washed with PBS. Cell suspensions in serum-free medium were seeded into each well at a concentration of $5x10^4$ and incubated with or without exogenous FliI at 37 °C for 15 and 30 minutes. At each time point, non-attached cells in the medium were removed by rinsing. Attached cells were fixed with ethanol and stained with 0.5% (w/v) crystal violet in 10% (v/v) ethanol for 5 minutes. Excess staining of crystal violet was washed and removed. Stained adherent cells were counted in 5 fields of view in each well and the cell adhesion indices were calculated in comparison to WT cells. The adhesion index was determined based on the equation below.

Adhesion index:

$$\frac{\%\text{ of adhesion test cells}}{\%\text{ of adhesion control cells}}$$

2.1.18 ELISA

The levels of cytokines and growth factors for human tumour necrosis factor alpha (TNF-α), mouse and human vascular endothelial growth factor (VEGF), human interleukin 10 (IL-10) and human interleukin 6 (IL-6) in culture medium (24 hours incubation) of CT26 WT and/or HT29 WT cells were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions, respectively (eBioscience (USA), PeproTech (USA), eBioscience (USA) and MABTech (Germany)).

2.1.19 Immunoperoxidase staining

Pre-incubated cells with or without exogenous recombinant FliI were cultured on 6-well plates and fixed in 4% (w/v) paraformaldehyde for 20 minutes, then, after washes, permeabilised with 0.5% (v/v) Triton x-100 for 15 minutes followed by washing steps. A blocking step was performed by incubation with 3% (v/v) hydrogen peroxide for 5 minutes. Cells were then incubated with primary antibodies (1:200 dilution of anti-PCNA and 1:100 dilutions of anti-α-SMA) (Dako, Denmark) for 1 hour at room temperature followed by washes. Dual link peroxidase (α-mouse/α-rabbit) was applied to the cells for 15 minutes and they were washed again. As a final step, DAB reagent was used to develop the colour on the slides and then cell nuclei were counterstained with haematoxylin and further analysed.

2.1.20 Immunocytochemistry

Pre-incubated cells with or without exogenous recombinant FliI were cultured on 8-well glass slide chambers, then washed briefly with PBS, fixed in 3% (w/v) formaldehyde in PBS for 10 minutes at room temperature, and again washed with PBS. For permeabilisation and blocking, the cells were treated with 10% (v/v) goat serum in 5% (w/v) BSA in PBS containing 1% FBS, 0.1% (v/v) Triton X-100 for 2 hours, followed by five washes in PBS. The cells were then incubated with primary antibody diluted in antibody diluent (Dako, Germany), 0.1% (w/v) BSA in PBS overnight at 4 °C in the dark, followed by washing steps. Rabbit monoclonal anti-FliI (ab108594; 1:100), anti-vinculin (ab73412; 1:200), anti-Ki-67 (ab92742; 1:200), anti-paxillin (ab32084; 1:100) and anti-cortactin (ab81208; 1:100) were used in this study based on previous experiments performed in the laboratory.

The cells were further incubated with corresponding either anti-mouse or anti-rabbit secondary fluorescence antibodies Alexa 488 and 594 (Molecular Probes Life Technologies, USA), then diluted 1:1000 in PBS containing 0.1% BSA for 1 hour. An additional wash was performed and cell nuclei were counterstained with Hoechst 33342, Trihydrochloride, Trihydrate (Life Technologies) or DAPI nuclei staining (Sigma, USA) diluted at 1:500 dilution. Glass slide chambers were removed and slides were coverslipped using an aqueous mounting medium. Cells were viewed using a Nikon D-Eclipse C1 confocal microscope (Nikon, Langen, Germany), using oil immersion and 40x objective. Positive stained cells were calculated in 5 fields of the microscope area of each well for each cell. Fluorescence intensity was measured based on the corrected total cell fluorescence (CTCF) using Image J software (McCloy et al., 2014).

2.1.21 Isolation of primary fibroblasts

Primary fibroblasts were isolated from mice skin using modified explant culture methods (Seluanov, Vaidya, & Gorbunova, 2010). Tissue fragments were cut into small pieces and transferred onto the scratched surfaces of 6-well plates (the wells were scratched using a scalpel). 300 µl of DMEM supplemented with 10% serum and a double concentration of penicillin/streptomycin was added into each well and placed in a humidified 37 °C, 5% CO_2 incubator for 24 hours. After 24 hours, 1 ml of complete medium was added into the wells and the cells allowed to grow. Cells' growth was observed after 6–8 days and established in the wells after 2 weeks (about 14–20 days). Cells were trypsinised using 0.25% of Trypsin-EDTA for seeding and were then ready to co-culture assay.

2.1.22 Co-culture with primary fibroblasts

Primary fibroblasts and either WT or knockdown of FliI of CT26 cells were co-cultured at a 1:1 ratio (30,000 cells for each cell type) in DMEM medium for 1 week (Bakhtyar et al., 2013). Fibroblast cells were seeded in 6-well plates and allowed to grow for 4 days, after which WT and knockdown of FliI of CT26 cells were co-cultured with the fibroblast cells for another 2 days. Cells were harvested for real-time PCR (qPCR) analysis to determine the expression of FliI in the system. Transwell coated with Matrigel was used in this system to separate different type of cells to further evaluate the expression of the myofibroblast marker, α-SMA, affected by FliI.

2.1.23 Tumour spheroid invasion assay

Spheroid tumour formation was established by seeding $1x10^4$ either WT CT26 or knockdown of FliI cells in 96-well low-adherent plates. Tumour spheroids were treated in medium containing 50 µg/ml of unspecific IgG (control) or 50 µg/ml of FnAb. Images were captured

at day 2, 5 and 10 using an inverted microscope at 10X magnification and the diameters of the spheroids were measured using Image J software.

2.1.24 Live/dead cell staining

Spheroids were then stained for live/dead staining using Calcein AM (Abcam, UK) and propidium iodide (Sigma, USA) with 45 minutes' incubation at 37 °C in the dark. The images and stacking properties of the spheroids were obtained using an A1 confocal microscope (Nikon, New York). Live cells were visualised with calcein AM (green) and dead cells were detected by propidium iodide (red).

2.2 *In vivo* Animal Study

Animal ethics approval for this study was obtained from the RMIT University Animal Ethics Committee (RMIT AEC # 19061).

In the ethics approval, part of the animal monitoring process included identifying signs of distress, panting or breathing difficulty, and/or major weight changes in the mice. Any elevated levels of these changes would result in animals being euthanised.

All mice were obtained at the same age, at 6 weeks old. It is important to record the initial body weight before tumour inoculation procedure; however variation weight between WT BALB/c mice and Flil $^{+/-}$ mice was not predicted.

2.2.1 Tumour nodules in the lung of CT26-induced colon cancer in animal models with different levels of expression of Flil I

Flil I heterozygous knockout (Flil$^{+/-}$) and WT BALB/c mice at 6 weeks old were kindly provided by A/Prof Allison Cowin from the Mawson Institute, UniSA, Adelaide. Mice genotyping has been previously described: in mice lacking one copy of the Flil gene, the

double knockout is embryonically lethal (H. D. Campbell et al., 2002; Cowin et al., 2007). All experimental mice were acclimatised in the RMIT animal facility for 48 hours on arrival. To established tumour nodules in the lung, 12 mice (n=6 from each genotype) were used. Firstly, each mouse was placed under a heat lamp for no more than 5 minutes. Restrained mice were injected intravenously with 100 µl of CT26 cells ($2x10^6$) in PBS via the tail vein. The mice were monitored for up to 4 hours post-injection. On day 14, the mice were killed by a $C0_2$ inhalation procedure. The lungs were collected for further analysis. Mice were bled via cardiac puncture, and serums were processed and stored at −80 °C for further cytokine analysis.

2.2.2 Effect of Flil I gene knockout on primary tumour growth

siRNA FliI CT26 cells with disruption of the FliI gene (FliI $^{-/-}$ cells) were used in tumour inoculation studies. BALB/c WT mice (n=20) at 6 weeks old were shaved on the right flank area and injected with either 100 µl WT CT26 cells ($2x10^6$) or siRNA FliI CT26 cells in PBS (10 mice for each condition). Mice were monitored up to 1 hour post-injection. Sizes of subcutaneous tumours were monitored and measured daily using electronic callipers. Mice were killed by a $C0_2$ inhalation procedure once the tumour reached a size of 1000 mm^3 (10 mm width x 10 mm length x 10 mm depth) or a maximum of day 21 post-injection. Primary tumours were surgically excised and fixed in 4% (w/v) paraformaldehyde (PFA) in PBS for further analysis.

2.2.3 Effect of Flil I gene knockout on metastatic tumour growth

siRNA FliI CT26 cells with disruption of the FliI gene (FliI $^{-/-}$) were also used in this metastatic model. Each BALB/c WT mouse was placed under a heat lamp for no more 5 minutes and restrained mice were injected intravenously with either 100 µl of WT CT26 cells ($2x10^6$) or

siRNA FliI CT26 cells in PBS via the tail vein. Each experimental group included 10 mice (n=10). The mice were monitored for up to 4 hours post-injection. After 14 days, mice were killed according to a $C0_2$ inhalation procedure. The lungs were collected for further analysis. Mice were bled via cardiac puncture, and serums were processed and stored at −80 °C for further cytokine analysis.

2.2.4 Therapeutic effects of FliI I–neutralising monoclonal antibody on lung tumour growth

In this particular animal model, a FliI-neutralising monoclonal antibody (FnAb) was administered via intranasal injection at a concentration of 50 μg/ml in 50 μl volume per mouse. FnAb was kindly prepared and provided by our collaborator, A/Professor Allison Cowin (UniSA). Rabbit anti-FliL FnAB was raised against the LRR domain of the FliI protein.

Twenty WT BALB/c mice including a vehicle control group at 6 weeks old were used in this experiment. Each mouse was placed under a heat lamp for no more 5 minutes and restrained mice were injected intravenously with 100 μl of CT26 cells ($2x10^6$) in PBS via the tail vein. The mice were monitored for up to 4 hours post-injection. On days 4 and 8, mice were administered with 20 μl FliI-neutralising monoclonal antibody (50 μg/ml) in PBS or unspecific IgG (50 μl) via intranasal injection using an isofluorane anaesthesia inhalation procedure. A control group received the antibody without the tumour cell inoculation procedure. After 14 days, mice were killed according to a $C0_2$ inhalation procedure. The lungs were dissected for histology analysis. Mice were bled via cardiac puncture, and serums were processed and stored at −80 °C for further cytokine analysis.

2.2.5 Haematoxylin and eosin staining

Paraffin-embedded sections of the primary tumours and organs were cut (4 μm) and the histological sections were stained with a standard haematoxylin and eosin (H&E) staining procedure. Histological images were analysed to determine the number of metastatic nodules in the lung.

2.2.6 Immunohistochemical staining (IHC)

Expression of Flil I, α-SMA, PCNA and cortactin (Abcam, UK) were quantified using the IHC technique. Histological sections were deparaffinised and heated in a microwave oven for 10 minutes in a pH 6.0, 0.1M citrate buffer for antigen retrieval. Subsequently, sections were blocked for endogenous peroxidase with 0.3% (v/v) H_2O_2 and for non-specific protein binding with 0.2% gelatin in BSA/PBS. Slides were immunostained with rabbit monoclonal anti-Flil (ab108594) (Abcam, UK), α-SMA (Dako, Denmark), anti-PCNA (Dako, Denmark) and anti-cortactin (ab81208) (Abcam, UK) monoclonal antibodies that were used in this study. After washing steps, slides were incubated with either anti-rabbit or anti-mouse secondary antibody (Dako, Denmark) for 30 minutes. Finally, DAB reagent was used to develop the colour on the slides and then cell nuclei were counterstained with haematoxylin. Images were analysed using the Image-Pro Plus Program (Media Cybernetics, USA) to quantify the expression of proteins of interest.

2.2.6.1 Immunoreactive score

Immunohistological sections were further scored to determine the level of expression of each tumour marker. The immunoreactive score (IRS) system was used, considering the percentage of positive cells and the intensity of the staining (Fedchenko & Reifenrath,

2014). IRS is mainly used for intracellular staining and in this study a slight modification was adopted for extracellular staining, which determines the percentage of the immuno-positive area within the tumour.

Table 2.3: The immunoreactive score (IRS) for immunohistology

A (percentage of positive cells/ area in tumour)	B (intensity of staining)	IRS score (multiplication of A x B)
0 = no positive cells/area	0 = no colour reaction	0–1 = negative
1 = <10% of positive cells/area	1 = mild reaction	2–3 = mild
2 = 10–50% positive cells/area	2 = moderate reaction	4–8 = moderate
3 = 51–80% positive cells/area	3 = intense reaction	9–12 = strongly positive
4 = >80% positive cells/area	**Final IRS score (A x B) = 0–12**	

Adapted and modified from (Fedchenko & Reifenrath, 2014).

2.2.7 Statistical analysis

All results are presented as mean ± SEM, N indicates the number of experiments performed for *in vitro* and *in vivo*. The effects between the groups were analysed using Student's unpaired t-test and one-way or two-way ANOVA, with *post hoc* analysis using Tukey's or Dunnet's multiple comparison test as appropriate; $p<0.05$ was considered statistically significant.

Chapter 3 Flightless I Regulates Cell Migration, Proliferation and Apoptosis

3.1 Introduction

Metastasis is the major cause of death in cancer patients therefore much research is required into therapeutic targeting of both tumour cell dissemination and inhibition of secondary tumour establishment in distant sites. Tumour dissemination involves invasion and metastasis, and research commonly focuses on the key regulators of tumour progression at a cellular level (Wells et al., 2013). In fact, the invasive and migratory capabilities of tumour cells and the support of stromal cells have become crucial factors in the management of cancer prognosis and treatment (Kramer et al., 2013). It is also important to provide a better understanding of the mechanisms involved in metastasis, which will allow targeting of cancer cells and stromal cells for anti-metastatic drug development.

Cell migration is a fundamental cellular function that underlies numerous diverse biological processes, including morphogenesis, gastrulation (Keller, 2005), tissue repair and immune response (Peter Friedl & Weigelin, 2008). It also contributes to pathological conditions such as tumour progression and cancer metastasis when there is deregulation of cell movement (Hood et al., 2002; Kramer et al., 2013). Migration and invasion involve cyclical changes in

cell morphology driven by the constant remodelling of the actin cytoskeleton into structures that coordinate cell migration. FA proteins are also involved and these are spatiotemporally regulated (Gardel, Schneider, Aratyn-Schaus, & Waterman, 2010; Tojkander et al., 2012). In order to migrate in response to extracellular signals, cells first assemble actin at the cell front, driving the extension of membrane protrusions called lamellipodia and filopodia (Partridge & Marcantonio, 2006). FliI associates with β-actin, so FliI is likely able to affect actin remodelling and to be involved in regulating cell adhesion to matrix proteins. Both of these phenomena are associated with changes in cell arrangement and motility. The tissue microenvironment, in relation to either cellular or non-cellular components, can affect cell motility by influencing the organisation and stiffness of the ECM (Erler & Weaver, 2009). The non-cellular components of the stroma, including growth factors and cytokines and changes in organisation of the ECM, have been shown to promote tumour cell motility and invasion (Tlsty & Coussens, 2006).

Several cellular changes have been found to occur in tumourigenesis, including alterations in cell proliferation, the cell cycle and apoptosis that also favour tumour cell survival and dissemination. These cancer hallmarks have been discussed widely, including deregulation of proliferative signals in cancer cells enabling uncontrolled cell proliferation and altered apoptosis/survival signalling that can influence cell survival (Hanahan & Weinberg, 2011).

In order to metastasise, cells must alter their attachment to the ECM, activate degradation of the ECM and penetrate into the basement membrane and also surrounding connective tissues, then intravasate into vessels and extravasate through the blood vessels at distant metastatic sites (Wells, 2006). Additionally, the tumour cell must escape immune surveillance. This complex process is mediated by the crucial first step of tumour cell

invasion. The ability of tumour cells to migrate and invade through ECM barriers determines the metastatic potential of these cells (Wells, 2006).

Monolayer scratch motility assays are an effective technique to determine cell migration properties (Nobes & Hall, 1999) and cell polarity (Etienne-Manneville & Hall, 2003), although this two-dimensional model may not necessarily provide information about tumour cell invasion through the ECM. In this chapter, the differences between the scratch motility behaviour of WT cells, FliI knockdown and cells overexpressing FliI are examined. In addition, flow cytometry is used to examine cell proliferation, apoptosis and cell cycles in these cells. Downstream functions of cytokines that play roles in promoting tumours are also investigated. Therefore, this chapter discusses the role of FIiI in affecting cell migration, proliferation, apoptosis and cell cycle by comparing WT cells and knockdown of FliI, and the effect of exogenous recombinant FliI.

3.2 Results

3.2.1 Expression of FliI

3.2.1.1 Expression of FliI in different types of cell lines

The expression of FliI was analysed in mouse and human colon carcinoma cell lines (CT26 WT and HT29 WT), mouse leukaemic monocyte macrophage cell lines (RAW 264.7), primary mouse fibroblasts and human embryonic kidney cells line (HEK 293T) by quantitative PCR analysis. Figure 3.1 show the levels of FliI expression in CT26 WT cells, HT29 WT cells and RAW 264.7 as compared to the mouse fibroblast cell lines and HEK 293T cells *in vitro*. HEK 293T and mouse fibroblast cells were used as positive control cells for FliI expression for

human and mouse cells, respectively. mRNA was quantified using the qPCR threshold cycle and normalised to a housekeeping 18S mRNA expression to correct for loading differences.

These results show that there is a readily detectable expression of FliI in both murine and human colon cancer cells and macrophages in comparison to the respective positive control cells. In mouse cells, CT26 WT and RAW cells showed similar levels of FliI compared to the positive mouse fibroblasts cells. Similarly, HT29 WT cells showed comparable expression levels of FliI to HEK293T cells. Interestingly, the overall expression of FliI was lower in the human cells compared to the mouse cells. A higher expression of FliI was observed in CT26 WT cells as compared to human colon cancer and normal cells, HT29 WT and HEK293T ($p<0.001$) (Figure 3.1).

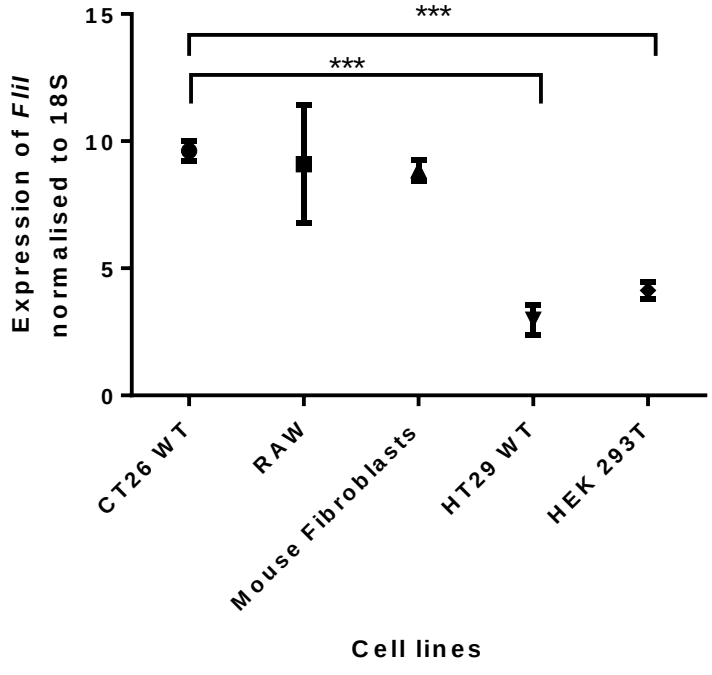


Figure 3.1: Quantitative PCR analysis showing expression of FliI in different cell lines. Expression of FliI in CT26 WT, RAW, primary mouse fibroblasts, HT29 WT and HEK 293T cell lines *in vitro*. Results are shown as expression of FliI normalised to 18S ± SEM (n=3). ***p<0.001 significantly different compared to CT26 WT (Tukey's multiple comparison test).

3.2.1.2 Expression of FliI in wild type, siRNA FliI and FliI-overexpressed CT26 and HT29 cells

Figures 3.2 and 3.3 show the expression of the FliI gene in WT, siRNA FliI and FliI-overexpressing CT26 and HT29 cells. FliI overexpressed in CT26 showed three-fold higher levels of FliI ($p<0.01$), while there was a marked reduction in the expression of FliI in siRNA FliI-treated CT26 cells ($p<0.05$). An unrelated gene Siah2 was used as a transfection control gene as a reference (Figure 3.2). The same pattern was also seen in FliI-overexpressing and siRNA FliI HT29 cells (Figure 3.3). HT29 cells showed similar three-fold increases in the level of FliI compared to mouse CT26 cells. There was a three-fold increase in the expression of FliI in FliI-overexpressing cells compared to HT29 WT cells ($p<0.05$), while reduced FliI expression was seen in siRNA FliI HT29 cells (Figure 3.3).

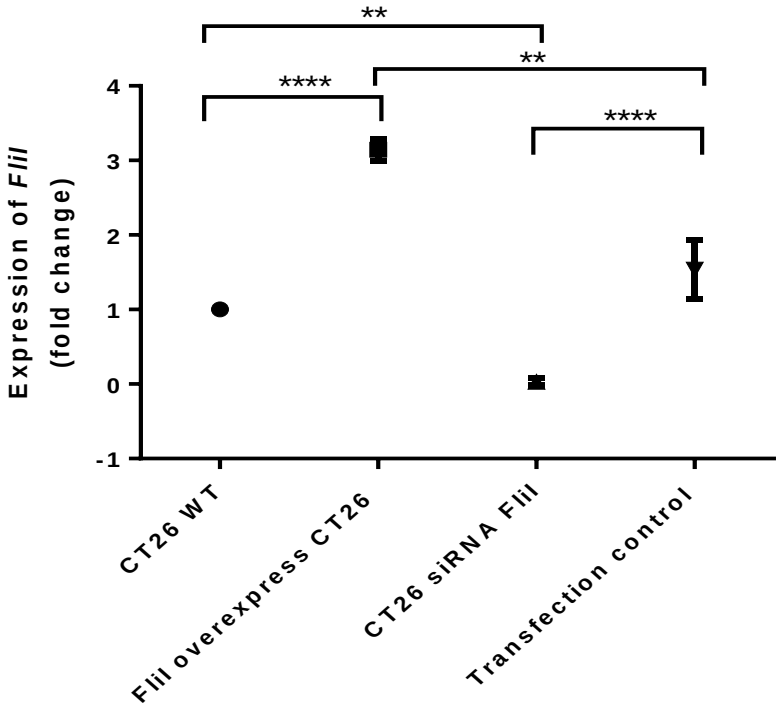


Figure 3.2: Quantitative PCR analysis showing expression of FliI in CT26 WT, FliI-overexpressing CT26, siRNA FliI CT26 and transfection control cells. Results are shown as n-fold change in expression of FliI ± SEM (n=3). **$p<0.01$ and *$p<0.05$ significantly different to control CT26 WT cells (Tukey's multiple comparison test).

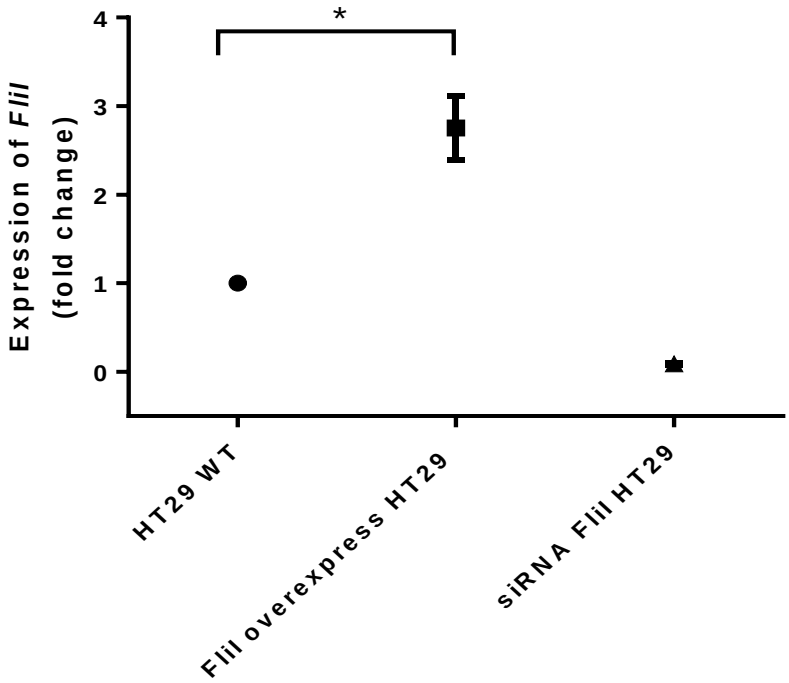


Figure 3.3: **Quantitative PCR analysis showing expression of FliI in HT29 WT and HT29 transfected with FliI (FliI-overexpressing HT29) and siRNA FliI (siRNA FliI HT29).** Results are shown as n-fold change of expression of FliI normalised to 18S (n=3). *p<0.05 significantly different to control HT29 WT cells (Tukey's multiple comparison test).

3.2.1.3 Detection of FliI expression by immunofluorescence

Expression of FliI was also detected in CT26 WT and siRNA FliI CT26 cells using immunofluorescence staining. Results show that the expression of FliI was reduced in siRNA FliI-treated CT26 cells compared to CT26 WT cells, as shown in Figure 3.4. Lower fluorescence intensity (green) was obtained in siRNA FliI CT26 cells showing less FliI expression, while increased fluorescence intensity in CT26 WT cells indicates abundant FliI expression in these cells. FliI was localised in both the nucleus and the cytoplasm associated with the actin-rich cytoskeleton (green fluoresecence) (Figure 3.4).

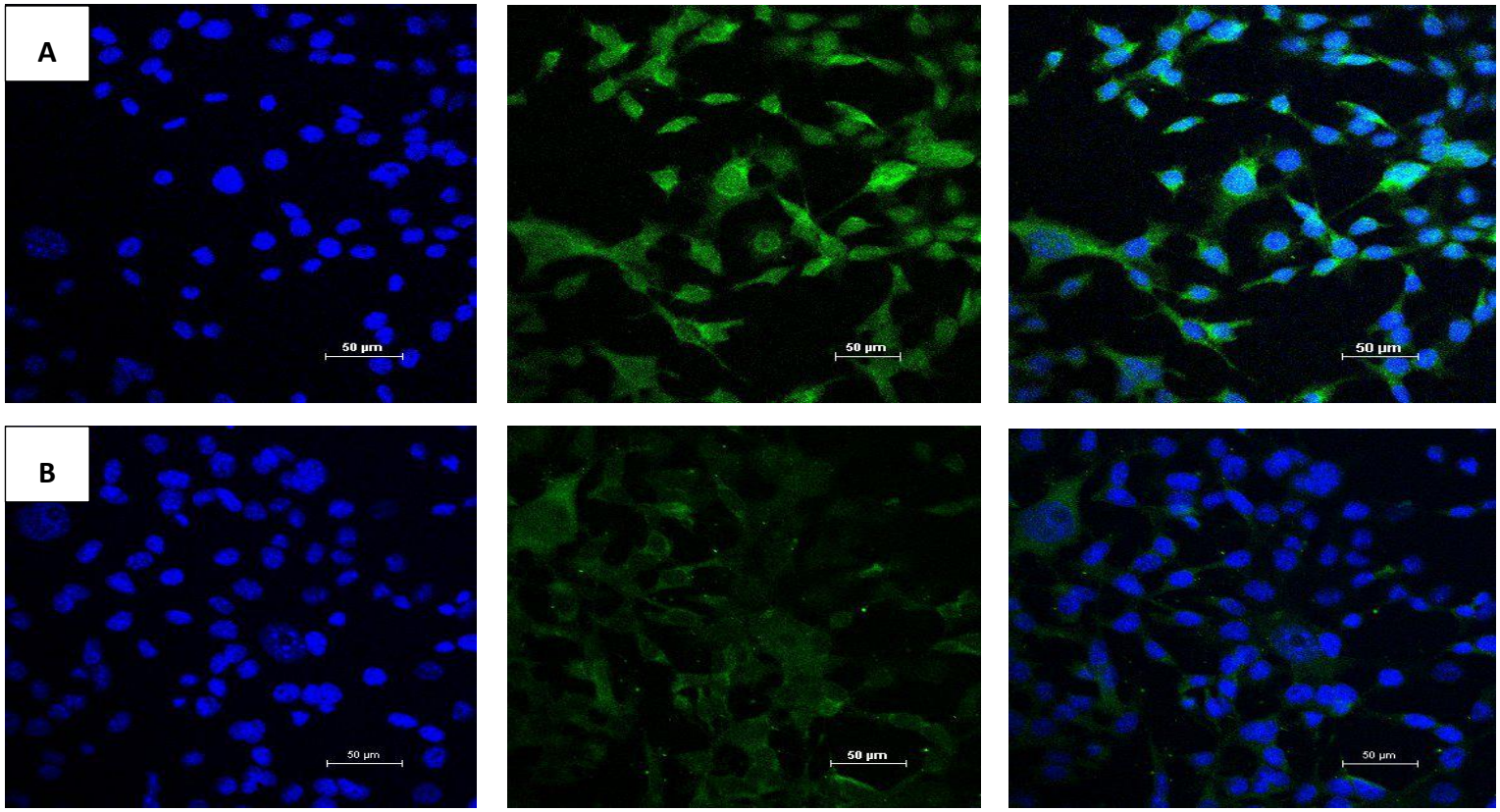


Figure 3.4: **Immunofluorescence staining of FliI in CT26 cells expressing different levels of FliI.** Immunofluorescence micrographs show expression of FliI in **(A)** CT26 WT and **(B)** siRNA FliI CT26 cells. Cells were immunostained for FliI (anti-FliI antibody) (green) and counterstained with Hoechst (blue). Images were taken using an A1 Confocal Microscope (Nikon) at 400X magnification.

3.2.2 Effect of FliI on cell migration

3.2.2.1 FliI regulates cell migration *in vitro*

Scratch assays were performed to study the migration properties of cancer cells expressing different levels of FliI *in vitro*. Results showed that treatment with siRNA FliI in CT26 cells significantly increased cell migration (90%) by reducing the area of the scratch as compared to CT26 WT ($p<0.01$) (75%) after 24 hours. In contrast, FliI-overexpressing CT26 cells showed reduced migration, with only 60% of the scratch closure compared to CT26 WT ($p<0.05$) (Figure 3.5).

Representative images of scratches in CT26 cell monolayers at 0 and 24 hour time points are shown in Figure 3.6, and image analysis was performed using a Scratch Assay Analyser (MiToBo program). Increased cell migration was observed in siRNA FliI-treated CT26 cells with an increase in scratch closure seen in images of segmentation analysis using Scratch Assay Analyser software. In contrast, reduced cell migration and less scratch closure were observed in FliI-overexpressing CT26 cells compared to CT26 WT cells.

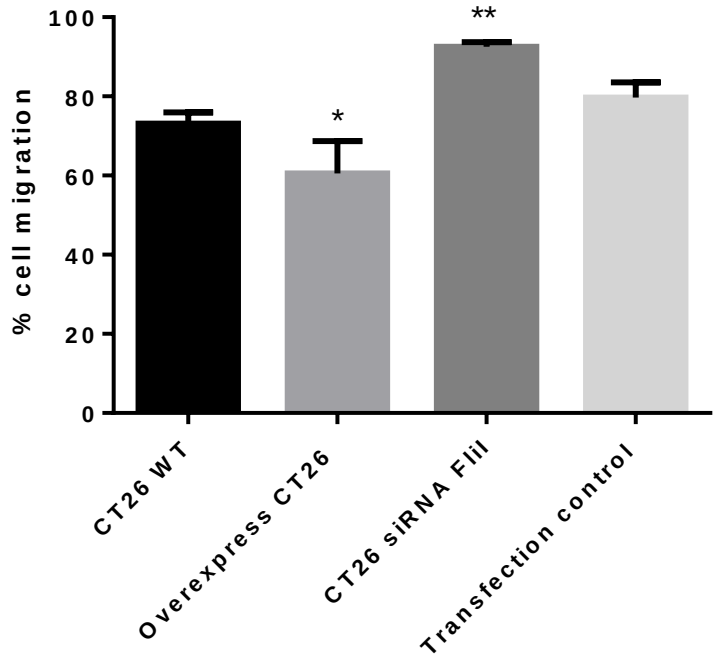


Figure 3.5: Cell migration in colon cancer cells expressing different levels of FliI *in vitro*. Percentages (%) of cell migration after 24 hours' incubation time with different levels of FliI expression in CT26 cells are shown as the scratch gap at 24 hours subtracting the value from the initial gap at 0 hour ± SEM (n=5).** $p<0.01$ and $p<0.05$ significantly different to control cells CT26 WT (Dunnett's multiple comparison test).

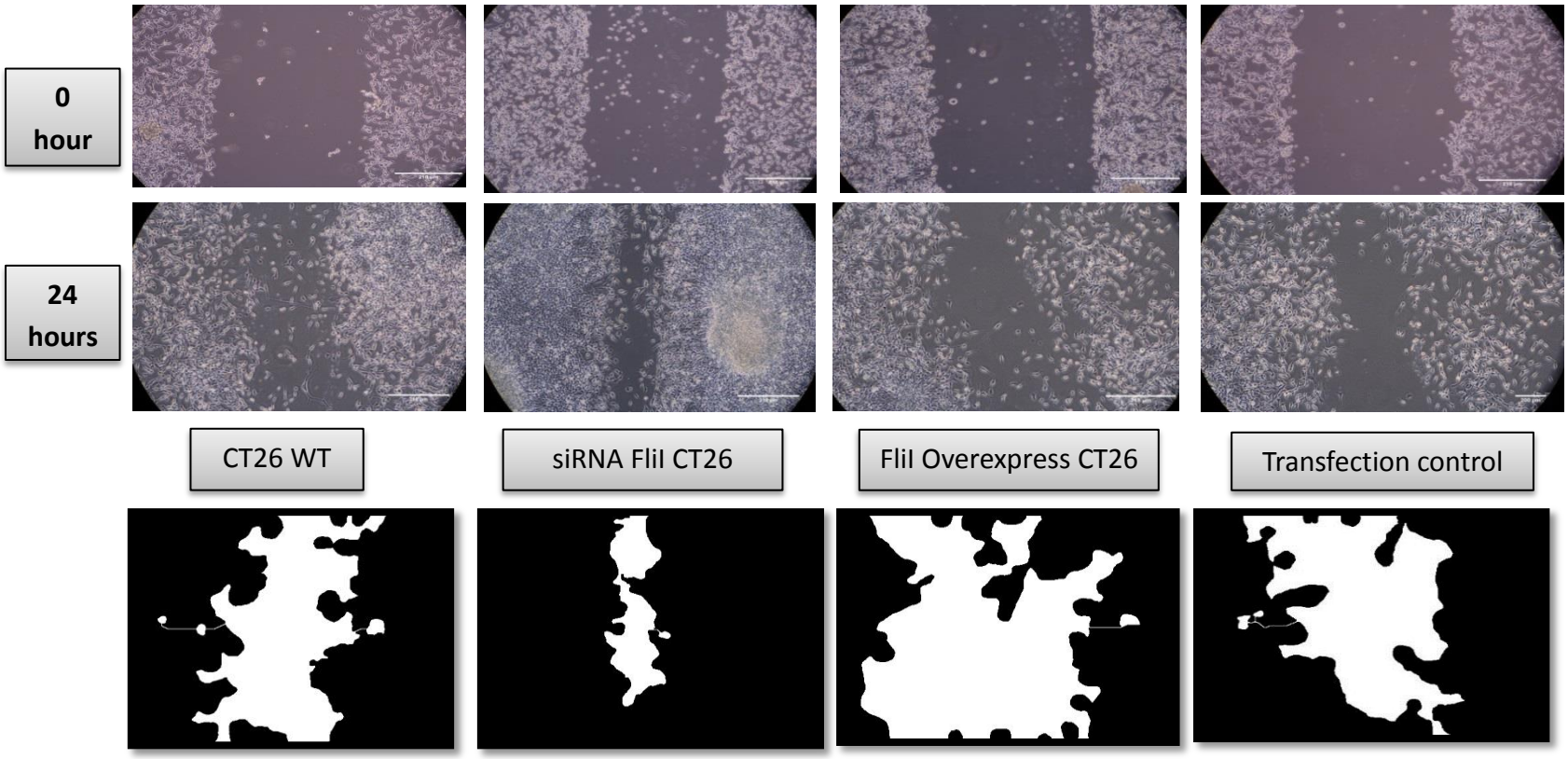


Figure 3.6: Monolayer scratch images in CT26 cells expressing different levels of FliI *in vitro*. Representative photographs of monolayer scratch images of CT26 WT, siRNA FliI CT26 cells, FliI-overexpressing CT26 and transfection control cells (siRNA Siah2 CT26) at 0 hour and 24 hours' incubation time. Images were taken using an inverted light microscope at 20X magnification. Images of segmentation analysis using the MiToBo program show the scratch gap (white area) and migratory cells (black area) of CT26 WT, FliI-overexpressing CT26, siRNA FliI CT26 cells and transfection control cells.

3.2.2.2 Exogenous recombinant FliI has no effect on cell migration *in vitro*

Scratch assays were also used to determine the effect of exogenous recombinant FliI (1 µg/ml) on CT26 cells, again using cells with different levels of FliI expression. Cell migration was measured in CT26 WT and siRNA FliI CT26 cells after treatment with recombinant FliI (1 µg/ml) *in vitro*. As with previous results, siRNA FliI CT26 cells showed significantly increased cell migration compared to CT26 WT cells after 24 hours ($p<0.05$) (Figure 3.7). The addition of recombinant FliI to CT26 WT and siRNA FliI CT26 cells had no significant effect on cell migration; however, there was a significant decrease in cell migration in the transfection control cells as compared to CT26 WT cells ($p<0.05$). Representative images of the scratch motility assay with the addition of exogenous FliI are shown in Figure 3.8.

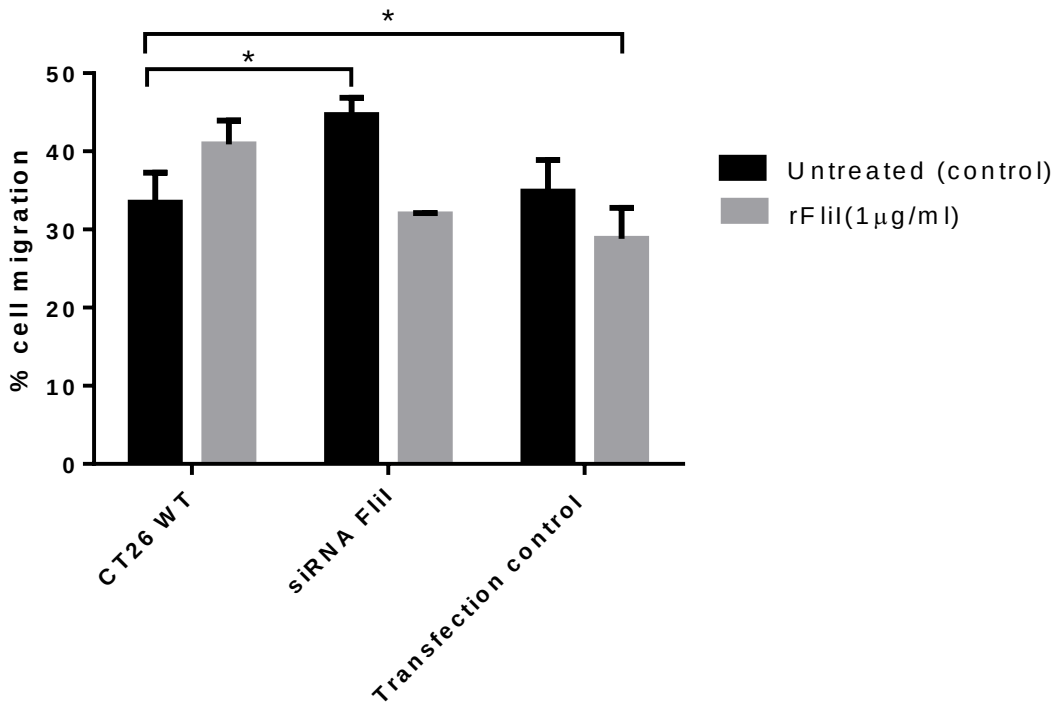


Figure 3.7: Colon cancer cell migration after exogenous FliI treatment *in vitro*. Percentages (%) of cell migration of CT26 WT and siRNA FliI CT26 cells with (rFliI 1 µg/ml) or without (untreated control) treatment with recombinant FliI (rFliI). Results are shown as % cell migration ± SEM (n=5). *$p<0.05$ significantly different to control cells CT26 WT (Dunnett's multiple comparison test).

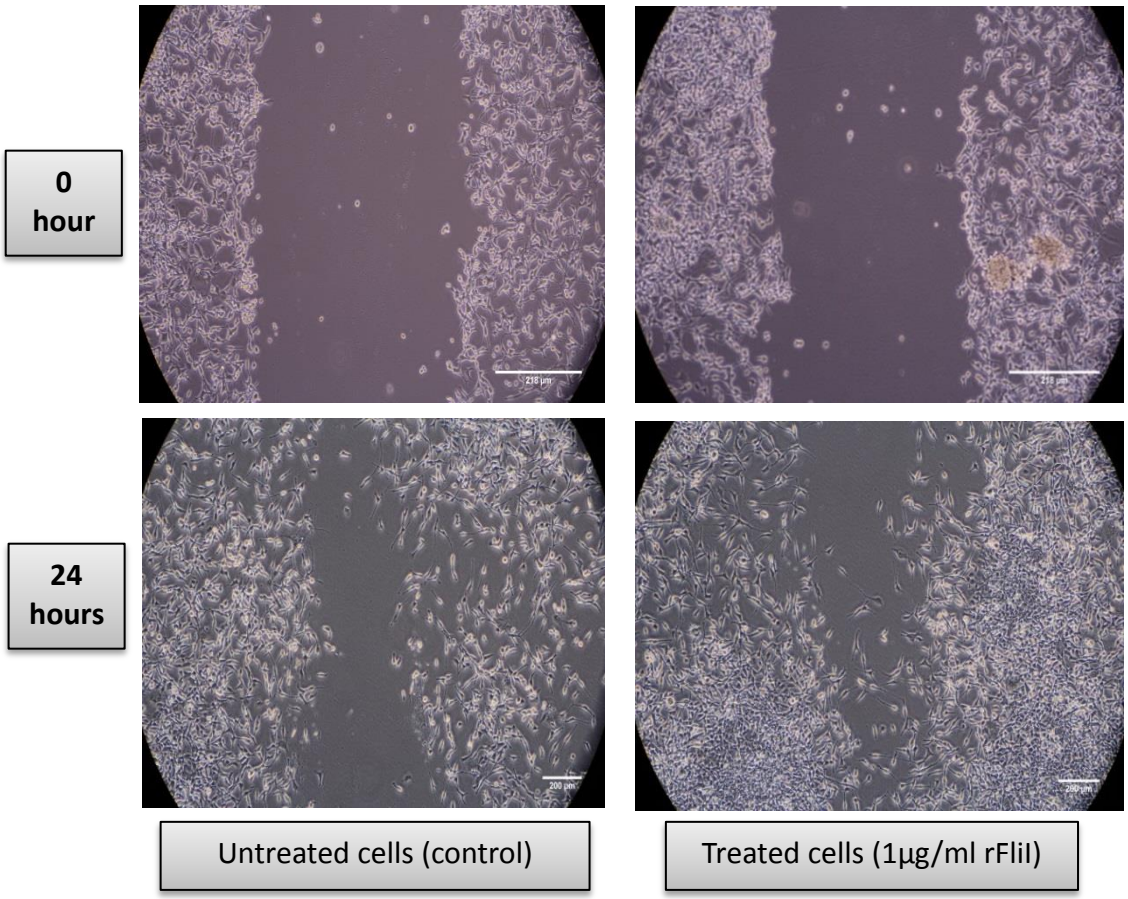


Figure 3.8: Images of CT26 cells in scratch assay after exogenous FliI treatment *in vitro*. Representative images of CT26 WT cells with (rFliI 1 µg/ml) or without (untreated control) treatment with recombinant FliI (rFliI) at 0 and 24 hours. Images show the scratch gap before treatment (0 hour) and after treatment with rFliI (24 hours). Images were taken using an inverted light microscope at 20X magnification.

3.2.3 Effect of exogenous FliI on cell apoptosis

3.2.3.1 Exogenous recombinant FliI increases apoptosis in CT26 cells

Stages of apoptosis, cell death and necrosis in CT26 colon cancer cells expressing different levels of FliI were measured by flow cytometry using 7AAD/Annexin V staining. Cells were treated with recombinant FliI for three different time periods (6, 24 and 48 hours) and then flow cytometry was carried out to look at apoptosis, cell death and necrosis. Findings

showed that adding exogenous recombinant FliI (1 μg/ml) to CT26 cells significantly increased the percentage of apoptosis at 24 hours post-incubation *in vitro* ($p<0.05$) (Figure 3.9). However, there was no difference in the percentage of cell death/necrosis in CT26 WT cells treated with exogenous recombinant FliI compared to the control cells (untreated) at the other time points examined (Figure 3.9).

Representative flow cytometry dot plots of early apoptosis, cell death and necrosis are shown in Figure 3.10. Untreated CT26 cells and cells treated with exogenous recombinant FliI (1 μg/ml) for 24 hours were collected and analysed by flow cytometry (Figure 3.10). Dot plots represent (A) Control ($7AAD^-$, Annexin V^-), (B) Cell death ($7AAD^+$, Annexin V^-), (C) Early apoptosis ($7AAD^-$, Annexin V^+) and (D) late apoptosis or necrosis ($7AAD^+$, Annexin V^+). Flow cytometry dot plots show that 15% of control CT26 cells underwent early apoptosis and this increased to 25.3% after incubation with exogenous recombinant FliI; 4.4% cell death and 6.1% cell necrosis were recorded in control CT26 cells and similarly, approximately 5.7% cell death and 6.3% necrotic cells were counted in CT26 cells treated with exogenous recombinant FliI.

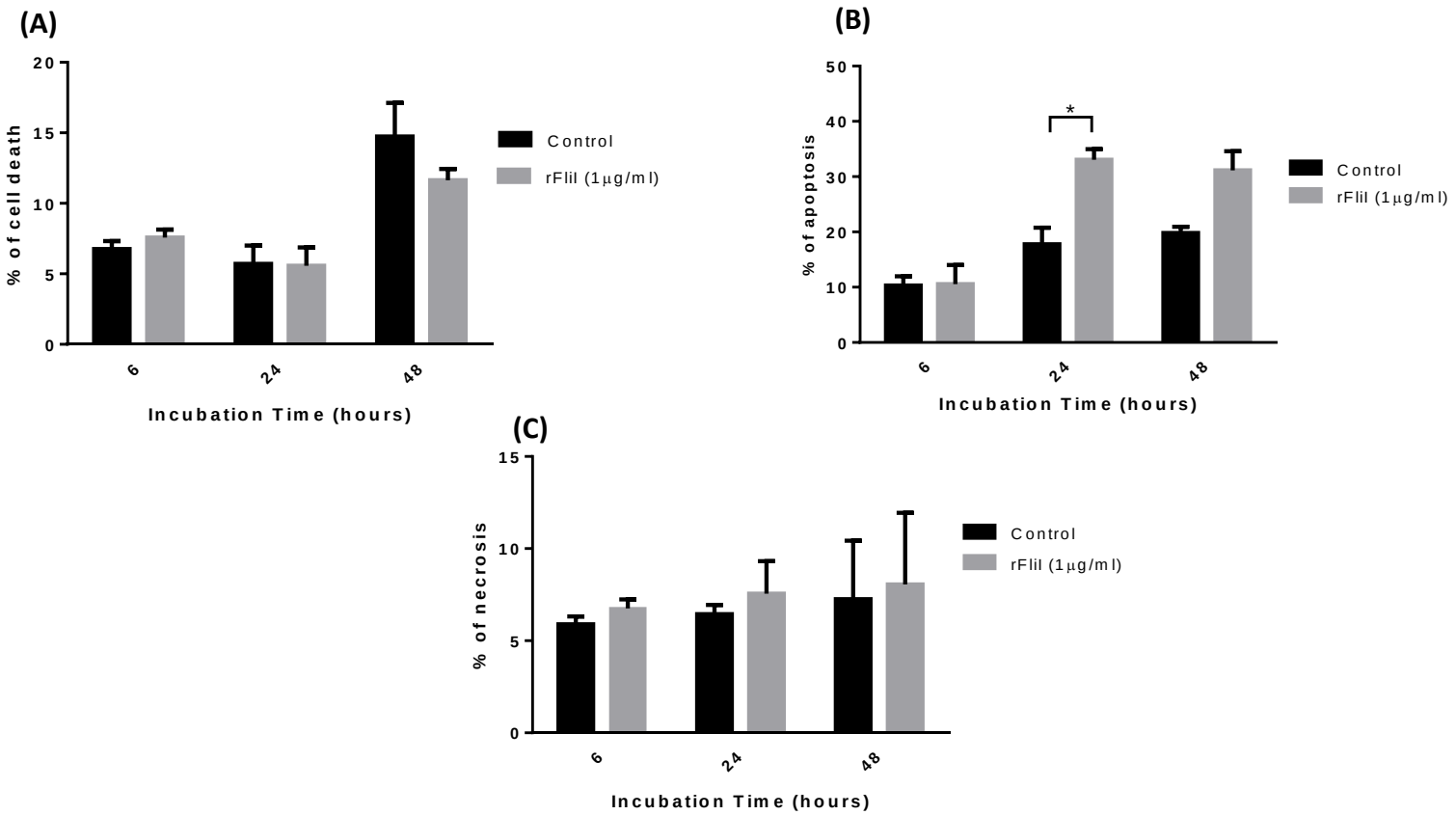


Figure 3.9: FliI effect on apoptosis in CT26 colon cancer cells *in vitro*. Induction of apoptosis, necrosis and cell death after treatment with exogenous recombinant FliI (rFliI; 1 µg/ml) shows percentages of **(A)** cell death ($7AAD^+$, Annexin V^-), **(B)** early apoptosis (Annexin V^+, $7AAD^-$) and **(C)** necrosis (Annexin V^+, $7AAD^+$) in untreated or treated (rFliI 1 µg/ml) CT26 cells over 6, 24 and 48 hours. Results are shown as mean ± SEM (n=3).*$p<0.05$ significantly different to untreated (control) cells (Tukey's multiple comparison test).

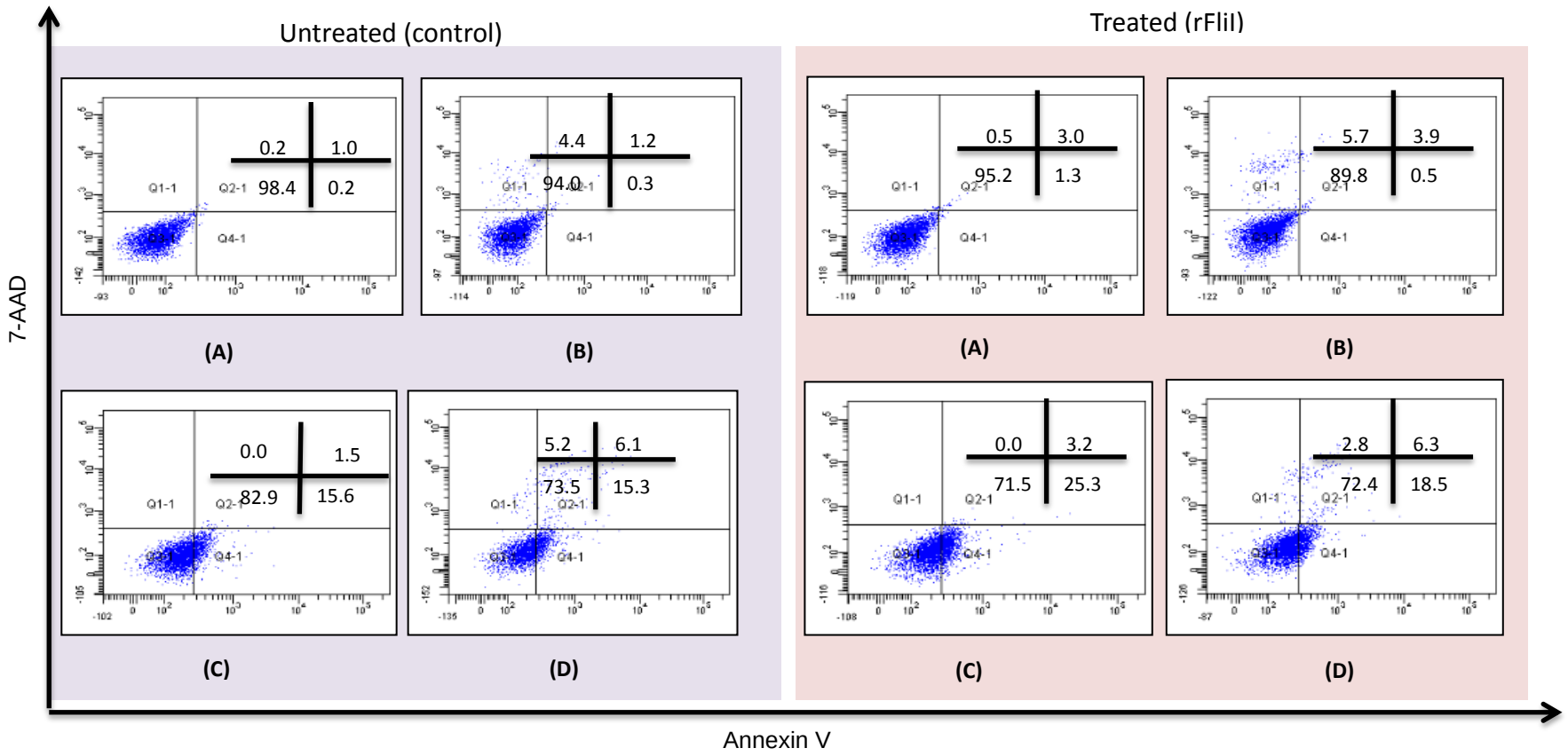


Figure 3.10: Representative flow cytometry dot plots showing apoptosis of CT26 colon cancer cells treated with exogenous FliI treatment *in vitro*. CT26 colon cancer cells were treated with recombinant FliI (1µg/ml) or control medium. Dot plots represent **(A)** control ($7AAD^-$, Annexin V^-) (Q3-1), **(B)** cell death ($7AAD^+$, Annexin V^-) (Q1-1), **(C)** early apoptosis ($7AAD^-$, Annexin V^+) (Q4-1) and **(D)** late apoptosis or necrosis ($7AAD^+$, Annexin V^+) (Q2-1). Flow cytometry analysis was collected on a FACSCanto (BD Biosciences) and analysed using FACS Diva Software (BD Biosciences).

3.2.3.2 No difference on cell apoptosis in knockdown of FliI using siRNA FliI in CT26 cells

To study the effects of knockdown of FliI (using siRNA) and addition of exogenous recombinant FliI (rFliI; 1 μg/ml), apoptosis was analysed by flow cytometry in untreated CT26 WT and siRNA FliI CT26 cells and/or cells treated with exogenous recombinant FliI (rFliI; 1 μg/ml). There is no significant change observed in the percentage of apoptotic cells (Annexin V^+/ $7AAD^-$) between the control scrambled siRNA and the FliI siRNA treated cells without or with treatment with exogenous rFliI (Figure 3.11).

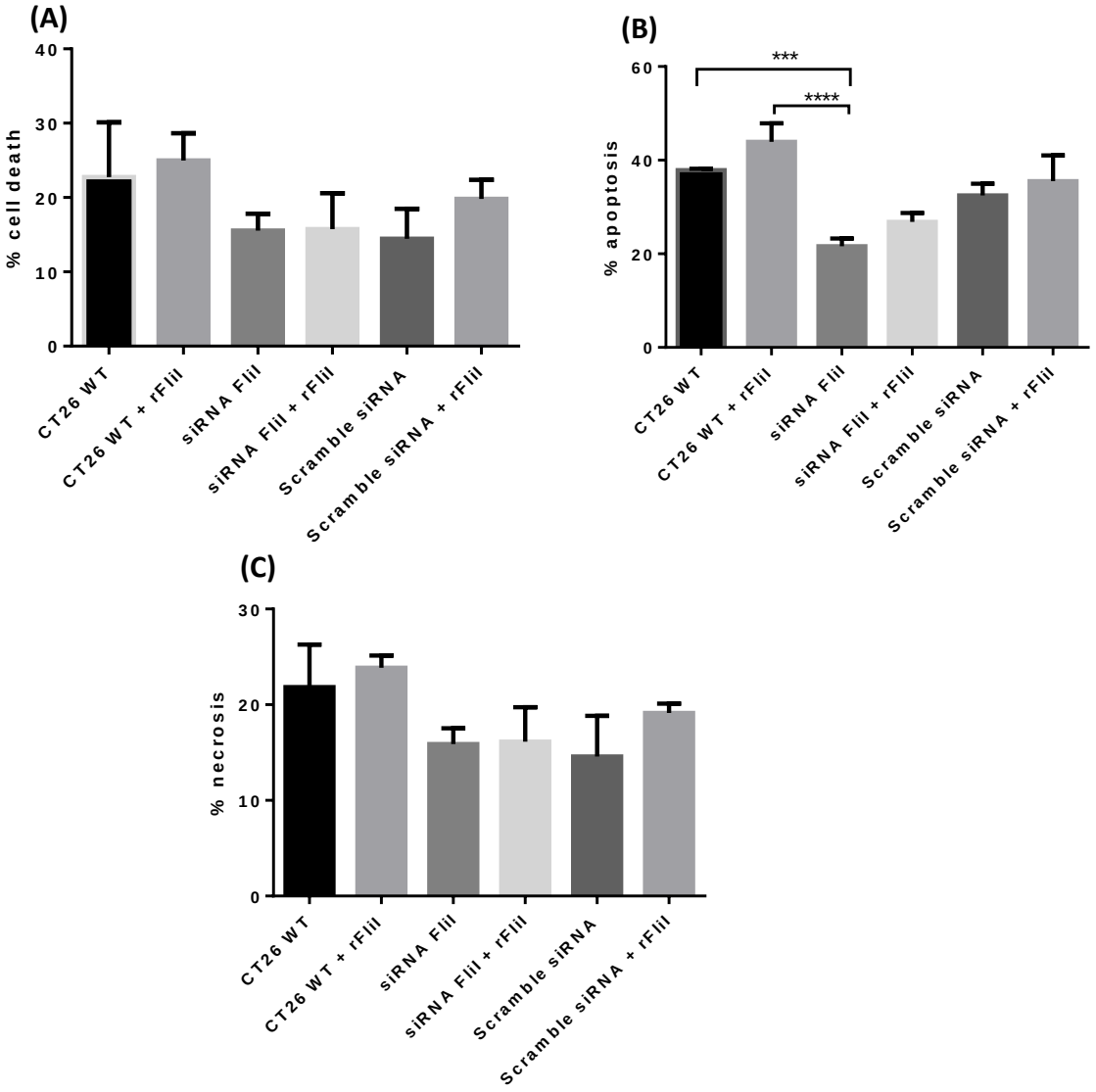


Figure 3.11: Apoptosisanalysis via flow cytometry in CT26 colon cancer cells expressing different levels of FliI and with recombinant FliI treatment (rFliI 1µg/ml) *in vitro*. Percentages (%) of **(A)** cell death ($7AAD^+$, Annexin V^-), **(B)** early apoptosis (Annexin V^+, $7AAD^-$) and **(C)** necrosis (Annexin V^+, $7AAD^+$) in CT26 WT, siRNA FliI CT26 and transfection control cells (scrambled siRNA) with and without exogenous recombinant FliI (rFliI) treatment. Results are shown as mean ± SEM (n=3).****p<0.0001 and ***p<0.001 significantly different to control cells CT26 WT (Tukey's multiple comparison test).

3.2.3.3 No difference in cellular apoptosis in knockdown of FliI in siRNA FliI HT29 cells

We found similar results in the reduction of apoptosis in siRNA HT29 cells as observed in CT26 cells (Figure 3.12). Apoptosis assays were carried out in untreated HT29 WT and siRNA FliI HT29 cell and cells treated with exogenous recombinant FliI (1 μg/ml). There was no significant change observed in the percentage of apoptotic cells (Annexin V^+/ $7AAD^-$) between the control scrambled siRNA and the FliI siRNA treated cells without or with treatment with exogenous rFliI (Figure 3.12). Results also show no difference in the percentages of cell death and necrosis in siRNA FliI HT29 cells as compared to scrambled siRNA cells with addition of exogenous recombinant FliI.

Representative flow cytometry dot plots representing early apoptosis, cell death and necrosis are shown in Figure 3.13. Untreated CT26 WT and siRNA FliI HT29 cells and cells treated with exogenous recombinant FliI (1 μg/ml) for 24 hours were collected and analysed by flow cytometry (Figure 3.13). Dot plots represent (A) control ($7AAD^-$, Annexin V^-), (B) cell death ($7AAD^+$, Annexin V^-), (C) early apoptosis ($7AAD^-$, Annexin V^+) and (D) late apoptosis or necrosis ($7AAD^+$, Annexin V^+). Flow cytometry dot plots show 37.7% of CT26 WT cells underwent early apoptosis and this decreased to 24.6% in siRNA FliI CT26 cells. There was no difference observed in the percentages of cell death and necrosis in CT26 WT cells (26.8% cell death and 27.1% cell necrosis) and in siRNA FliI CT26 cells (19.7% cell death and 19.8% necrotic cells).

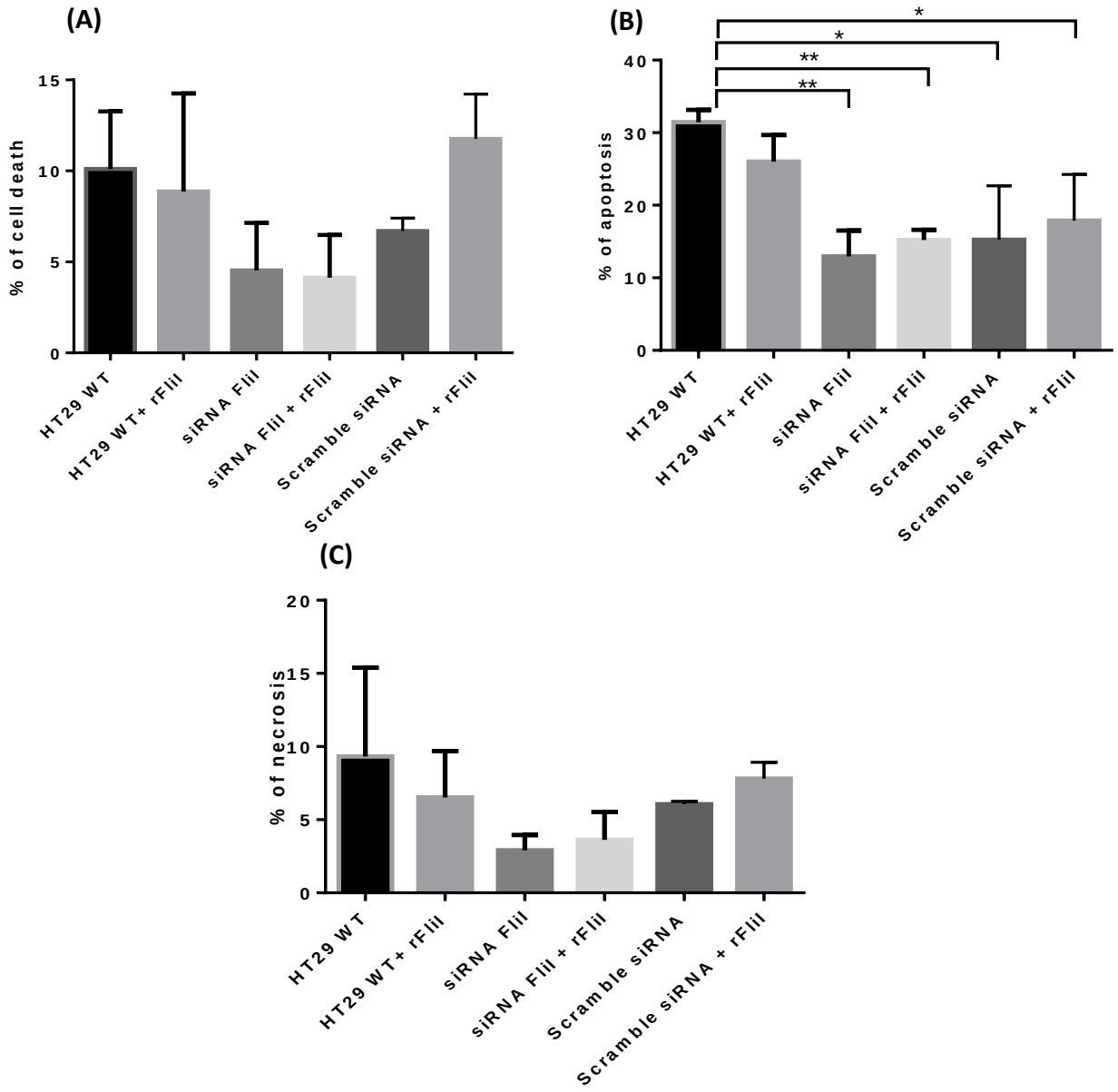


Figure 3.12: Apoptosis analysis via flow cytometry in HT29 colon cancer cells expressing different levels of FliI and with exogenous FliI treatment (rFliI 1µg/ml) *in vitro*. Percentages (%) of **(A)** cell death ($7AAD^+$, Annexin V^-), **(B)** early apoptosis (Annexin V^+, $7AAD^-$) and **(C)** necrosis (Annexin V^+, $7AAD^+$) in HT29 WT, siRNA FliI HT29 cells and transfection control cells (scrambled siRNA) with and without exogenous recombinant FliI (rFliI). Results are shown as mean ± SEM (n=3). ***p<0.05 significantly different to control cells HT29 WT (Tukey's multiple comparison test).

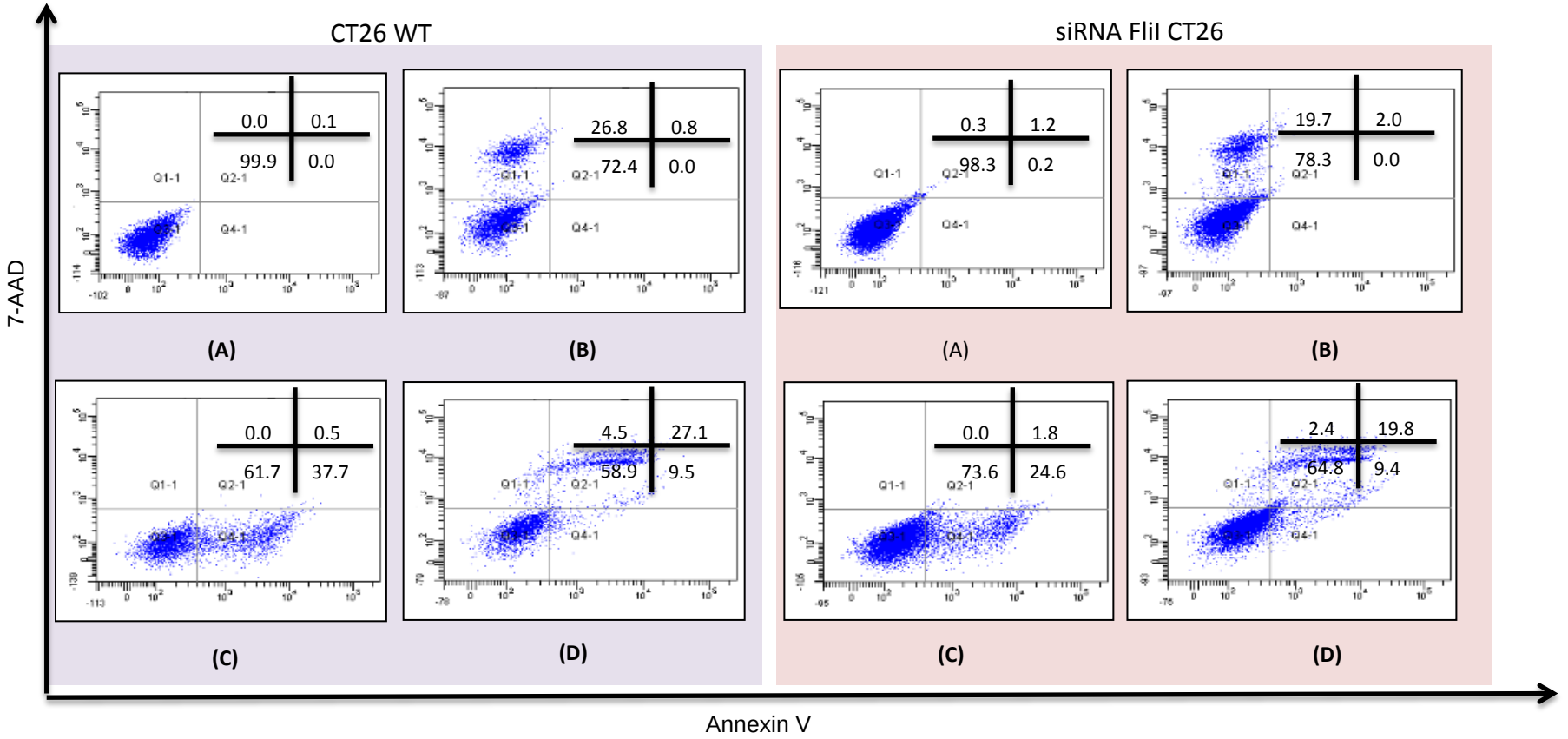


Figure 3.13: Representative flow cytometry dot plots showing apoptosis of CT26 colon cancer cells expressing different levels of FliI treated with exogenous FliI (rFliI 1µg/ml) *in vitro*. CT26 WT and siRNA FliI CT26 cells were treated or left untreated with recombinant FliI. Dot plots represent **(A)** control ($7AAD^-$, Annexin V^-) (Q3-1), **(B)** cell death ($7AAD^+$, Annexin V^-) (Q1-1), **(C)** early apoptosis ($7AAD^-$, Annexin V^+) (Q4-1) and **(D)** late apoptosis or necrosis ($7AAD^+$, Annexin V^+) (Q2-1). Flow cytometry analysis was performed on a FACSCanto (BD Biosciences) and analysed using FACS Diva Software (BD Biosciences).

3.2.4 Effect of FliI on cell proliferation

3.2.4.1 Knockdown of FliI increased cell proliferation *in vitro*

Intracellular PCNA expression in human colon cancer HT29 cells was assessed by flow cytometry to determine the effect of FliI on cell proliferation *in vitro*. Our results show that knockdown of FliI significantly increased cell proliferation (MFI = 65.80 ± 7.607) as compared to HT29 WT cells (MFI = 34.87 ± 5.080) ($p<0.05$). Addition of exogenous recombinant FliI (rFliI) to HT29 cells had no significant effect on cell proliferation compared to HT29 WT control cells after 24 hours (Figure 3.14). The expression of PCNA in HT29 WT and siRNA FliI HT29 cells treated with exogenous rFliI slightly increased in HT29 WT cells (MFI = 54.60 ± 4.392) while it decreased in siRNA HT29 cells (MFI = 47.40 ± 4.339); however, this is not a statistically significant difference compared to HT29 WT control cells.

Representative flow cytometry histograms show PCNA expression in (A) HT29 WT, (B) HT29+ rFliI (1 µg/ml), (C) siRNA FliI HT29 and (D) siRNA FliI HT29 + rFliI (1 µg/ml) cells (Figure 3.15). 37.7% proliferation in HT29 WT cells was counted and the percentage increased to 67.3% in siRNA FliI HT29 cells. Adding exogenous recombinant FliI increased PCNA expression in HT29 WT cells (57.9%) while siRNA FliI HT29 cell showed reduced PCNA expression (44.1%) (Figure 3.15).

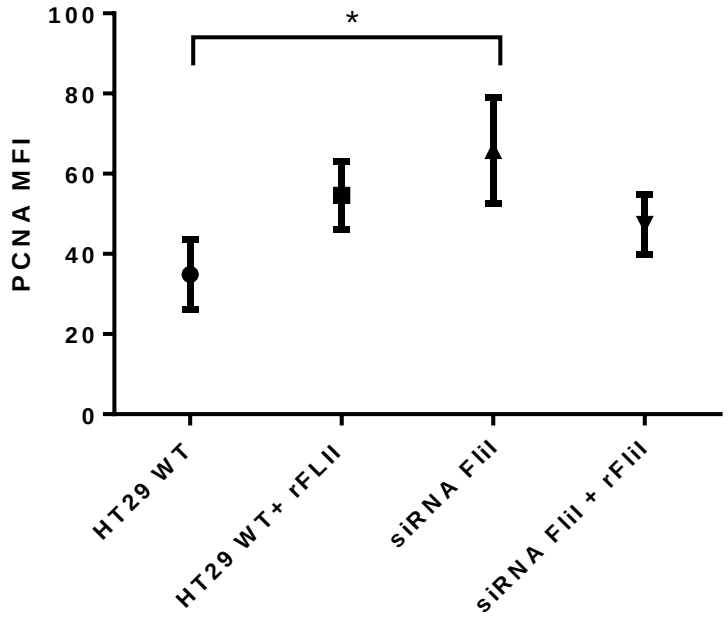


Figure 3.14: PCNA expression in HT29 cells expressing different levels of FliI and after treatment with exogenous recombinant FliI (rFliI) analysed by flow cytometry. Intracellular expression of PCNA in HT29 WT and siRNA FliI HT29 cells treated with or without rFliI (1 µg/ml). PCNA expression is shown as PCNA mean fluorescence intensity (MFI) ± SEM (n=3). *p<0.05 indicate statistical significance between each experimental group (Tukey's multiple comparison test).

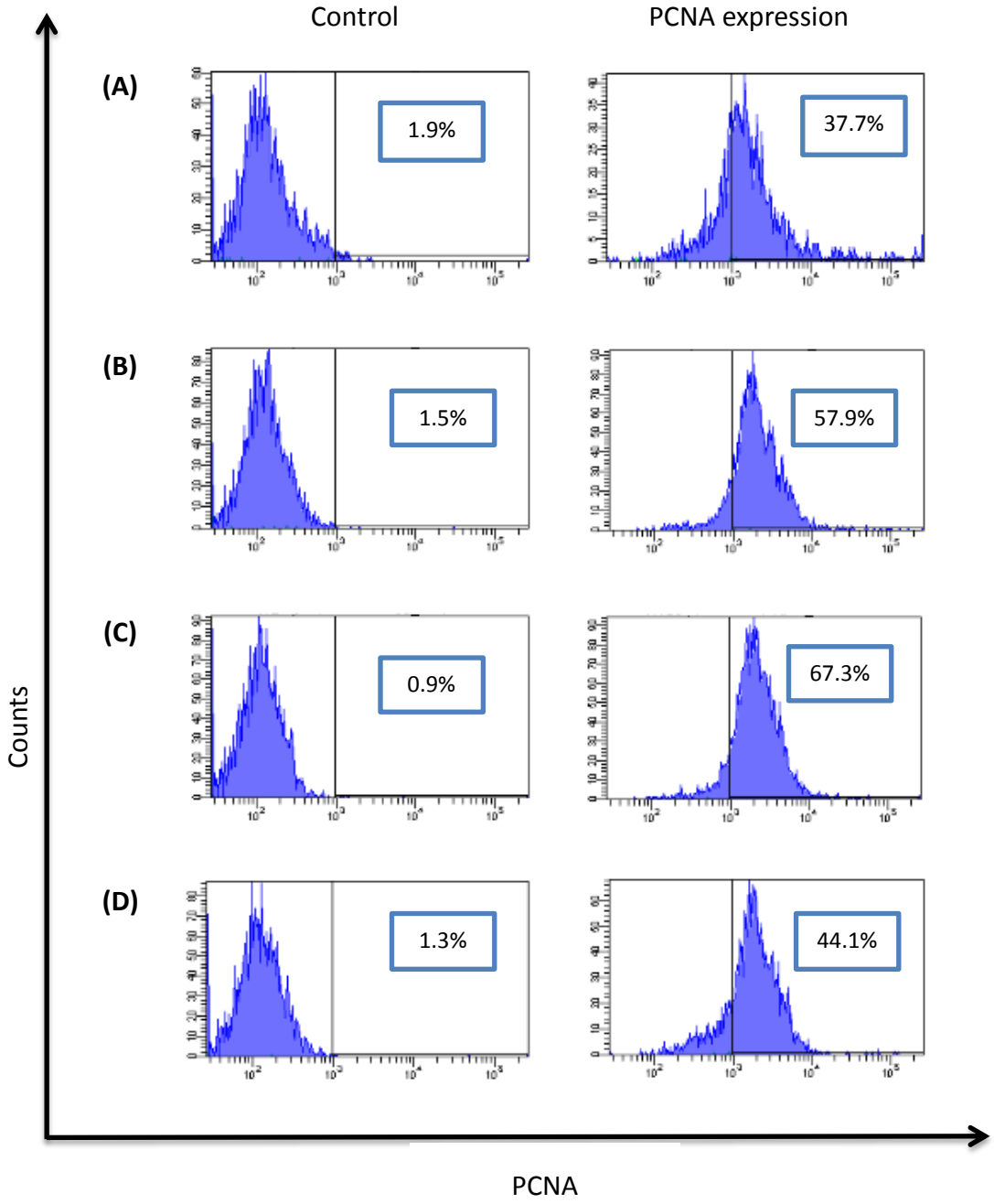


Figure 3.15: Representative flow cytometry histograms showing PCNA expression in HT29 cells expressing different levels of FliI or with added FliI protein. Representative flow cytometry histograms show PCNA expression in **(A)** HT29 WT, **(B)** HT29 WT+ rFliI (1 µg/ml), **(C)** siRNA FliI HT29 and **(D)** siRNA FliI HT29 + rFliI (1 µg/ml). Histograms on the left represent control (secondary antibody alone) and on the right represent cells stained intracellularly for PCNA with secondary FITC-labelled antibody. Results were analysed by flow cytometry (FACSCanto, BD Biosciences) and analysed using FACSDiva Software (BD Biosciences).

Immunoperoxidase staining of PCNA in HT29 WT and siRNA FliI HT29 with and without recombinant FliI treatment showed an increased number of positive cells stained with PCNA in siRNA FliI HT29 compared to HT29 WT cells (Figure 3.16). The number of proliferating cells (nucleus stained brown) also increased after the addition of exogenous recombinant FliI (1 µg/ml rFliI) in HT29 WT cells. However, a reduction in PCNA-positive cells was observed after the addition of exogenous recombinant FliI (1 µg/ml rFliI) in siRNA FliI HT29 cells (Figure 3.16).

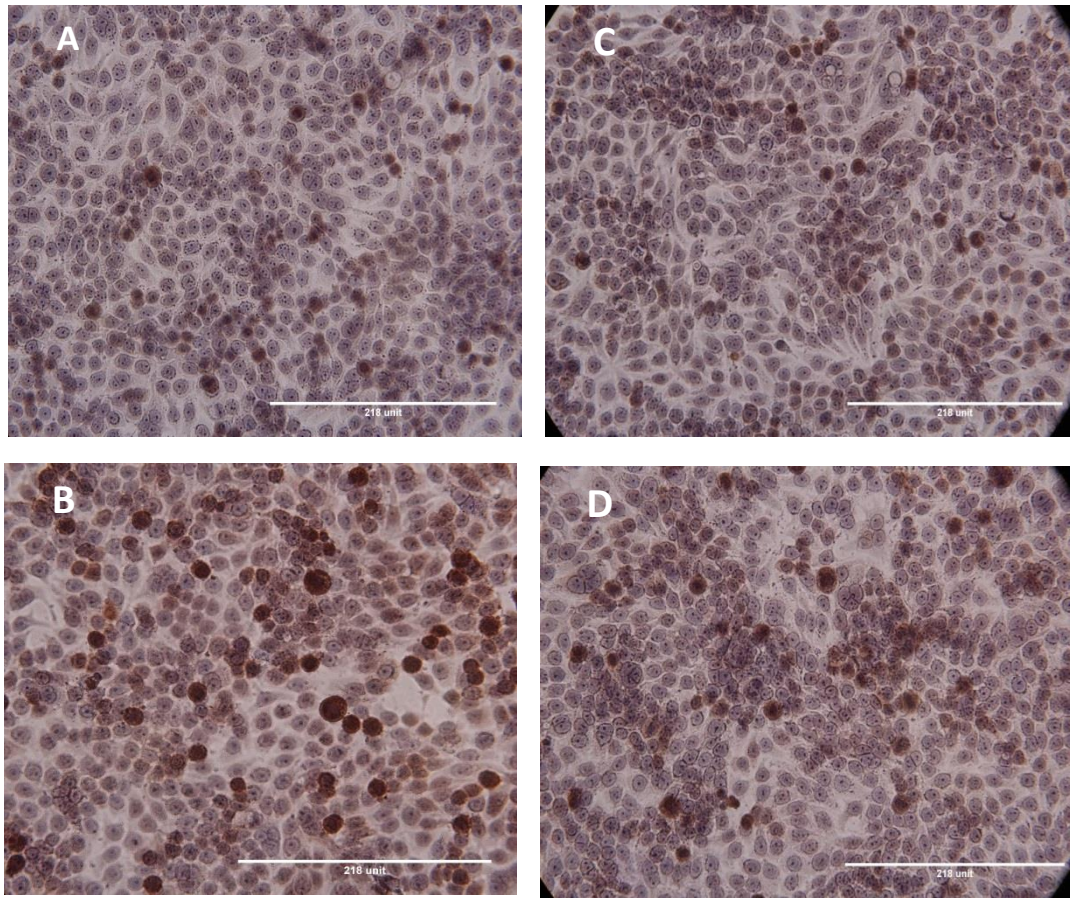


Figure 3.16: PCNA expression in HT29 cells expressing different levels of FliI with and without treatment with exogenous recombinant FliI (rFliI) analysed by immunoperoxidase staining. Representative images of immunoperoxidase staining of PCNA in **(A)** HT29 WT, **(B)** siRNA FliI HT29, **(C)** HT29 WT + exogenous recombinant FliI (rFliI; 1 µg/ml) and **(D)** siRNA FliI HT29 + exogenous recombinant FliI (rFliI; 1 µg/ml). Images taken using an inverted light microscope at 20X magnification.

3.2.4.2 FliI regulates Ki-67 expression in colon cancer cells *in vitro*

In order to determine the effect of FliI on cell proliferation in mouse colon cancer cells, CT26 WT and siRNA FliI CT26 cells were immunostained against an additional proliferation marker, Ki-67. Ki-67 positive CT26 cells were counted using Image J software. Similar to the immunofluorescence staining of PCNA, there showed an increased positive staining of Ki-67 in siRNA FliI CT26 cells compared to CT26 WT cells ($p<0.01$) (Figure 3.17). Positive stained cells were counted in 5 fields per well from three independent experiments.

Figure 3.18 shows representative images of immunofluorescence staining of Ki-67 in CT26 WT and siRNA FliI CT26 cells. There were more cells positive for Ki-67 (green fluorescence) in siRNA FliI CT26 cells compared to the staining seen in CT26 WT cells.

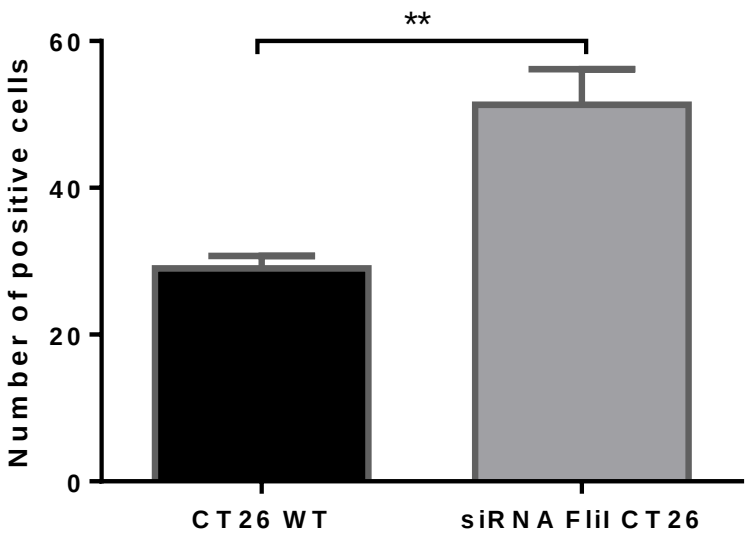


Figure 3.17: Quantification of Ki-67 expression in CT26 cells expressing different levels of FliI. Number of positive cells stained by immunoperoxidase with proliferation marker Ki-67 in CT26 WT and siRNA FliI CT26 cells were counted using Image J software in 5 fields per well from three independent experiments. Results are shown as mean ± SEM (n=3). **$p<0.01$ indicates statistical significance compared to control cells CT26 WT (Student's unpaired t-test).

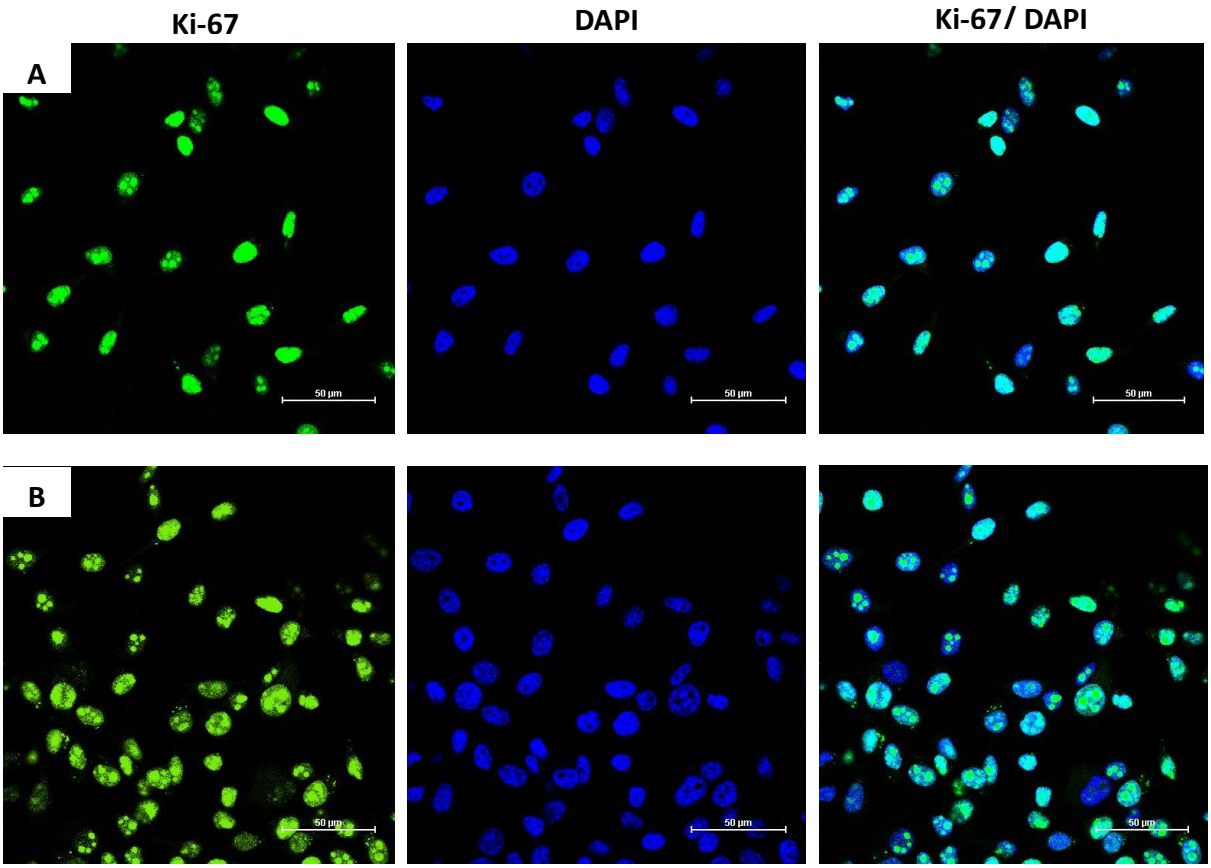


Figure 3.18: Immunofluorescence staining of Ki-67 in CT26 cells expressing different levels of FliI. Representative images of immunofluorescence staining of Ki-67 expression (green) in **(A)** CT26 WT and **(B)** siRNA FliI CT26 cells counterstained with DAPI (blue). Photos taken using an A1 confocal microscope at 60X magnification.

3.2.5 Effect of FliI on cell cycle

3.2.5.1 FliI showed no effect on the cell cycle of CT26 colon cancer cells *in vitro*

Cell cycle analysis of CT26 colon cancer cells expressing different levels of FliI was performed using propidium iodide (PI) staining and flow cytometry. Analysis was also performed on CT26 WT and siRNA FliI-treated CT26 cells with or without addition of exogenous recombinant FliI (1 µg/ml). Cells were treated with 1 µg/ml of rFliI for 24 and 48 hours, and stained with PI staining for cell cycle analysis using flow cytometry (Figure 3.19). Cell counts in each cell cycle phase were measured using FlowJo software analysis.

There was no difference in the cell count in the different phases of the cell cycle at 24 and 48 hours' incubation time between CT26 WT cells and siRNA FliI CT26 cells regardless of exogenous recombinant FliI treatment. No significant difference in the cell count was observed in the GI phase (DNA preparation for synthesis phase), S phase (DNA synthesis and replication) or G_2M phase (cell growth and mitosis) at 24 and 48 hours' incubation time (Figure 3.19).

Representative flow cytometry dot plots and histogram of cell cycle analysis using PI staining are shown in Figure 3.20.

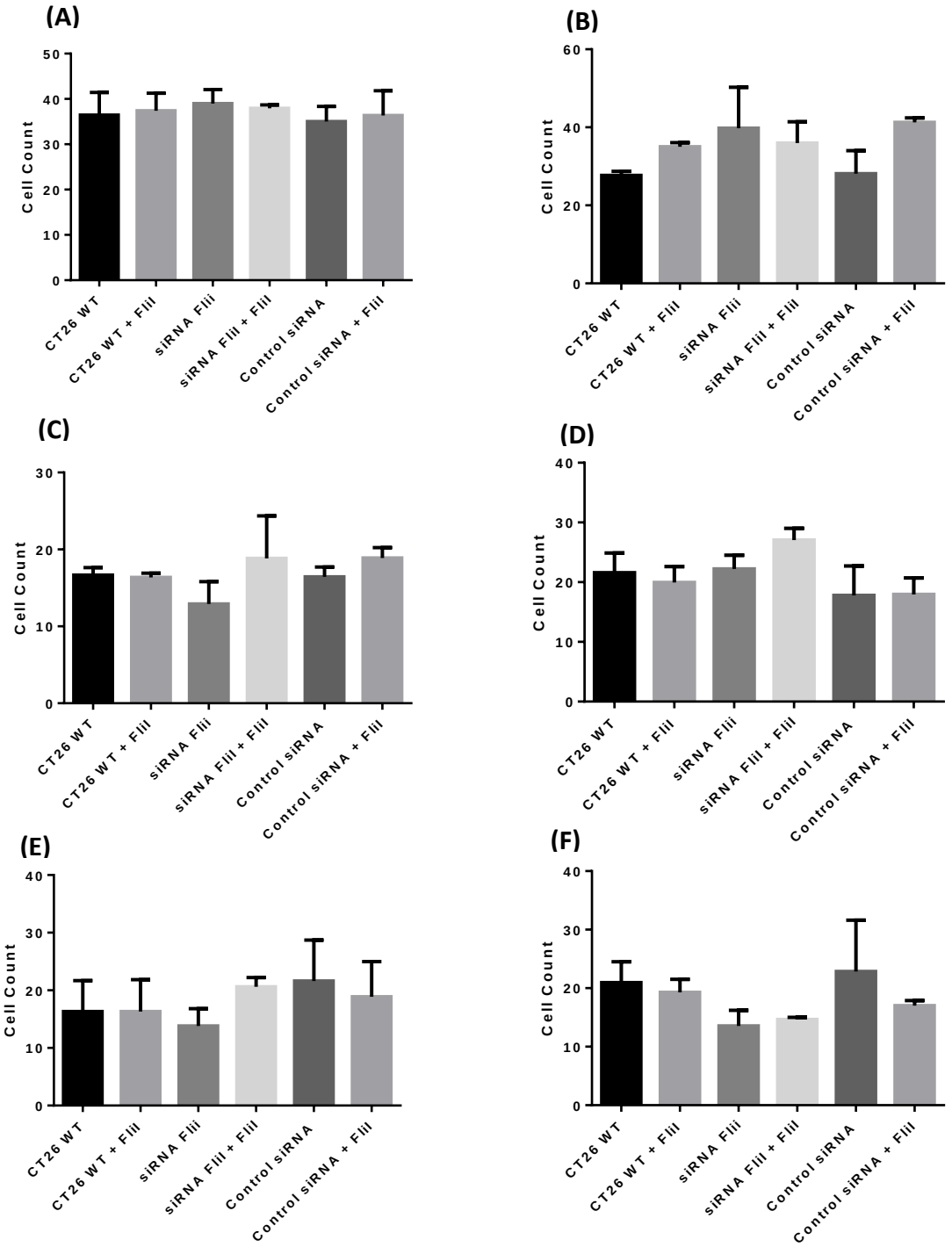


Figure 3.19: Cell cycle analysis using in CT26 cells expressing different levels of FliI with or without treatment with exogenous recombinant FliI (rFliI 1µg/ml) *in vitro*. Effect of FliI on phases of the cell cycle in CT26 WT, siRNA FliI CT26 and transfection control cells (siRNA Siah2 CT26), **(A)** and **(B)** G1 phase, **(C)** and **(D)** S phase, and **(E)** and **(F)** G_2M phase at 24 **(A, C, E)** and 48 **(B, D, F)** hours' incubation time. Phases were determined using propidium iodide staining and flow cytometry. Results are shown as mean ± SEM (n=3). *p<0.05 indicates statistical significance to control CT26 WT cells (Dunnet's multiple comparison test). No significant differences were observed in any treatment.

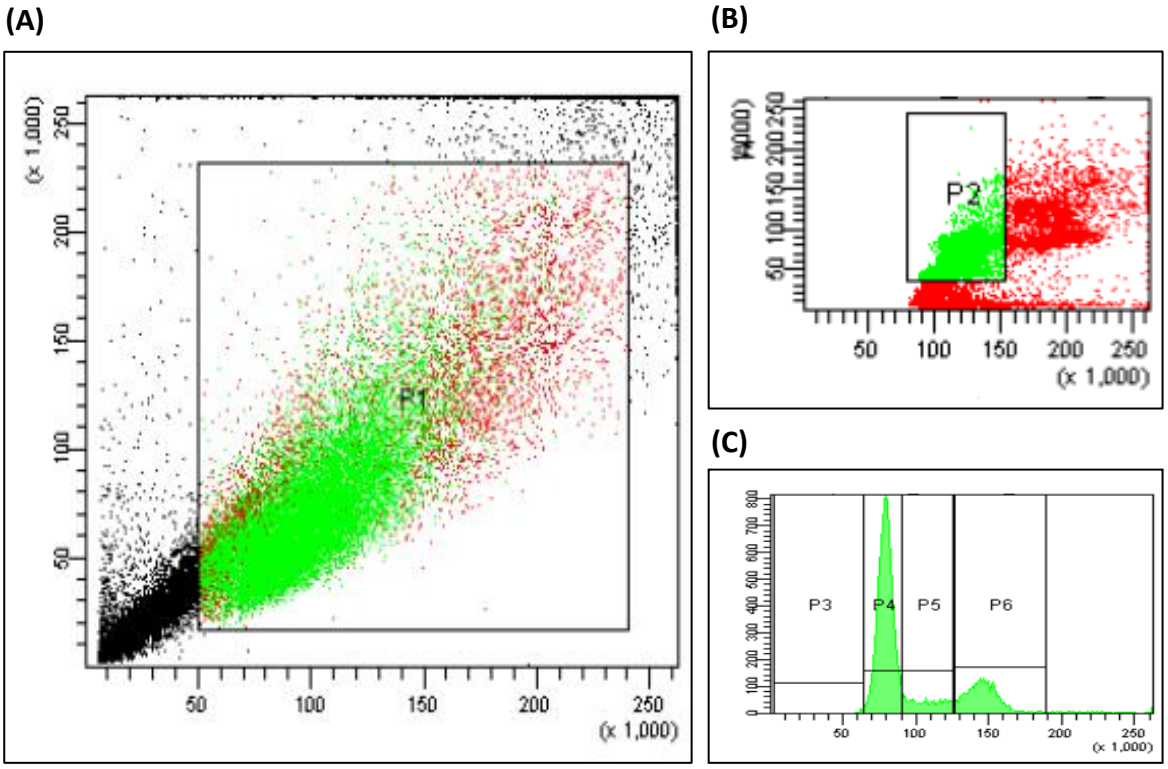


Figure 3.20: Representative cell cycle dot plots using flow cytometry analysis *in vitro.* (A) gated cell populations (P1) of the basis of FSC and SCC scatter, **(B)** selected within another gate (P2) and **(C)** applied to a histogram analysing PI fluorescence. The histogram shows the markers for identifying cells in P3 (non-dividing), P4 (G1 phase), P5 (S phase) and P6 (G_2M phase). Results were obtained using flow cytometry (FACSCanto, BD Biosciences) and analysed using FACSDiva Software (BD Biosciences).

3.2.6 Effect of FliI on colon cancer cell cytokine secretion

3.2.6.1 Knockdown of FliI reduced while exogenous recombinant FliI increased the levels of TNF-α, VEGF and IL-10 *in vitro*

Analysis of cytokines that play a role in cancer progression and potential roles in inflammation or inhibition, such as TNF-α, VEGF, IL-6 and IL-10, were determined in colon cancer cell supernatants using ELISAs (Figure 3.21). Colon cancer cell supernatants were obtained from HT29 WT and siRNA FliI-treated HT29 cells without and with addition of exogenous recombinant FliI (1 µg/ml) after 24 hours' incubation. Levels of TNF-α ($p<0.01$),

VEGF ($p<0.05$) and IL-6 ($p<0.01$) were significantly reduced in siRNA FliI-treated HT29 cell supernatants compared to the levels in HT29 WT cells. However, no difference was observed in the levels of IL-10 in any of the conditions. It was also found that addition of exogenous recombinant FliI significantly increased the secretion of TNF-α in HT29 WT cell supernatants ($p<0.05$) and increased the level of IL-6 in siRNA FliI HT29 cell supernatants ($p<0.001$).

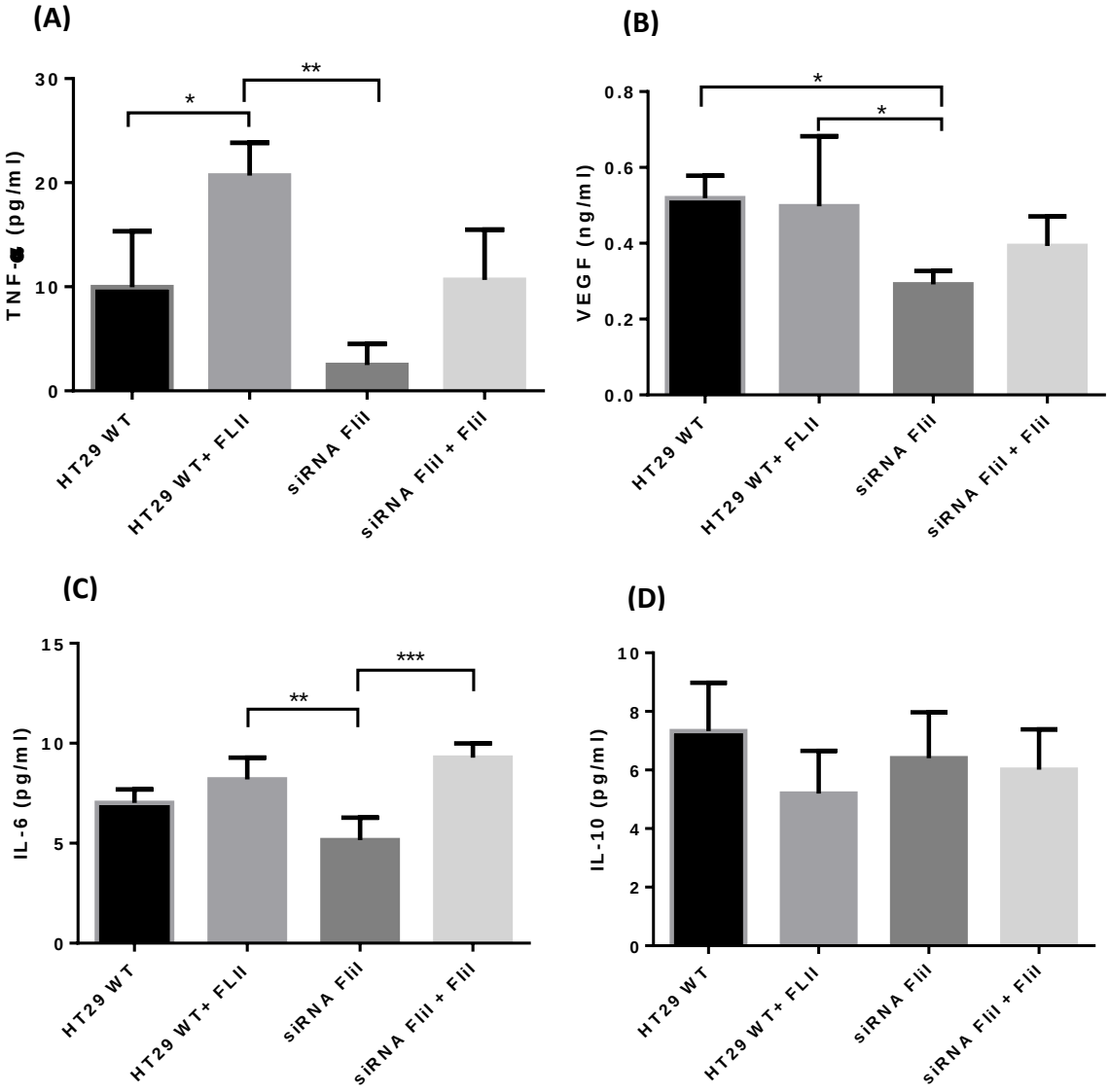


Figure 3.21: Cytokine levels in HT29 cell culture supernatants from cells expressing different levels of FliI and after treatment with exogenous recombinant FliI (rFliI 1µg/ml). Supernatants from human HT29 colon cancer cells expressing different levels of FliI as well as after treatment with recombinant FliI (1 µg/ml) were analysed for **(A)** TNF-α, **(B)** VEGF, **(C)** IL-6 and **(D)** IL-10 by ELISA assay. Results are shown as mean (pg/ml) ± SEM (n=3). ***p<0.001, **p<0.01 and *p<0.05 indicate statistical significance between experimental groups (Tukey's multiple comparison test).

3.3 Discussion

3.3.1 Role of FliI in cell migration

To enable investigation into the role of FliI in colon cancer growth and metastasis, it is important to establish baseline mRNA levels of FliI in the cell lines used in experiments. PCR results showed that there was detectable expression of FliI in mouse and human colon carcinoma cell lines (CT26 and HT29 cells) that were higher than in control cultured cells, mouse fibroblasts and HEK 293T cells, respectively. FliI in both cell lines was then knocked down using FliI siRNA or overexpressed using transfection with a FliI expression plasmid and expression confirmed with qPCR analysis. This enabled us to begin studies to determine the role of FliI in cell migration, proliferation, invasion and cell cycles, and its effect on cytokine release.

As a member of the actin-remodelling family of gelsolin-like proteins, FliI is involved in cell motility, adhesion and contraction (Davy et al., 2000). It is worthwhile to emphasise the difference between migration and invasion in order to perhaps explain the mechanism of FliI in affecting the migration and invasion of cancer cells (which will be the basis of later chapters). Based on their definition, migration occurs on 2D surfaces in the absence of any obstructive fibre network (ECM), while invasion involves movement of cells through a 3D matrix in which cells must modify their shape and remodel the ECM to enable them to protrude through the cell membrane (as lamellipodia or invadopodia) and move through the matrix. Therefore, invasion requires cell attachment and adhesion to successfully invade the matrix (Kramer et al., 2013).

Cell migration is crucial in determining cell motility, and deregulation of this process could contribute to pathological conditions such as tumour angiogenesis and metastasis (Ridley et

al., 2003). In this study, it has been confirmed that knockdown of FliI in the cell increased 2D migration while overexpression of FliI reduced cell migration. Studies done previously (Cowin et al., 2007) showed that FliI deficiency (using siRNA knockdown) in fibroblast monolayers derived from mice skin increased the rate of wound closure in a scratch assay as compared to WT and FliI overexpressing fibroblasts. Our studies confirm that this mechanism seems to apply in the tumour cell lines used in this study. In addition, the same study also showed that FliI-deficient ($FliI^{+/-}$) mice showed improved wound healing *in vivo* via increasing epithelial migration and proliferation. A previous study by Mohammed et al. (Mohammad et al., 2012) suggested that actin filament ends are not capped in migrating cells where FliI has been knocked down, and that FliI retards the turnover of actin filaments and thus can be associated with slower migration. They further suggested that FliI mediated cell migration via FA-regulated actin-capping activity. *In vivo* studies show a significant increase in CD151, integrin β1 protein and laminin protein expression in FliI heterozygous mice. Increases in these factors are associated with the escalation of cellular migration and contraction (Zlatko Kopecki et al., 2009). Conversely, the overexpression of FliI decreased the expression of integrin receptors, causing a reduction in cell migration and proliferation, and the atypical removal of ECM proteins (Zlatko Kopecki et al., 2009).

3.3.2 FliI affects cell apoptosis and cytokine release

The importance of programmed cell death by apoptosis in acting to prevent or suppress cancer progression has been well established in previous studies (de Bruin & Medema, 2008; Hanahan & Weinberg, 2000; Lowe, Cepero, & Evan, 2004). Resistance to apoptosis can allow tumour cells to survive and undergo tumour progression. However, there are also findings pointing to the ability of cancer cells to switch their susceptibility to apoptosis, and

this may play a key role in tumour development (J. Adams & Cory, 2007; Lowe et al., 2004). It has been shown that activation of a number of downstream pro-apoptotic effector pathways via growth-deregulating oncoproteins was likely to induce apoptosis (Evan & Vousden, 2001). In this study, we found that adding of exogenous FliI to WT cells increased apoptosis, while knockdown of FliI significantly decreased the percentage of cells undergoing apoptosis. Cancer cells can undergo evolution to escape from this destructive machinery and suppression of apoptosis mechanisms is common in many tumour cells (Evan & Vousden, 2001). However, the mechanisms underlying this process are still unclear.

In contrast to apoptosis, necrotic cell death has been commonly shown to release pro-inflammatory factors into the tumour microenvironment that can promote tumour progression (Hanahan & Weinberg, 2011) and areas of tumour necrosis are often an indicator of poor prognosis. Necrotic cells have also been shown to enhance recruitment of immune cells which are capable of promoting tumour development and inducing angiogenesis (Galluzzi & Kroemer, 2008; Grivennikov, Greten, & Karin, 2010). In addition, by releasing regulatory factors such as IL-1α cells undergoing necrosis may directly stimulate adjacent viable cells to proliferate and thus potentiate neoplastic progression (Grivennikov et al., 2010).

Even though these findings show that knockdown of FliI reduced cellular apoptosis and increased cell proliferation, which are suggestive of FliI having an anti-tumour activity, we also show that these cells produced less TNF-α, VEGF and IL-6, which are known to have potent effects in promoting cancer progression. It has been shown that cells can have an immediate response on stimulation of growth factors which actively trigger cell migration (Maheshwari et al., 1999; Ware et al., 1998). Furthermore, addition of exogenous

recombinant FliI increased apoptosis and also showed an increase in the level of inflammatory cytokines measured in this study.

3.3.3 Effect of FliI on cell proliferation and cell cycle

Our data suggests that knockdown of FliI increased cell proliferation, as shown by nuclear staining of the proliferation markers PCNA and Ki-67. FliI has previously been found to regulate cell proliferation via FliI localisation to β-tubulin-based structures which are then involved in cell division (Davy et al., 2001). Studies by collaborators (Cowin et al., 2007) have shown that siRNA knockdown of FliI in fibroblasts and keratinocytes increased cell proliferation, similar to our results. Reduced proliferation and migration were also observed in fibroblasts overexpressing FliI, resulting in impairment of the healing process. In fact, it has been postulated that FliI negatively regulates cell proliferation and migration via an actin-severing function (Cowin et al., 2007).

PCNA is widely used as a tumour marker, but there are conflicting results in the correlation between expression of PCNA and tumour progression and prognosis (S.-C. Wang, 2014). In addition to having a role in proliferation, PCNA is also required for DNA repair, which is also involved in tumour progression (Toschi & Bravo, 1988). A high percentage of proliferating cells often correlates with aggressive tumours in various types of cancer, while other tumours grow relatively slowly due to a high rate of cell loss via apoptosis (Arends et al., 1994). This indicates that in certain tumour types, increased levels of both apoptosis and mitotic activity may reflect aggressive tumour growth.

Despite some of the differences observed in this study, there are a number of limitations that need to be considered. Overexpression of FliI in tumour cells was only partially established, since the cells did not produce the expected large increases in levels of FliI

expression. Other laboratories, including our collaborators' laboratory and that of Dr Rachael Murray (QUT), have also experienced problems with FliI overexpression, which seem to relate to correct processing of the protein in cells that have incorporated the FliI plasmid. This aspect of the project requires further study. The establishment of cell lines that significantly overexpress the FliI gene will greatly aid in further studies in this area.

3.4 Conclusion

In this chapter we have explored a number of *in vitro* experimental approaches in order to elucidate the role FliI may play in the regulation of four well-established tumour growth promoters: cell migration, proliferation, apoptosis and the secretion of tumour-enhancing cytokines. The effects observed remain difficult to interpret, as FliI appears to regulate both pro- and anti-tumour functions, and it is evident that there are complexities involved in the functions of FliI that demand further and extensive examination. Similar findings have been made in relation to wound healing, in that FliI can correlate with enhanced adhesion and reduced migration, which would both tend to be seen as tumour inhibiting. However, the *in vitro* findings thus far continue to support the hypothesis that FliI may play a potential role in tumour growth and progression, and so provide a rationale for further exploration. The cellular effects of FliI on two other important tumour-regulating factors, cell adhesion and invasion, will be discussed in Chapter 4. In conjunction with the findings reported in this chapter, this work provides motivation to pursue these investigations via an *in vivo* cancer model.

Chapter 4 Flightless I Mediates Cell Invasion and Adhesion

4.1 Introduction

Cancer cell migration and invasion into adjacent tissues and extravasation into blood/lymphatic vessels are necessary for metastasis, and acquisition of a migratory phenotype of invasive carcinoma cells has been shown in several types of human cancers (W. Wang et al., 2005). Invasion and metastasis are dependent on the ability of tumour cells to modify and degrade the constituents of the ECM around them (Hoon et al., 2006). Metastasis then involves two major phases, the physical translocation of tumour cells from the primary tumour microenvironment to distant tissues and then the establishment of secondary tumours at these sites (Chaffer & Weinberg, 2011). Protrusions of the cell membrane into the ECM by invading cells, invadopodia, are important in this process since several pro-invasion activities are centred at these sites. These activities include branched actin assembly close to the membrane, cell-signalling molecules and adhesion sites for the ECM, and secretion of proteases that lead to degradation and remodelling of the ECM (Weaver, 2006).

Invadopodia formation has been observed in highly invasive cancer cells and has therefore been implicated in tumour cell metastasis (Yamaguchi et al., 2005). Since invadopodia are enriched in actin regulatory proteins, adhesion molecules, signalling/adaptor proteins, membrane-remodelling proteins and matrix-degrading proteases, it was considered

worthwhile to further investigate the possible role of FliI in modifying important FA proteins which may affect the dynamics of FA complexes and so have an impact on invadopodia formation.

In this study, because of the limitations of using 2D cell-culture systems showing cell migration, a more physiological 3D culture environment was used to obtain a better understanding of the possible regulation by FliI of cell invasion and adhesion. A co-culture system was also used in conjunction with transwell invasion assays using ECM-coated surfaces overlaid with a porous filter (Kramer et al., 2013). This assay was used to determine whether changes in FliI influence on invasiveness of the cancer cells had an impact on FA protein expression and to look for the involvement of invadopodia. Therefore, this chapter discusses the effect of intracellular expression levels and the exogenous addition of FliI in the regulation of cell invasion and adhesion in order to further elucidate the involvement of FA proteins and regulation of invadopodia in promoting cancer progression and metastasis.

4.2 Results

4.2.1 Effect of FliI expression on cell invasion

4.2.1.1 Knockdown of FliI reduced cell invasion and exogenous FliI increased invasiveness of cancer cells

The effect of FliI in inducing the invasiveness of cancer cells was determined using a Matrigel invasion assay. CT26 WT and siRNA FliI CT26 cells treated with or without exogenous recombinant FliI (rFliI; 1 μg/ml), were analysed. Results show that knockdown of FliI greatly inhibited cell invasion, while addition of exogenous FliI increased the percentage of cells invading through the membrane in both WT and siRNA knockdown cells. The data

shown in Figure 4.1 is represented in two ways; (A) indicates the percentage of cell invasion and (B) the invasion index (ratio of invading cells compared to control cells).

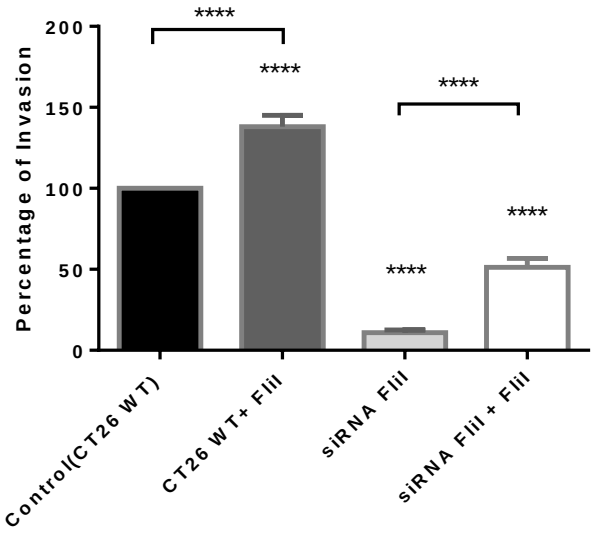


Figure 4.1: **3D cell invasion of CT26 colon cancer cells expressing different levels of FliI and after recombinant FliI treatment *in vitro*.** Quantification of cell invasion (determined as % mean of invading cells in FliI-treated cells compared to the mean in control cells) in CT26 WT and siRNA FliI CT26 cells with or without recombinant FliI. Results are shown as mean ± SEM (n=3). Significant differences from control cells (WT CT26) shown as ****p<0.0001, (Dunnett's multiple comparison test).

Images from an inverted microscope at 40X magnification show that there were few knockdown-treated cells invading through the Matrigel matrix and membrane as compared to WT cells, while the addition of exogenous FliI appeared to increase the invasiveness of cells (Figure 4.2).

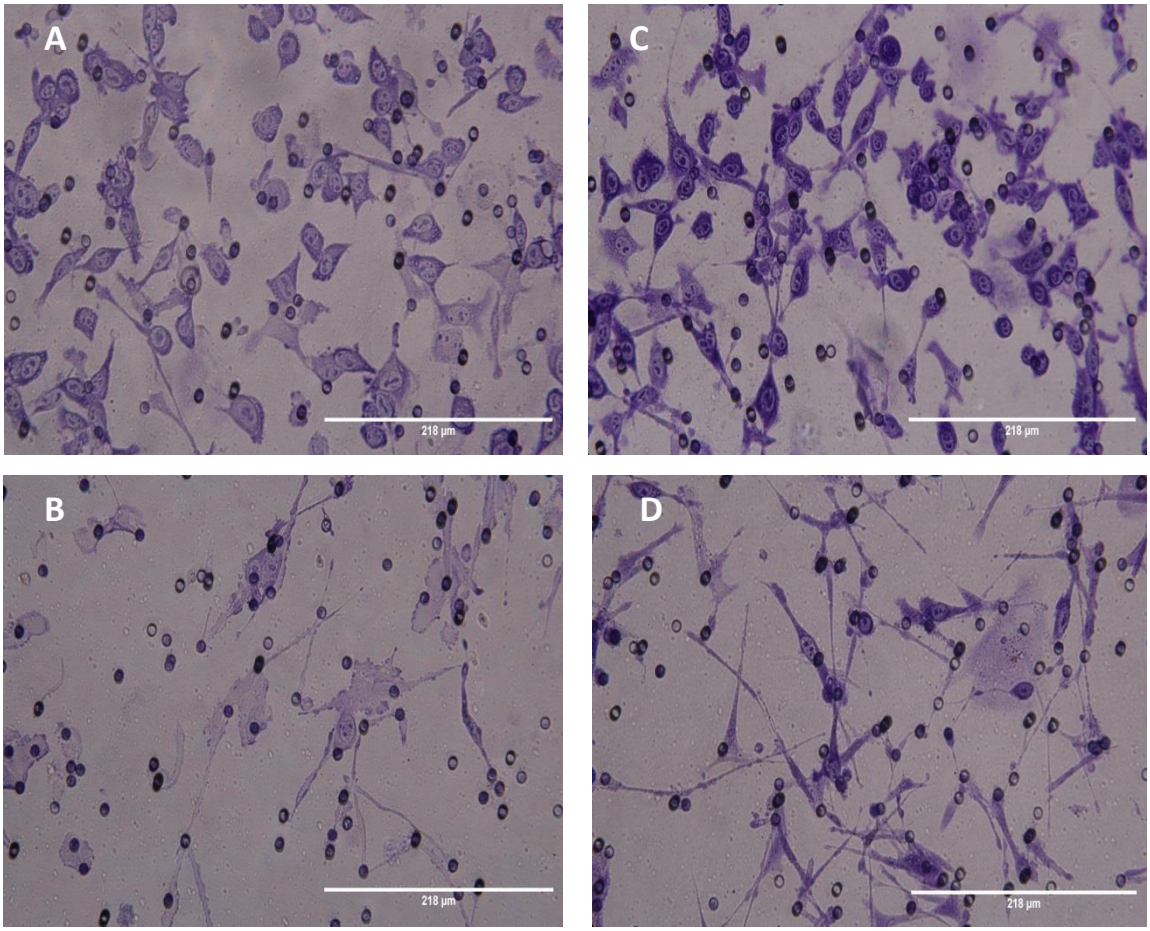


Figure 4.2: Toluidine blue staining of invading colon cancer cells expressing different levels of FliI and after exogenous FliI treatment. Representative phase micrographs of cells **(A)** CT26 WT and **(B)** siRNA FliI CT26, **(C)** CT26 with exogenous recombinant FliI and **(D)** siRNA FliI CT26 with exogenous recombinant FliI. Invading cells were fixed in 2.5% paraformaldehyde and stained with toluidine blue (40X magnification).

4.2.2 Effect of FliI on invadopodia marker expression

4.2.2.1 Knockdown of FliI downregulates invadopodia marker cortactin in colon cancer cells *in vitro*

Total cell fluorescence after immunofluorescence staining for cortactin was used as the main marker of invadipodia and was determined in CT26 WT and siRNA FliI CT26 cells. Higher expression levels of cortactin was found in CT26 WT cells compared to siRNA FliI-treated CT26 cells, which showed a significantly lower level of cortactin fluorescence. Graphs showing total fluorescence intensity for cortactin in WT and siRNA FliI-treated CT26 cells are shown below (Figure 4.3).

Immunofluorescence photomicrographs showing expression of cortactin are shown in Figure 4.4 comparing CT26 WT and siRNA FliI CT26 cells.

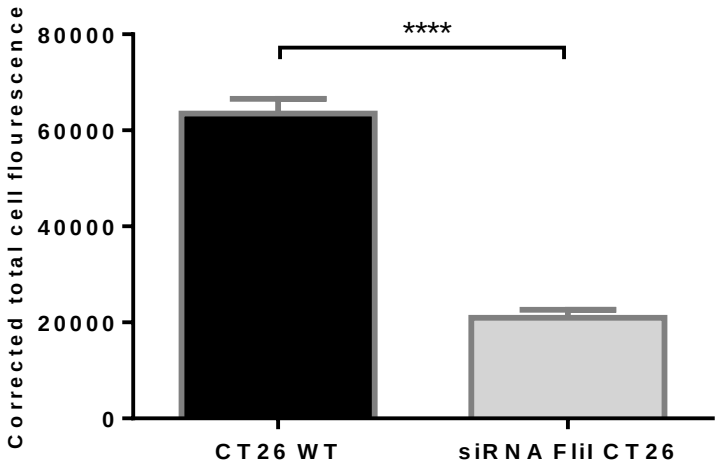


Figure 4.3: Cortactin expression in colon cancer cells expressing different levels of FliI. Expression of the invadopodia marker cortactin, based on total fluorescence in CT26 WT and siRNA FliI CT26 cells. Results are shown as corrected total cell fluorescence mean ± SEM (n=3). **** shows $p<0.0001$ is statistically significant between experimental groups (Student's unpaired t-test).

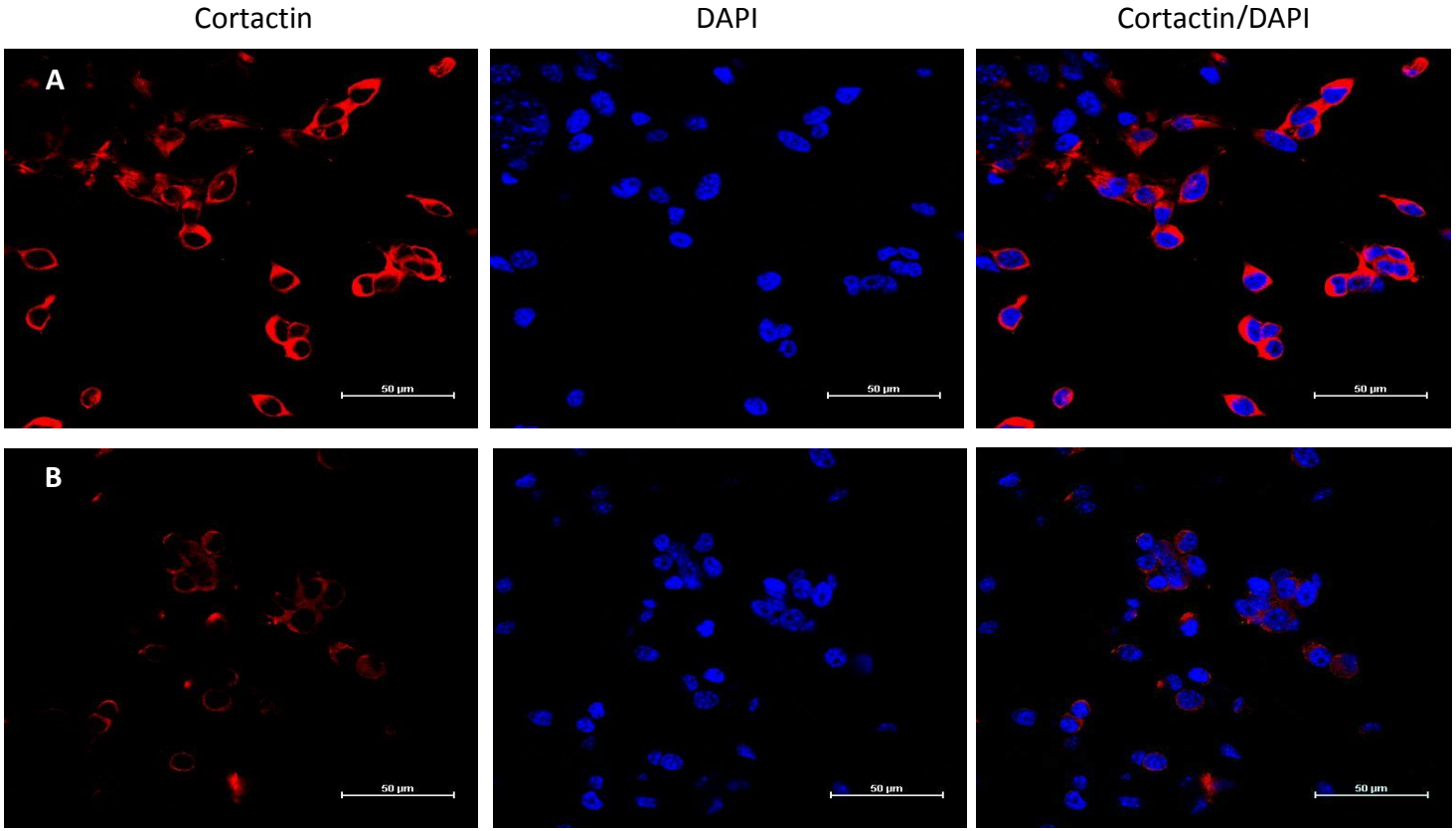


Figure 4.4: Cortactin staining in CT26 colon cancer cells expressing different levels of FliI detected by immunofluorecsence. Representative immunofluorescence micrographs showing expression of cortactin in **(A)** CT26 WT and **(B)** siRNA FliI CT26 cells. Cells were immunostained for invadopodia marker cortactin (red) and counterstained with DAPI (blue) to show nuclei. Images were taken using an A1 confocal microscope (Nikon) at 600X magnification.

4.2.3 Effect of FliI expression on cell adhesion

4.2.3.1 Knockdown of FliI increased cell adhesion

Cell adhesion assays were performed to determine the possible effect of FliI expression levels in regulating cell adhesiveness. Results showed that knockdown of FliI using siRNA increased the ability of the cells to adhere to both types of coating matrix that were used to mimic ECM, which were poly-L-lysine (Figure 4.5(A)) and laminin (Figure 4.5(B)). Adhesion was determined at 15 and 30 minutes after cell seeding. Addition of exogenous FliI decreased cell adhesiveness of siRNA FliI-treated CT26 cells at 15 and 30 minutes' incubation time on laminin; however, there was no effect on cell adhesion of the poly-L-lysine substrate. Representative images of phase-contrast micrographs of adherent cells (CT26 WT and siRNA FliI CT26) on the poly-L-lysine substrate, in the presence and absence of exogenous FliI, are shown in Figure 4.6.

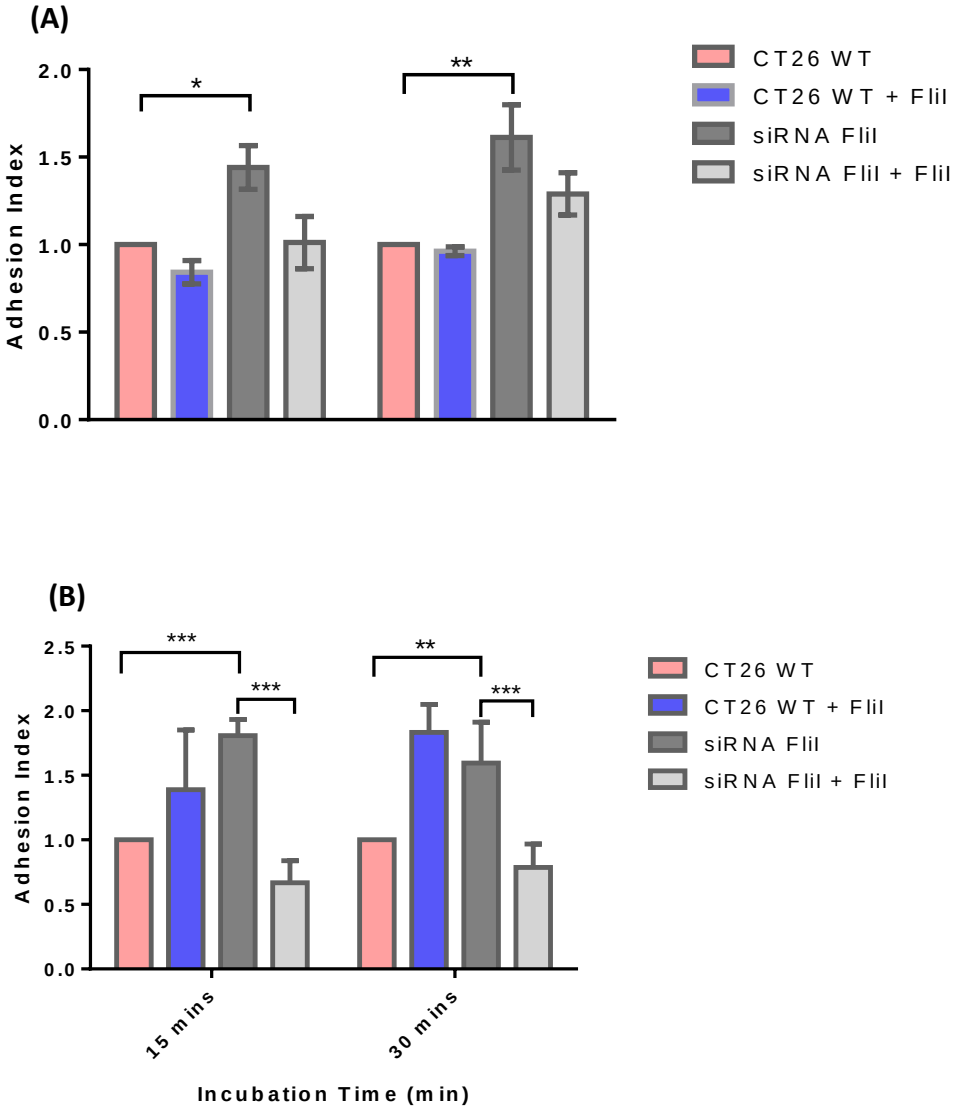


Figure 4.5: Cell adhesion assay in colon cancer cells expressing different levels of FliI and after exogenous FliI treatment. Cell adhesion index using **(A)** poly-L-lysine and **(B)** laminin coating materials at 15 and 30 minute time points in CT26 WT and siRNA FliI CT26 cells with and without exogenous recombinant FliI. Results are shown as mean ± SEM (n=3). ***p<0.001, **p<0.01, *p<0.05 show significant difference to control cells (CT26 WT) using Dunnett's multiple comparison test.

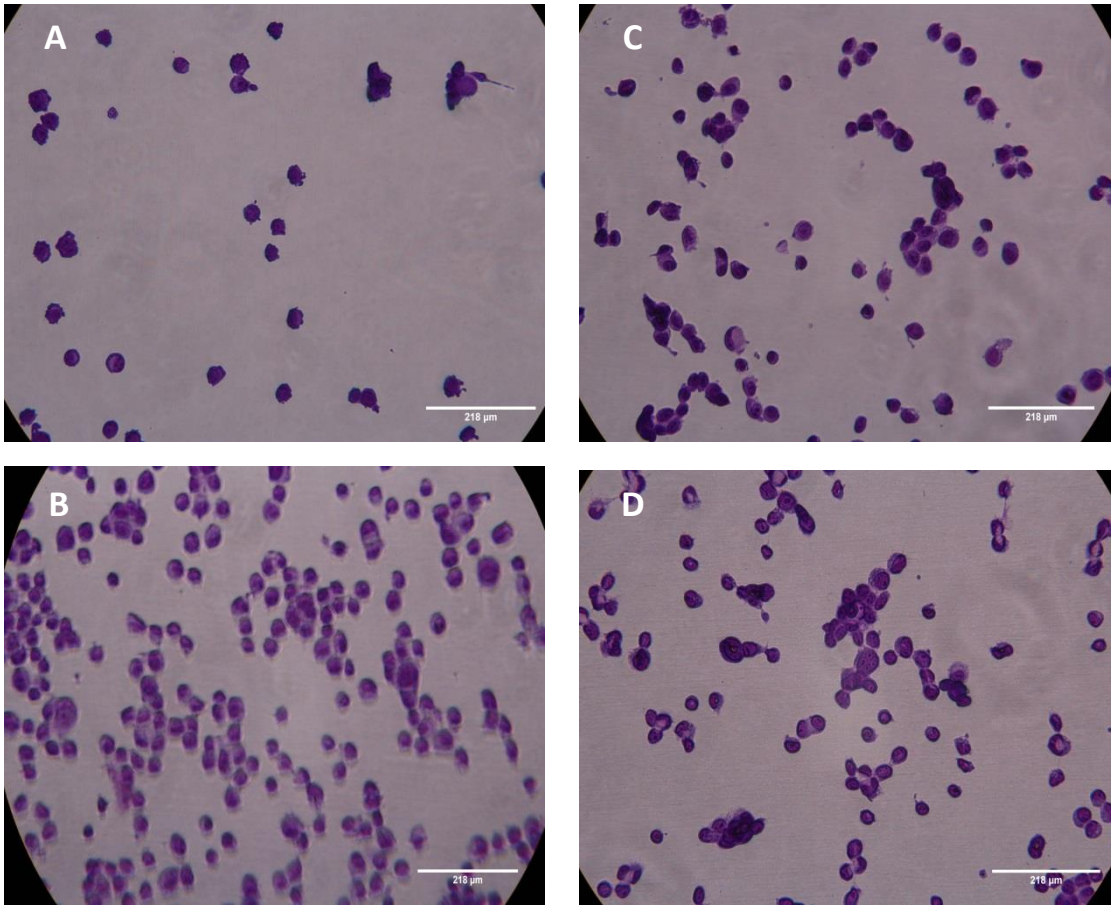


Figure 4.6: Toluidine blue staining of adherent colon cancer cells expressing different levels of FliI and after recombinant FliI treatment. Representative phase micrograph of adherent cells **(A)** CT26 WT and **(B)** siRNA FliI CT26, **(C)** CT26 WT with exogenous recombinant FliI and **(D)** siRNA FliI CT26 with exogenous recombinant FliI on poly-L-lysine substrate. Adherent cells were fixed in 2.5% formaldehyde, stained with toluidine blue and viewed under a light microscope at 20X magnification.

4.2.4 Effect of FliI on focal adhesion protein expression

4.2.4.1 Knockdown of FliI upregulates FA protein paxillin in colon cancer cells *in vitro*

Immunofluorescence staining of the FA protein paxillin showed an upregulation of the expression of this FA protein in siRNA FliI-treated CT26 cells compared to CT26 WT cells (Figure 4.7). Statistical analysis of paxillin expression levels showed a highly significant

difference ($p<0.001$) in comparison to CT26 WT cells. Representative images of immunofluorescent staining for paxillin are shown in Figure 4.8.

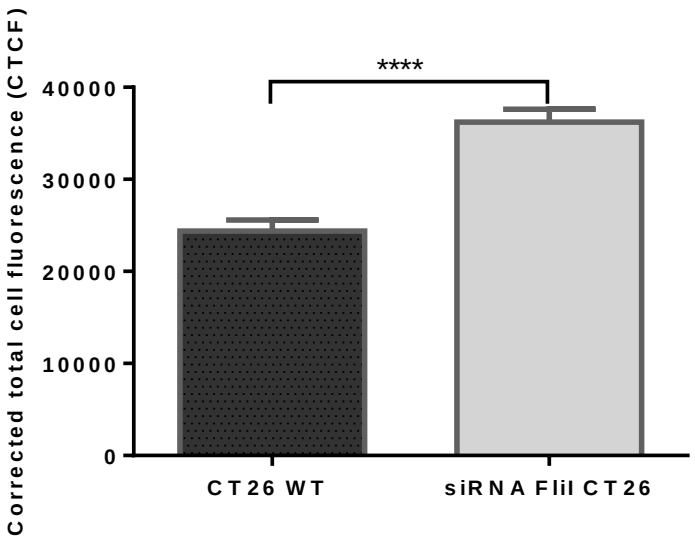


Figure 4.7: Paxillin expression in CT26 colon cancer cells expressing different levels of FliI *in vitro*. Expression of FA protein paxillin, based on total cell fluorescence, comparing CT26 WT and siRNA FliI-treated CT26 cells. Results are shown as mean corrected cell fluorescence ± SEM (n=5). ****$p<0.0001$, significant difference to control cells CT26 WT (Student's unpaired t-test).

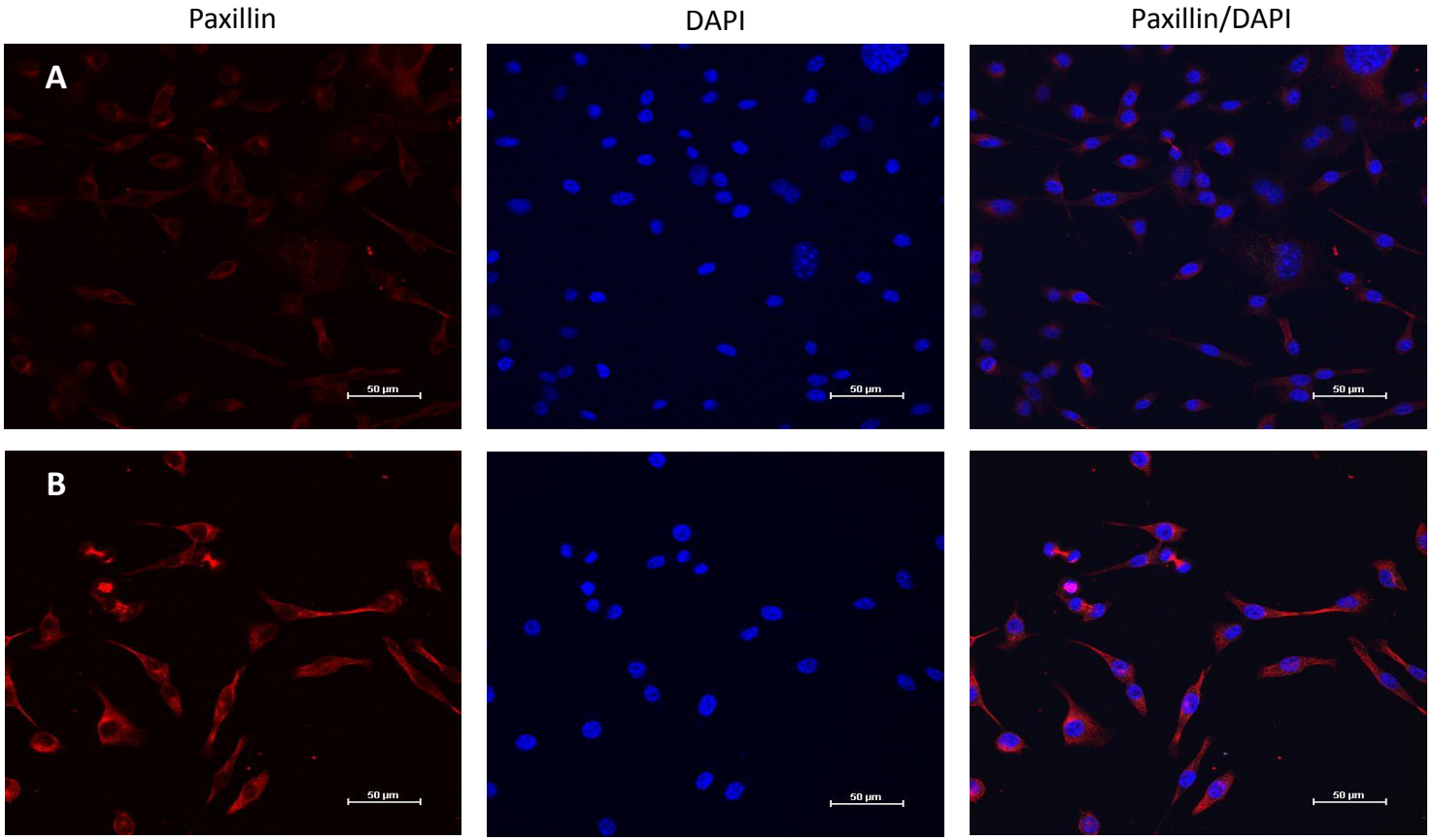


Figure 4.8: Paxillin staining in CT26 colon cancer cells expressing different levels of FliI determined by immunofluorecsence. Representative immunofluorescence micrographs showing expression of paxillin in **(A)** CT26 WT and **(B)** siRNA FliI CT26 cells. Cells were immunostained for FA protein paxillin (red) and counterstained with DAPI (blue). Images were taken using an A1 confocal microscope (Nikon) (400X magnification).

4.2.4.2 Knockdown of FliI upregulated vinculin expression in colon cancer cells *in vitro*

Immunofluorescence staining for the FA protein vinculin also showed a higher level of expression in siRNA FliI-treated CT26 cells compared to CT26 WT cells (Figure 4.9). Respresentative images of immunofluorescence staining for vinculin in CT26 WT and siRNA FliI-treated CT26 cells are shown in Figure 4.10.

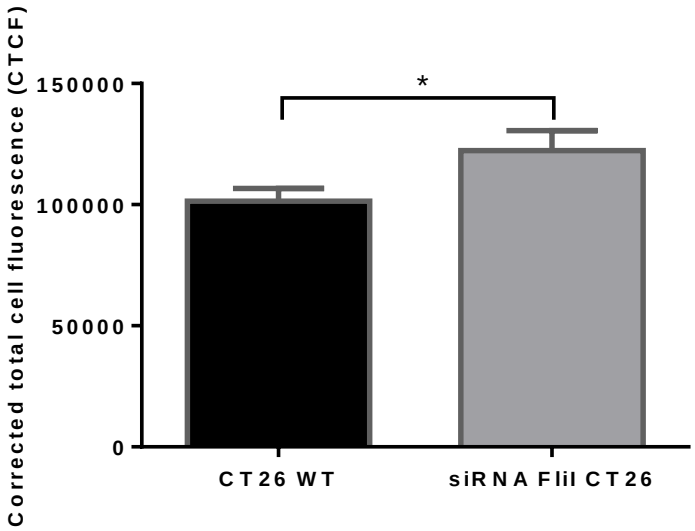


Figure 4.9: Vinculin expression in CT26 colon cancer cells expressing different levels of FliI. Expression of FA protein vinculin, based on total cell fluorescence, in comparing CT26 WT and siRNA FliI-treated CT26 cells. Results are shown as mean corrected cell fluorescence ± SEM (n=5). *p<0.05 shows significant difference to control cells (CT26 WT) (Student's unpaired t-test).

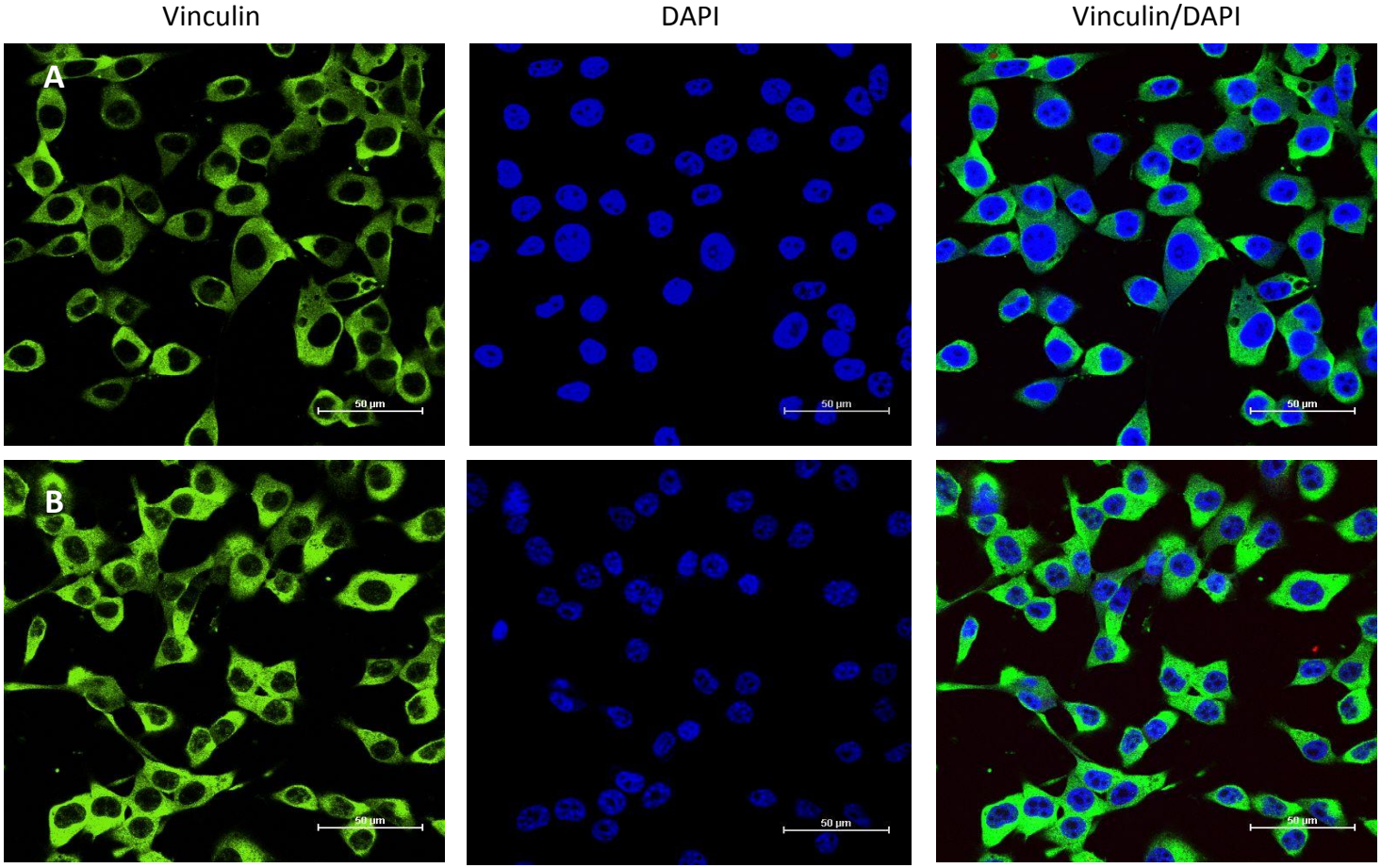


Figure 4.10: Immunofluorescence staining of vinculin in CT26 colon cancer cells expressing different levels of Flil. Representative immunofluorescence images showing expression of vinculin in CT26 WT **(A)** and siRNA Flil-treated CT26 cells **(B)**. Cells were immunostained for vinculin (green) and counterstained with DAPI (blue). Images were taken using an A1 confocal microscope (Nikon) at 600X magnification.

4.2.5 Co-culture system with primary fibroblasts

A number of stromal cells in the tumour microenvironment can enhance growth and facilitate the ability of cancer cells to metastasise to distant organs. Stromal cells have the ability to create a surrounding microenvironment that favours tumour growth and spread to secondary sites. In order to obtain a better understanding of the effect of fibroblasts within the tumour microenvironment and the role of FliI in this microenvironment, co-culture systems with primary fibroblasts (FIB) were established and the expression of FliI in the system was determined. A 1:1 ratio of cancer cells (either CT26 WT or siRNA FliI CT26) and FIB were co-cultured for 24 hours. Controls, which were CT26 WT and FIB only, were also included. The expression of FliI in the co-culture system was determined using quantitative RT-PCR. There was an approximate four-fold increase in the expression of FliI in the co-culture of WT and primary fibroblasts in comparison to single cultures of either CT26 WT or fibroblast cells (Figure 4.11).

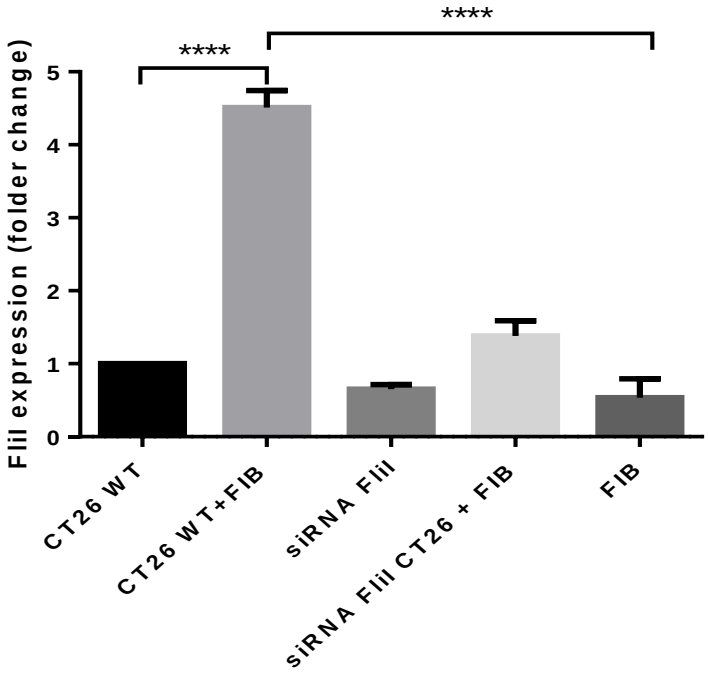
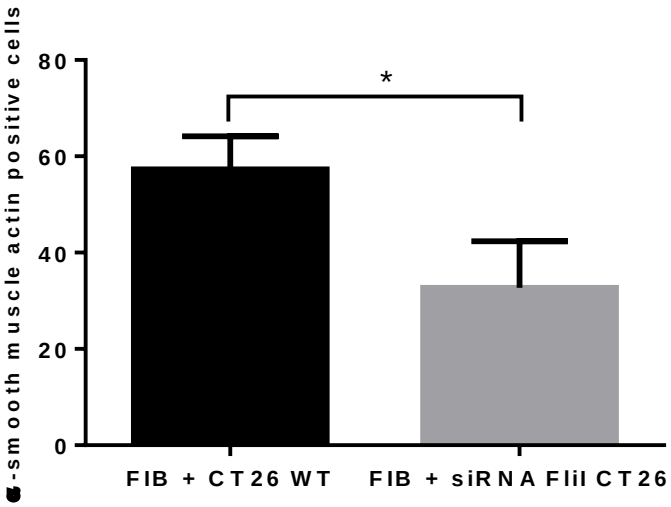


Figure 4.11: FliI expression in co-cultures of primary fibroblasts with colon cancer cells expressing different levels of FliI. Quantitative PCR analysis showing expression of FliI in co-cultures of primary fibroblasts (FIB) with and without CT26 WT and siRNA FliI CT26 cells compared to single cultures. Results are shown as FliI expression (n-fold change) ± SEM (n=3). ****p<0.0001 significant difference to control cells (WT CT26) (Tukey's multiple comparison test).

Expression of the myofibroblast marker α-SMA was also evaluated to determine whether the expression of FliI had an effect on the differentiation of fibroblasts into their activated form, α-SMA-positive myofibroblasts. Transwells coated with Matrigel were used in these experiments to separate the two types of cells in the co-culture system. Interestingly, there was a significant decrease in the number of α-SMA-positive cells in the siRNA FliI CT26 co-cultures, suggesting fewer myofibroblasts were activated in the presence of CT26 cells with reduced FliI expression (Figure 4.12). Greater α-SMA expression was observed in CT26 WT cell co-cultures, as shown in representative images of the immunostaining (Figure 4.12).



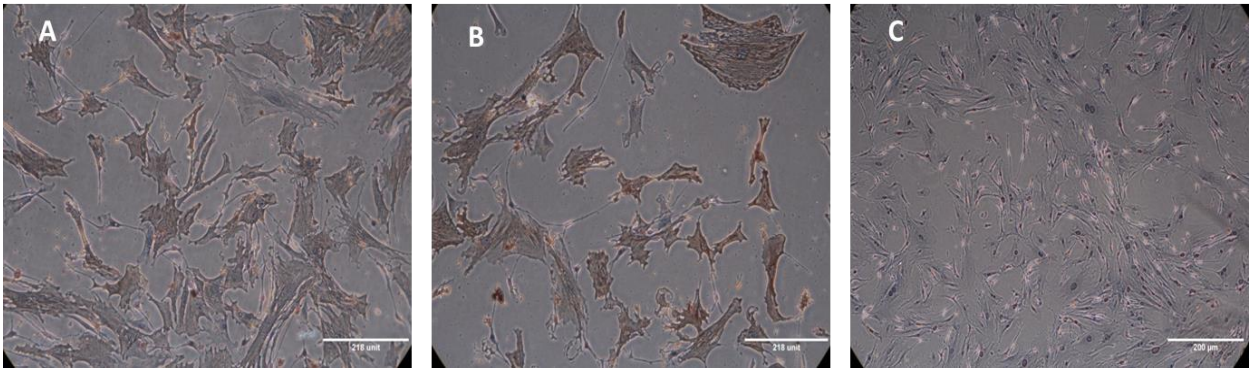


Figure 4.12: α-SMA expression in primary fibroblasts (FIB) co-cultured with colon cancer cells expressing different levels of FliI detected by immunoperoxidase staining. Co-cultures of primary fibroblasts with **(A)** CT26 WT and **(B)** siRNA FliI CT26 cells were stained with anti-α-SMA antibody. (C) Negative control of α-SMA of primary fibroblasts cells. Results are shown as α-SMA-positive cells ± SEM (n=3). *p<0.05 significant difference to control cells (CT26 WT) (Student's unpaired t-test).

4.3 Discussion

4.3.1 Role of FliI in cell invasion

3D matrix invasion assays are an established method to determine the capacity of particular cells to invade the ECM and these assays have been frequently used in studies of cancer cell

invasion, for example colon cancer (F. Zhang et al., 2013) and human melanoma cell invasion (Kreiseder et al., 2013). In this assay, ECM is coated onto a membrane that has pores of a size that block non- or less invasive cells from migrating through the membrane. However, invasive cells can move through the ECM layer by degrading the matrix, then penetrate the 8 μm pores and adhere to the bottom of the membrane. Our findings indicate that knockdown of FliI greatly inhibited cell invasion in Matrigel-coated invasion membranes as compared to WT cells. In contrast, the addition of exogenous FliI significantly increased the invasiveness of the cells, allowing them to protrude through the membrane. On wounding of cell monolayers in 2D, it has been shown that FliI is involved in lamellopodia formation in keratinocytes and remodelling of the ECM at the wound site (Cowin et al., 2007). Our findings suggest that FliI may have different effects on different cell types and also have a different effect on 3D invasion compared to 2D cell motility, which does not involve penetration of the ECM. Invasive tumour cells have been demonstrated to present dysregulated cell motility in response to extracellular signals from growth factors and cytokines (Wells, 1999) and our results suggest that FliI expression in cancer cells may contribute to increased invasiveness.

Our results also indicate that FliI might play a role in reorganisation of the cytoskeleton to create a protrusive actin meshwork, suggesting invadopodia formation in cancer cells; however, this needs to be further investigated. FliI has recently been reported to be a novel P-Rex1-enriched binding partner where FliI is required for P-Rex1-driven cell contraction that may in turn lead to cell migration (Marei et al., 2016). P-Rex1 induces Rac-1 downstream effects where Rac-1 has the ability to stimulate cell-to-cell adhesion, reduce cell migration and aid in ECM degradation by regulation of MMP release (G. Jin et al., 2000). Understanding the mode of

migration and invasion is important in designing strategies to target evolving cancer cells and block their movement through tissues and organs (Kramer et al., 2013). Migratory and invasive properties of cancer cells both involve dramatic reorganisation of the actin cytoskeleton and the concomitant formation of membrane protrusions which are required for invasive growth (Yilmaz & Christofori, 2009). However, the molecular processes underlying such cellular changes are still only poorly understood and the involvement of various migratory organelles, including lamellipodia, filopodia, invadopodia and podosomes, still requires better molecular and functional characterisation.

In summary, our results suggest that it may be beneficial to target and block FliI with the ultimate aim of limiting cell penetration of a barrier matrix (such as the basement membrane) and thus play a role in inhibiting metastasis.

4.3.2 Knockdown of FliI downregulates cortactin expression

Cancer cell invasion and metastasis are considered major negative prognostic factors, being associated with increased malignancy and decreased patient survival. Therefore, better understanding of the intracellular mechanisms controlling metastasis is crucially important to designing future therapies.

In comparisons between WT and knockdown of FliI in CT26 cells, our results showed that knockdown of FliI significantly downregulated expression of cortactin. Cortactin is involved in cell protrusions via lamellopodia and invadopodia, and it is present at dynamic sites of actin assembly (Yamaguchi & Condeelis, 2007). Therefore, cortactin is thought to act as an important regulator and a specific marker for invadopodia based on its capability to promote protease

secretion at the active adhesion site (Clark & Weaver, 2008). Furthermore, cortactin has the ability to bind to several cytoskeletal proteins through its C-terminal SH3 domain, which links it to numerous processes, including endocytosis, lamellipodial protrusion and directed cell migration (Ammer & Weed, 2008).

Overexpression of cortactin has been shown to increase ECM degradation via increased secretion of MMP-2 and MMP-9 (Clark & Weaver, 2008; Clark et al., 2007), and has also been shown to promote cell cycle progression through a RhoA-dependent pathway in cancer cells (Croucher, Rickwood, Tactacan, Musgrove, & Daly, 2010). Increased secretion of MMPs in tumour cells is closely related to invasive capability, increased metastasis and poor prognosis in several types of cancers (Buday & Downward, 2007; Stetler-Stevenson, Hewitt, & Corcoran, 1996). A previous study by (Bowden et al., 2006) suggested that cortactin and p-phosphotyrosine signalling is crucial for activation of invadopodia proteases leading to degradation of the ECM, thus mediating cell invasion. Cortactin expression has been shown to regulate membrane trafficking and adhesion dynamics that could alter the secretion of ECM from late endosomal/lysosomal compartments, while cortactin knockdown (KD) cells demonstrated defects in both cell motility and lamellipodia functions (B. H. Sung, Zhu, Kaverina, & Weaver, 2011).

In addition to invadopodia, cell adhesion via FAs is an important factor in governing cell motility and invasion. In our study, reduced expression of cortactin was observed with knockdown of FliI, suggesting that reduced FliI expression may inhibit cell invasion via reduced formation of invadopodia, although further experiments are required to confirm this. Elevated levels of cortactin expression have been shown to correlate with increased aggressiveness of head and

neck squamous cell carcinoma (HNSCC) cells and the cortactin levels were found to directly correlate with size of tumour, degree of vascularisation and cell proliferation in an orthotopic HNSCC *in vivo* model (Clark et al., 2009).

4.3.3 FliI regulation of cell adhesion

Previously, FliI has been shown to regulate cell adhesion, actin remodelling and intracellular signalling by co-localisation with actin and microtubule-based structures (H. D. Campbell et al., 2002; Davy et al., 2001; Z. Kopecki & Cowin, 2008). Previous studies on fibroblasts and keratinocytes by Professor Cowin's laboratory have suggested that in wound healing, FliI may increase cell adhesion by affecting FA proteins. The present study shows that knockdown of FliI in cancer cells increased cell adhesion; however, exogenous recombinant FliI had no significant effect on adhesiveness of the cells. Invasive cells have often been found to be less adhesive, and this is associated with higher motility and higher metabolic activity (Turner & Sherratt, 2002). In accordance with the effect of FliI on cell adhesion, we also observed a significant reduction of invasive cells in knockdown cells.

Recently, it has been shown that overexpression of FliI impaired the formation of tight junctions in mouse skin, which delayed the formation of the epidermal barrier that is required for repair (Zlatko Kopecki et al., 2014). During wound healing, FliI was reported to regulate adhesion via hemidesmosome formation and integrin-mediated cellular adhesion molecules (Zlatko Kopecki et al., 2009; Thiery, 2003). In fact, knockdown of FliI was also shown to increase adhesion in migratory cells via actin monomer assembly. However, a more recent study by Kopecki et al. (2015) suggested that FliI expression did correlate with increased 3D invasiveness in a

squamous cell cancer line. These findings suggest that the role of FliI in adhesion and invasion may be dependent on cell type and may be altered in transformed cells.

In addition, the adhesion process of epithelial cells to the ECM involves linkages to the actin cytoskeleton. This adhesion complex involves the adaptor proteins talin, paxillin and vinculin, and the ternary complex of pinch, parvin and integrin-linked kinase (ILK) (Yilmaz & Christofori, 2009). Therefore, expression of these adaptor proteins, specifically paxillin and vinculin, was also determined and is discussed in the next section of this study. Cell adhesion to neighbouring cells and/or to the surrounding ECM or stroma, the tumour microenvironment, also plays a critical role in tumour progression by regulating cell proliferation and survival, which enable tumour cell dissemination and metastasis. The local tumour microenvironment, including its molecular composition and organisation, density and rigidity of the stromal ECM, has in turn been shown to influence tumour malignancy (Deakin et al., 2012).

4.3.4 Knockdown of FliI increased focal adhesion protein expression: paxillin and vinculin

Previously, FliI was found to be localised in FAs, which, as mentioned, have been shown to play important regulatory roles in cell migration (Mohammad et al., 2012). FliI may affect adhesion via hemidesmosome formation, and the integrin-mediated adhesion of migratory keratinocytes which overexpress FliI has been shown to impair hemidesmosome formation, resulting in decreased wound re-epithelisation (Zlatko Kopecki et al., 2009). FliI has been reported to co-localise with integrin-binding proteins such as actin and talin (Davy et al., 2001), vinculin (Zlatko Kopecki et al., 2009; Mohammad et al., 2012) and paxillin (Zlatko Kopecki et al., 2009), which are all known to be actively involved in adhesion-dependent signalling pathways. These actin-

binding proteins are suggested to form a cytoskeletal complex directly linking the integrin receptors to the actin cytoskeleton.

In this study, we found that expression of paxillin and vinculin was increased by siRNA knockdown of FliI in cancer cells, suggesting upregulation of FA proteins in association with FliI. Previous studies on non-cancer cells showed that reduced expression of p-paxillin in $FliI^{Tg/Tg}$ fibroblasts (which overexpress FliI) resulted in impaired FA turnover (Z. Kopecki et al., 2011). Analysis of adhesion dynamics during 3D migration identified a role for paxillin in regulating the adhesion process, including assembly, stabilisation and disassembly. Paxillin-depleted cells have been found to show a decreased rate of adhesion disassembly, possibly due to decreased FAK activity (Deakin & Turner, 2011). In addition, paxillin may influence Rac1 and RhoA activity of the Rho-GTPase signalling pathway during 3D cell migration (Deakin & Turner, 2008), suggesting a mechanism for increased cell migration in knockdown cells. In addition, FliI has been shown to regulate FA turnover through inhibition of the phosphorylation of paxillin in an Rac1-dependent manner (Z. Kopecki et al., 2011). However, in contrast vinculin expression has been shown to inhibit cell migration by stabilising FAs via binding of inositol phospholipids, which play an important role in FA turnover (Saunders et al., 2006). This situation could be explained by the dynamics of FA complexes, and the proportions of phosphorylated and non-phosphorylated paxillin may affect the adhesion complex and function (Zaidel-Bar et al., 2007).

There are thus contradictions regarding a direct correlation between paxillin expression/phosphorylation and cancer aggressiveness depending on tissue type and the specific roles of paxillin (Brown & Turner, 2004). A study by (Zaidel-Bar et al., 2007) showed that cells which overexpress a phosphomimetic paxillin mutant had enhanced lamellipodial

protrusions and stabilised the formation of FA complexes, whereas non-phosphorylated paxillin enhanced fibronectin fibrillogenesis. Increased expression of paxillin has also been found to be directly correlated with the expression of HER2/3 receptor in aggressive breast cancer lines and grade III human breast tumors (Vadlamudi, Adam, Tseng, Costa, & Kumar, 1999). However, decreased paxillin levels have been shown in canine and feline mammary tumours (Scibelli et al., 2003) and lung cancer (Salgia et al., 1999), both of which are highly metastatic cancers.

As mentioned in the previous chapter, several studies have previously suggested that paxillin expression was reduced in colorectal cancer, correlating with poor prognosis and cancer progression (Deakin et al., 2012). Our results suggest that reducing FliI expression may reduce tumour metastasis and result in a better prognosis. This may be via increasing adhesion through FA protein pathways. However, the effect of FliI expression may vary with cell and tissue type.

Paxillin phosphorylation may play a role in cancer progression regulated by integrin and growth factor receptors, both of which play roles in tumourigenesis and invasion (Brown & Turner, 2004). Invasiveness of cancer cells may be dependent on paxillin phosphorylation, which is associated with the formation of a novel signalling-invadopodia complex that includes paxillin, cortactin and PKC, but not FAK (Bowden et al., 1999).

Importantly, other cytoskeletal proteins such as alpha-actinin and zyxin, integrin linkers such as vinculin and talin, and signalling molecules such as FAK, paxillin and ERK have also been shown to be involved in invadopodia/pedosome formation (Linder & Aepfelbacher, 2003; Weaver, 2006). These proteins are commonly located in ring-adherent structures of the actin core, which consists of several adhesion components and is responsible for mediating invadopodial

protrusion, and correlates with invadopodium ECM degradation (Branch, Hoshino, & Weaver, 2012). Interestingly, recent findings also revealed that paxillin could promote cell survival via direct interactions with vinculin, FAK and p210BCR/ABL (Deakin et al., 2012), and regulation of cell-survival signalling through the apoptosis inhibitory protein bcl-2 (Sheibani, Tang, & Sorenson, 2008). In addition, vinculin has also been shown to be implicated in modulating the apoptosis-signalling pathway and has been claimed to exert tumour-suppressing functions in a rat adenocarcinoma model (Subauste et al., 2004; Ziegler et al., 2006).

4.4 Conclusion

The cellular effects of FliI as an important tumour-regulating factor, specifically cell invasion and adhesion, and important biomarkers involved in both processes, specifically cortactin, paxillin and vinculin, have been discussed in this chapter. FliI appears to negatively regulate expression of the FA proteins paxillin and vinculin, suggesting an effect on cell adhesion that may promote tumour spread. FliI knockdown appears to enhance adhesiveness and inhibit invasiveness, suggesting a possible tumour-suppressing role. Similarly, decreased cortactin expression in FliI knockdown cells could also explain their apparent reduced invasiveness in 3D cultures.

The next chapter will discuss the role of FliI in regulating tumour growth and dissemination of tumour cells *in vivo* via the establishment of primary and lung nodule murine colon tumour models.

Chapter 5 Flightless I Modulates Cancer Growth and Metastasis in Colon Cancer Models

5.1 Introduction

Tumourigenesis is a multi-step process of cellular changes, including modification of cell proliferation and cell adhesion, and regulation of apoptosis. These changes may initiate cancer cell dissemination, and also influence tumour cell motility and migration. In order for tumour cells to metastasise, several mechanisms are involved, including angiogenesis, enhanced degradation of the ECM and infiltration of tumour cells through the basement membrane and surrounding connective tissues, followed by intravasation and extravasation through the blood vessels at secondary sites, escape from immune system surveillance and, finally, establishment of distant metastatic sites (Wells, 2006).

The importance of the tumour microenvironment or tumour stroma in driving tumour progression and invasion leading to metastasis is now accepted (Quail & Joyce, 2013). The contribution of stromal cells to cancer development is not limited to their effects on primary tumour growth, but has also been shown to be involved in the establishment of both pre-metastatic and metastatic microenvironments at distant sites (Hanahan & Weinberg, 2011). In fact, the stroma is also important in the context of drug delivery and drug efficacy, and may be useful as a biomarker to predict the tumour response to chemotherapy (Hale et al.,

2013). In addition, many published papers have provided data that has demonstrated the interaction between tumour cells and the stroma, which may either promote or in some cases limit cancer progression (Erler & Weaver, 2009; Hawinkels et al., 2014; Pietras & Östman, 2010).

Invasive cancer cells are capable of adapting and exploiting their tissue microenvironment, leading to cellular and molecular changes of the surrounding cells, including epithelial cells and also cells in the stroma such as inflammatory cells, fibroblasts and capillaries. These changes include promotion of angiogenesis, attracting immune-cell infiltration (mainly macrophages), stimulating the differentiation of CAFs, which are derived from local fibroblasts, and the release of cytokines, growth factors and proteases that are involved in remodelling of the ECM (Sounni & Noel, 2013). Specifically, the complex tumour microenvironment at the primary tumour site mainly consists of endothelial cells of both the blood and lymphatic circulation, bone marrow derived–cells (BMDCs) such as macrophages, neutrophils, mast cells and mesenchymal stem cells, while in the metastatic niche activated stromal cells and TAMs are commonly present, and are associated with greater release of proteases, chemokines and growth factors that mediate cell intravasation (Joyce & Pollard, 2009).

Recently, FliI has been shown to promote the progression of a chemically induced cutaneous squamous cell carcinoma (SCC) in an animal model, possibly by increased tumour cell invasion (Z Kopecki et al., 2015). Reduction of tumour cell apoptosis was also observed in the same study. In the present study, primary tumours and disseminating murine colon tumour models were established to determine the effects of FliI expression in tumour growth and establishment at secondary sites, respectively.

This chapter also discusses the role of FliI I in regulating tumour growth and the establishment of lung nodules using mice with different levels of expression of FliI; that is, using WT and FliI heterozygous knockout (FliI $^{+/-}$) mice. In addition, tumour growth was studied in mice where WT tumour cells were pre-treated with siRNA FliI colon carcinoma (CT26) cells prior to inoculation into the mice. This allowed us to examine the effect of tumour cell FliI expression independent of the level of FliI expression in the host animal, and thus FliI expression in the tumour microenvironment. Knockdown of FliI in this way may tell us something about the early effect of FliI deficiency on tumour establishment, even though the effect of knockdown is likely to be short-lived. The effects of each model in modulating the tumour growth and tumour microenvironment were determined by examining the expression of tumour markers of proliferation: PCNA; myofibroblast activation; α-SMA expression and invasion of invadopodia marker; and cortactin localisation. The effects of FliI expression in the host animal and in the tumour cells were thus studied to determine the effects of FliI on cancer progression and establishment of tumour nodules in the lung.

5.2 Results

5.2.1 Effect of expression of Flightless I in mice injected intravenously with tumour cells

5.2.1.1 Bodyweight monitoring

In this model, FliI heterozygous +/− (FliI $^{+/-}$) mice with reduced FliI expression and WT BALB/c mice were used to determine the effects of lower FliI expression on the development and establishment of lung nodules, by determining the number of tumour nodules in the lung 14 days post tumour inoculation with colon cancer cells (CT26 WT cells). Inoculation was made by intravenous injection via the mouse tail vein. Bodyweight

measurement of the mice showed a consistent increment throughout the 14 days post-inoculation of cancer cells (CT26 WT cells) and mice were also monitored to ensure that there was no sign of distress or respiratory discomfort.

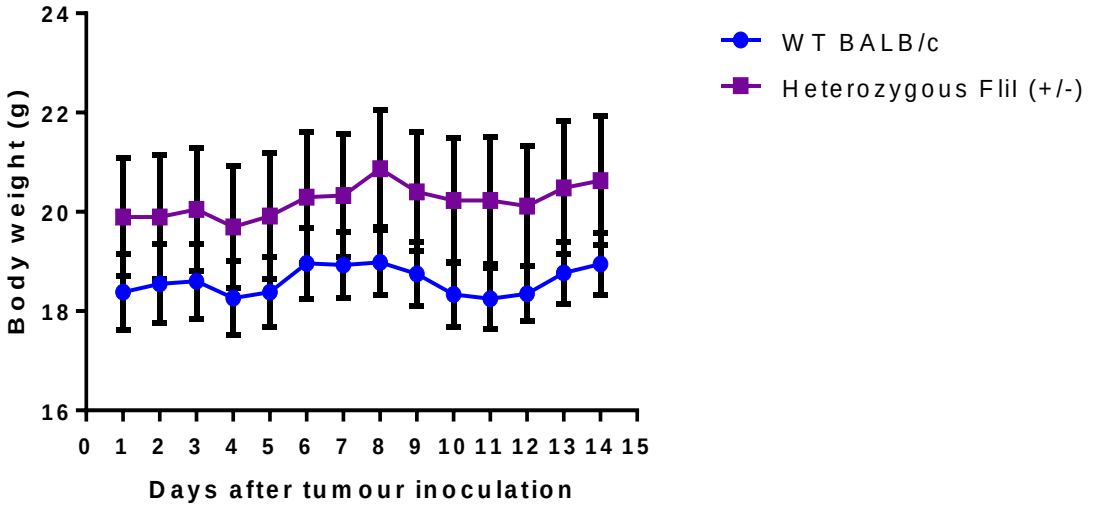


Figure 5.1: Monitoring of body weight in BALB/c mice expressing different levels of FliI I after intravenous inoculation of colon cancer cells. BALB/c mice expressing different levels of FliI (WT BALB/c and heterozygous FliI $^{+/-}$) were injected intravenously with CT26 WT cells to induce lung tumours. Bodyweights are shown of WT BALB/c and FliI heterozygous +/− (FliI $^{+/-}$) mice was monitored for 14 days after tumour inoculation with CT26 WT colon cancer cells. Results are shown as mean weight ± SEM (n=6 each group).

5.2.1.2 Inhibition of FliI expression reduced development of nodules in the lung

FliI$^{+/-}$ heterozygous mice with reduced FliI expression were used in this metastatic model in comparison to WT BALB/c mice. Cancer cells (CT26 WT) were inoculated via tail-vein injection and allowed to invade and develop nodules in the lung during 14 days post-injection. There was a reduction in the number of microscopic metastatic nodules observed in the lungs of heterozygous mice (BALB/c FliI +/−) compared to WT BALB/c mice ($p<0.05$) after 14 days of tumour inoculation with colon cancer cells (CT26 WT). Histological sections

stained with H&E staining were used to count the number of nodules present in the lungs of both groups of mice.

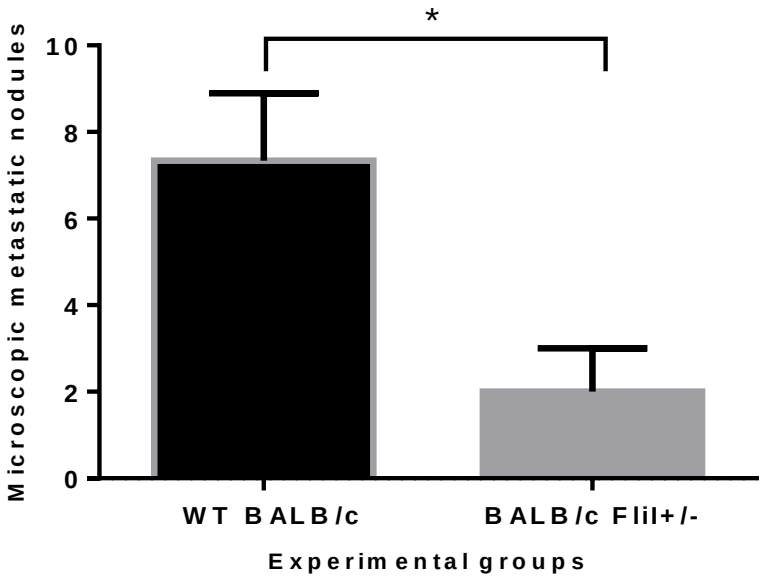


Figure 5.2: Microscopic tumour nodules in BALB/c mice expressing different levels of FliI after inoculation with colon cancer cells. BALB/c mice expressing different levels of FliI (WT BALB/c and heterozygous $FliI^{+/-}$) were injected intravenously with CT26 WT and the numbers of microscopictumour nodules counted after H&E staining. Number is shown of microscopic nodules present in the lung in WT BALB/c and FliI heterozygous mice (n=6 each group). Results are shown as mean ± SEM. *p<0.05 is significantly different from control group (Student's unpaired t-test).

5.2.1.3 Histological sections and FliI expression in lung nodules

Histological analysis of nodules established in the lungs showed an increased number of tumour nodules in the lungs of WT BALB/c mice; however, very few to no tumour nodules were found in the lungs of $FliI^{+/-}$ heterozygous mice (Figure 5.3), 14 days post tumour inoculation with colon cancer cells (CT26 WT). Histological sections stained with H&E were used to count the number of tumour nodules present in the lungs in the two experimental groups.

Representative staining of FliI in lung nodules showed higher expression in the tumours of WT BALB/c mice and lower expression in the tumours of $FliI^{+/-}$ heterozygous mice (Figure 5.4). The expression of FliI in positive control tissue, skeletal muscle, is also shown in Figure 5.4.

Representative staining for cortactin (Figure 5.5) and α-SMA (Figure 5.6) in the lung nodules also showed greater expression of both cortactin and α-SMA in the tumours of WT BALB/c mice compared to the tumours in $FliI^{+/-}$ heterozygous mice.

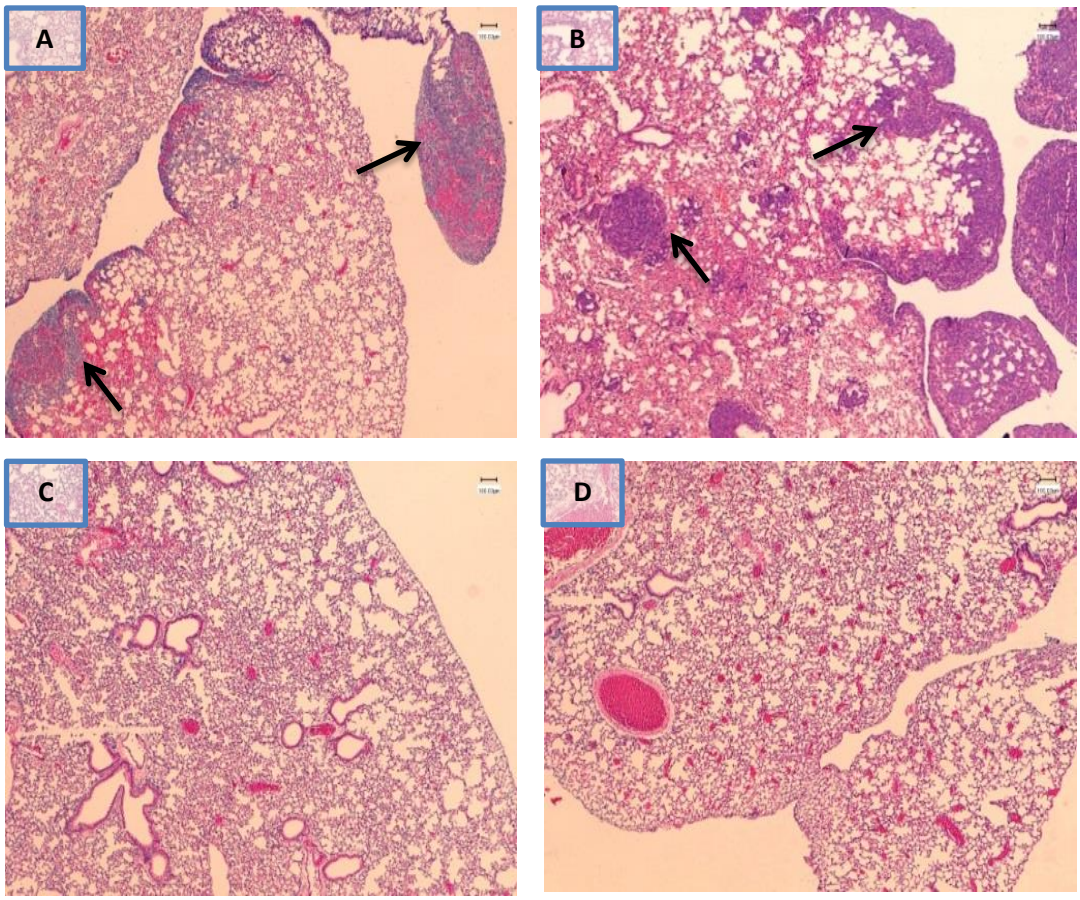


Figure 5.3: Microscopic tumour nodules in BALB/c mice expressing different levels of Flil after induction of lung nodules with colon cancer cells. Representative H&E staining of lung sections of **(A, B)** WT BALB/c and **(C, D)** Flil heterozygous (+/−) mice (4X magnification). Arrows indicate tumour nodules, which stain darkly in the lung tissue. In Flil heterozygous mice, nodules were rarely seen and the large pink/red structures observed here are blood vessels.

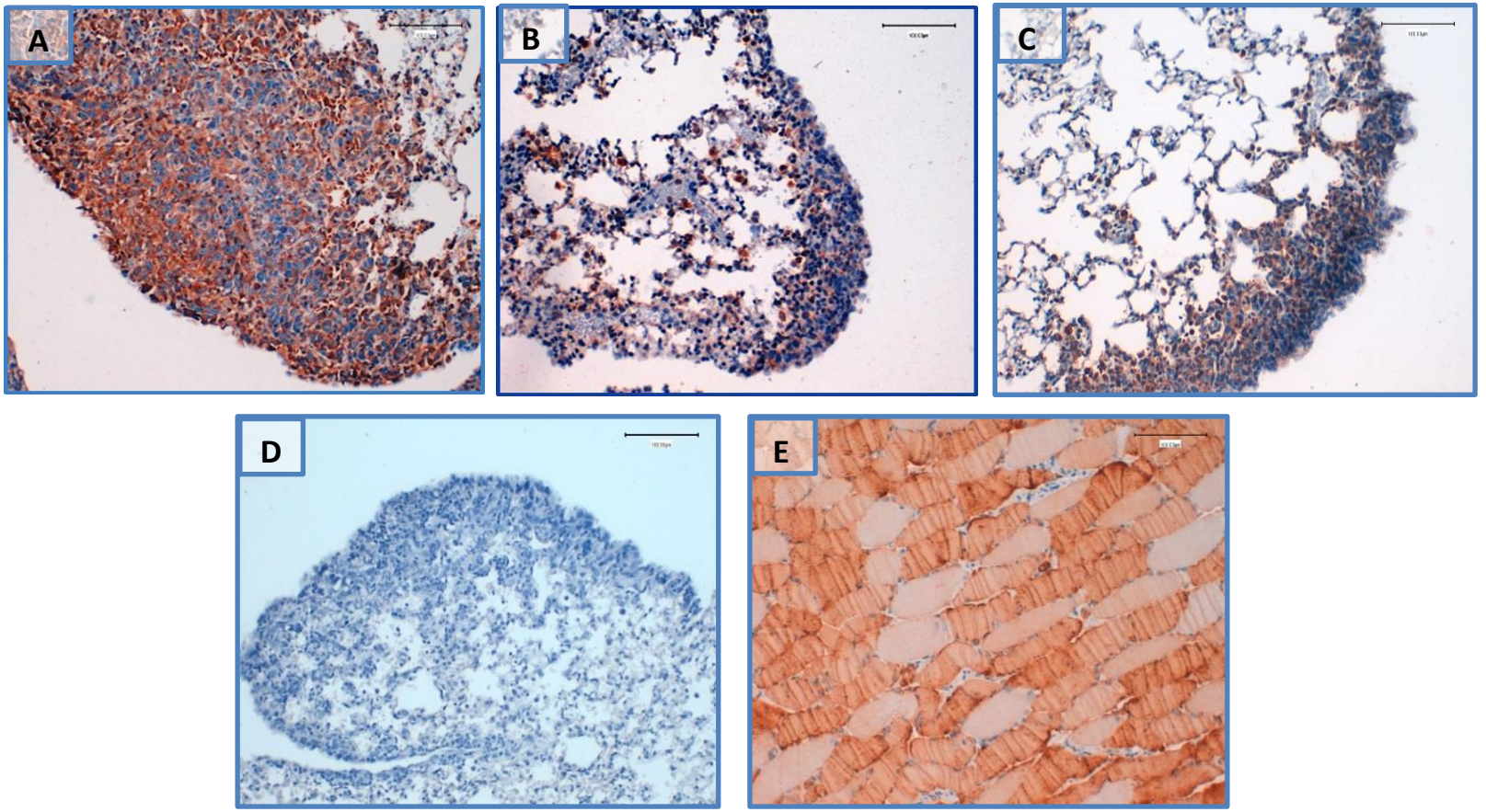


Figure 5.4: FliI expression in tumour nodules in BALB/c mice expressing different levels of FliI after induction with colon cancer cells, shown by immunohisctochemical staining. Representative images of immunohistochemical staining of FliI in the lungs in tumour nodules of **(A)** WT BALB/c mice, **(B, C)** $FliI^{+/-}$ heterozygous mice (20X and 40X magnification) and **(D)** a negative control are shown. **(E)** Positive control staining of FliI using mouse skeletal muscle (40X magnification).

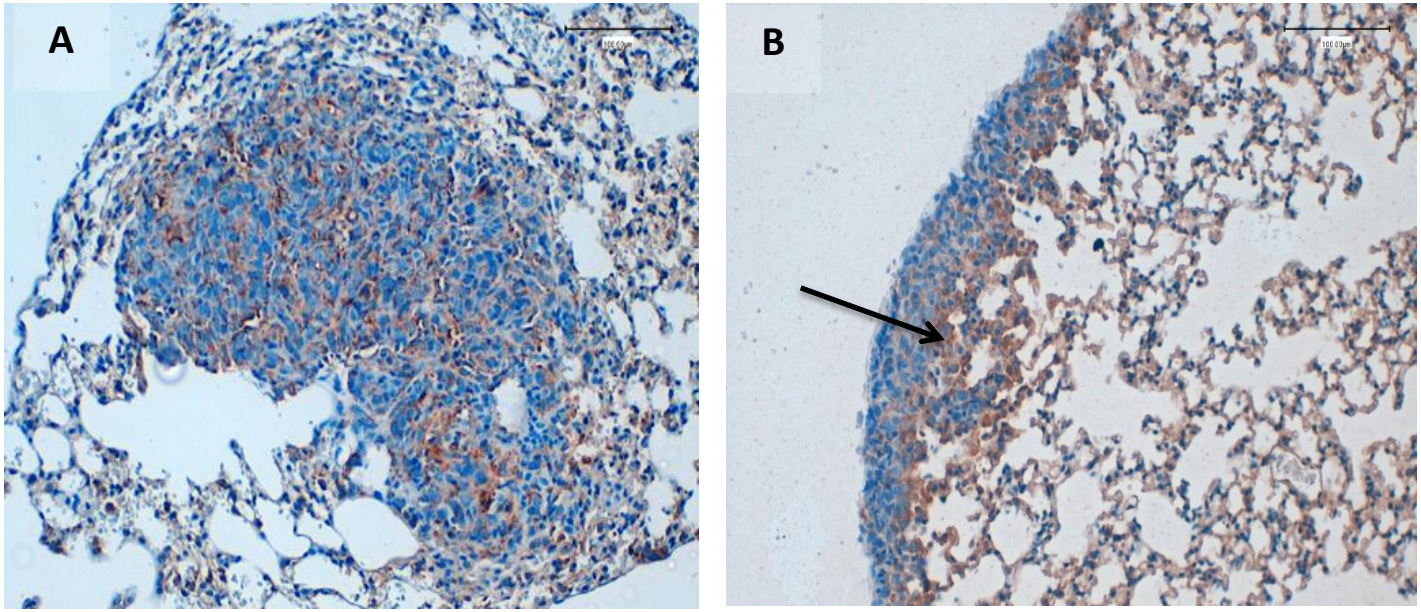


Figure 5.5: Cortactin expression in tumour nodules in BALB/c mice expressing different levels of FliI after induction using colon cancer cells. Representative images of immunohistochemical staining of cortactin in the lungs in tumour nodules of **(A)** WT BALB/c mice and **(B)** heterozygous mice (20X magnification). The densely packed cells at the periphery of the lung in **B** show a small aggregation of cancer cells close to the surface of the lung (arrow).

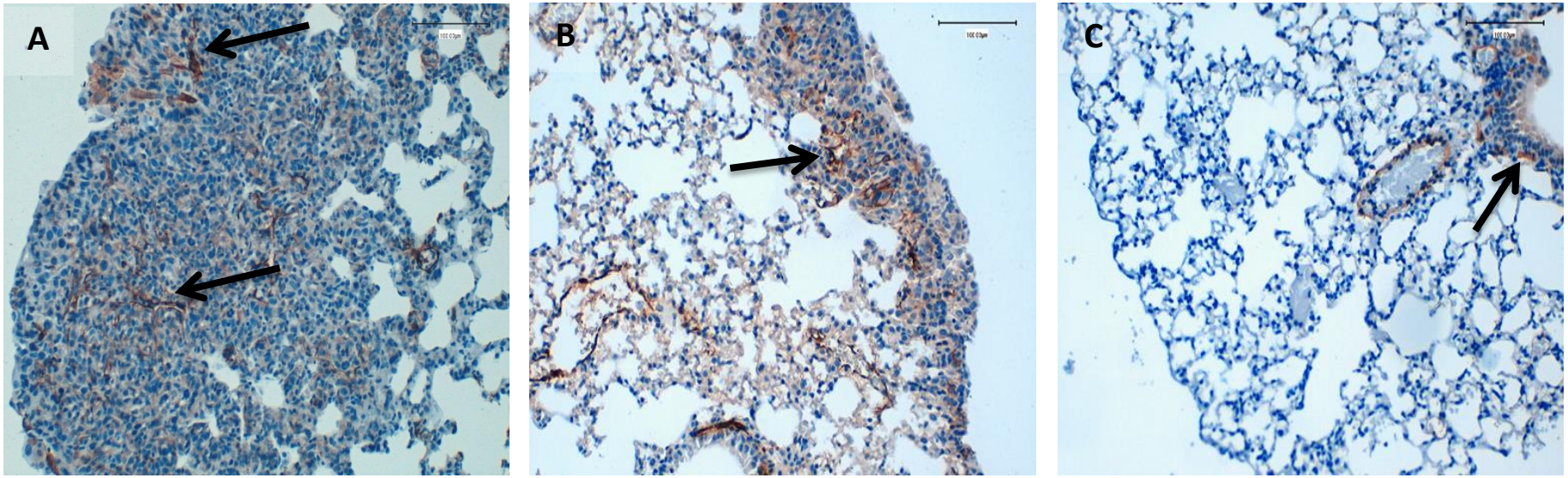


Figure 5.6: Alpha smooth muscle actin (α-SMA) expression in tumour nodules in BALB/c mice expressing different levels of FliI. Representative images of immunohistochemical staining of α-SMA in the lungs in tumour nodules of **(A, B)** WT BALB/c mice and **(C)** heterozygous mice (20X magnification). Arrows indicate expression of α-SMA around tumour cells. The pattern of expression of α-SMA shows expression in fibroblasts present in the stroma (rather than in tumour cells).

5.2.2 Effect of knockdown of Flil I in cancer cells on primary tumour growth in animal model

5.2.2.1 Inhibition of FliI expression in cancer cells reduced the size of primary tumours

Primary tumours were induced by injecting CT26 WT and siRNA FliI-treated CT26 cells into the right flank of the WT BALB/c mice. Primary tumours were measured daily for a maximum of 21 days or until tumours reached 1000 mm^3 in size. It was observed that smaller tumours were grown in mice inoculated with siRNA FliI-treated CT26 cells compared to mice inoculated with CT26 WT cells, with a significant difference observed at days 10 ($p<0.01$) and 12 ($p<0.05$) (Figure 5.7) post-inoculation with colon cancer cells. In contrast, larger primary tumours were observed in WT BALB/c mice inoculated with CT26 WT colon cancer cells. At 14 days post-inoculation of colon cancer cells, there was no significant difference in tumour size observed between the two groups.

Representative images of primary tumour formation on the right flank of WT BALB/c mice inoculated with CT26 WT or siRNA FliI CT26 cells at days 12, 14 and 20 are shown in Figure 5.8. Larger tumours were grown on the right flank of the WT BALB/c mice inoculated with CT26 WT cells, while smaller primary tumours were observed in mice inoculated with siRNA FliI-treated CT26 cells (Figure 5.8).

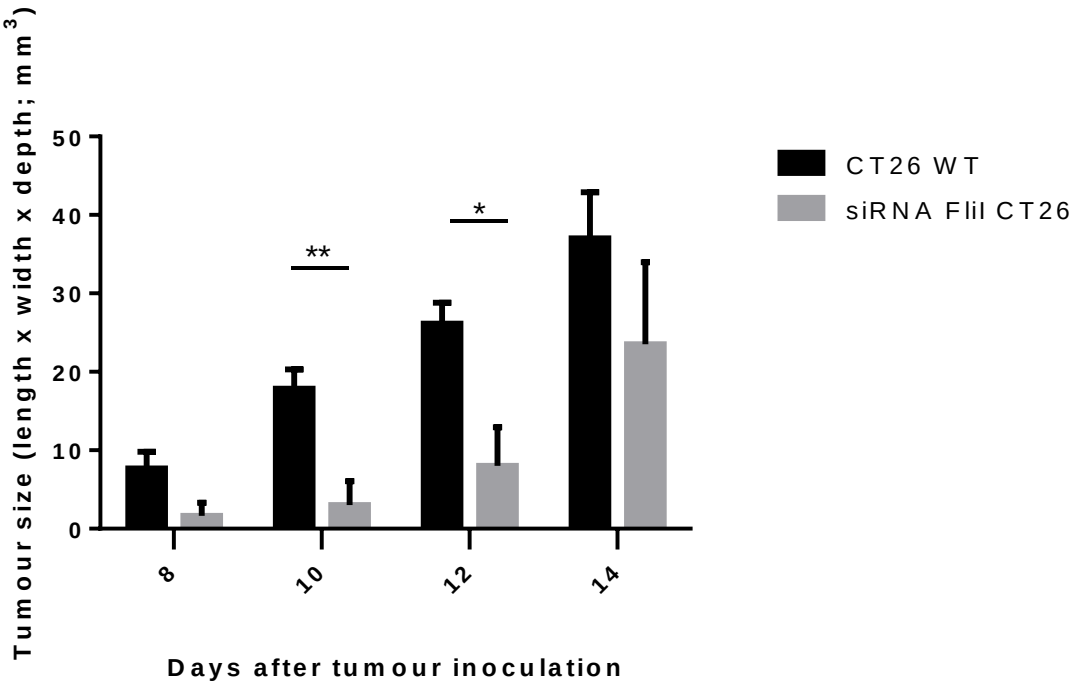


Figure 5.7: Measurement of primary tumours in BALB/c mice inoculated with colon CT26 cells expressing different levels of FliI expression *in vivo*. The size of primary tumours on the right flank of the mice at days 8, 10, 12 and 14 days in BALB/c mice inoculated with CT26 WT and siRNA FliI CT26 colon cancer cells. Results are shown as mean ± SEM (n=10 each group). **p<0.01 and *p<0.05 are significantly different from control group (Dunnet's multiple comparison test).

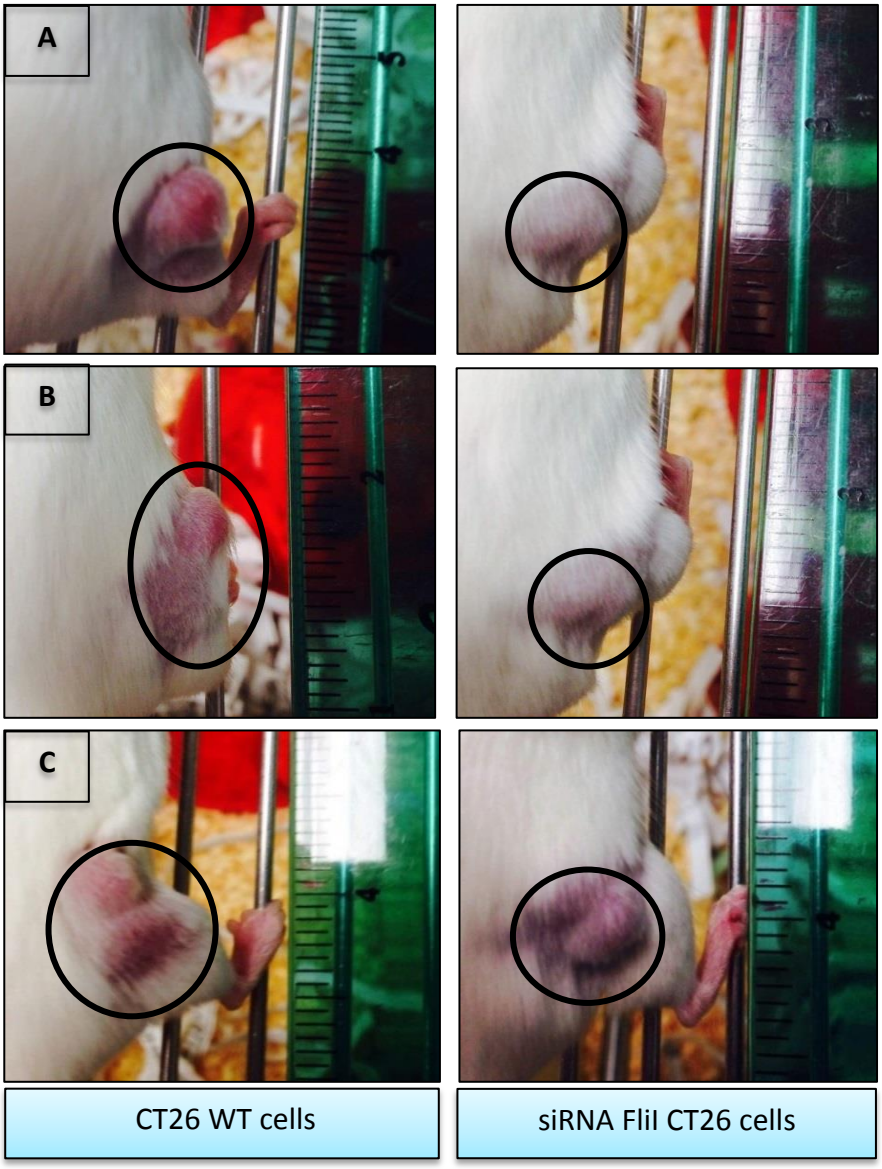


Figure 5.8: Primary tumours in BALB/c mice inoculated with WT or siRNA FliI-treated CT26 cells. Representative images of primary tumours in BALB/c mice after inoculation with CT26 WT and siRNA FliI CT26 cells at days **(A)** 12, **(B)** 14 and **(C)** 20 days post-inoculation.

5.2.3 Effect of Flil I expression on tumour growth in BALB/c mice

5.2.3.1 Bodyweight monitoring

In this model, WT BALB/c mice were inoculated either with CT26 WT cells or siRNA Flil-treated CT26 cells intravenously via the tail vein. Mouse bodyweight was constantly recorded throughout 14 days post-inoculation of cancer cells (CT26 WT and siRNA Flil CT26 cells) and indicated that mice remained healthy during this experimental period (Figure 5.9). There was no sign of distress or respiratory discomfort in the mice.

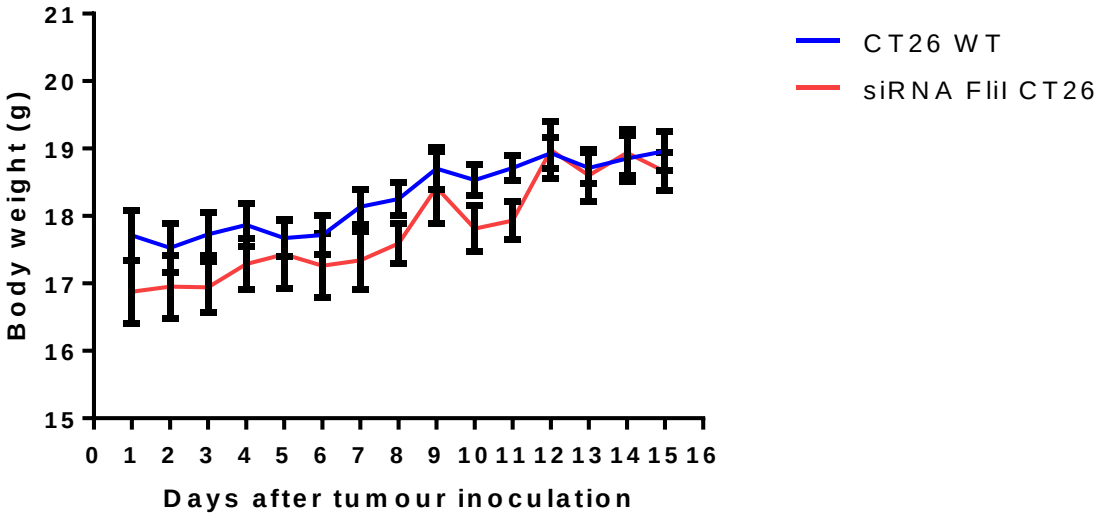


Figure 5.9: Monitoring of bodyweight in BALB/c mice inoculated with cells expressing different levels of Flil. BALB/c mice were injected intravenously with CT26 cells expressing different levels of Flil to induce lung tumour nodules. Bodyweight is shown in BALB/c mice after inoculation with CT26 WT and siRNA Flil CT26 cells for 14 days post-inoculation. Results are shown as mean ± SEM (n=10 each group).

5.2.3.2 Inhibition of FliI expression in cancer cells reduced tumour nodules in the lung

Seeding of tumour nodules in the lung was induced by injecting CT26 WT cells and siRNA FliI-treated CT26 cells via the tail vein in BALB/c mice. Mice were then monitored for 14 days post-inoculation. At day 14, the mice were killed and the lungs were removed for further histological analysis. The number of microscopic nodules present in the lung was counted in lung sections after H&E staining. A significantly lower number of lung nodules was observed in mice inoculated with siRNA FliI-treated CT26 cells compared to mice inoculated with CT26 WT cells, which showed greater tumour numbers ($p<0.001$) (Figure 5.10).

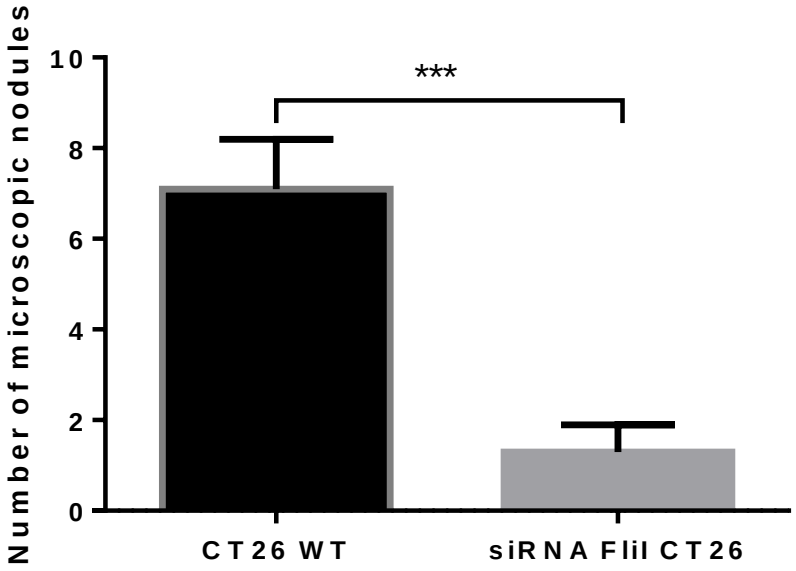


Figure 5.10: Quantification of microscopic tumour nodules in BALB/c mice inoculated with colon CT26 cancer cells expressing different levels of FliI. BALB/c mice were injected intravenously with CT26 cells expressing different levels of FliI and the number of microscopic lung nodules counted after H&E staining. Results are shown as mean ± SEM (n=10 each group). ***p<0.001 shows significant difference from control group (Student's unpaired t-test).

5.2.3.3 Histology of lung nodules

Histological analysis of the lungs from injected mice showed that an increased number of and larger tumour nodules were present in mice inoculated with CT26 WT cells compared to mice inoculated with siRNA FliI-treated CT26 cells (Figure 5.11), 14 days post tumour inoculation. Histological sections stained with H&E were used to count the number of tumour nodules present in the lungs of both experimental groups.

Representative histological images of H&E staining of nodules in the lung show more and larger-sized tumours in WT BALB/c mice inoculated with CT26 WT cells, while fewer and smaller tumours are observed in WT BALB/c mice inoculated with siRNA FliI CT26 cells (Figure 5.11).

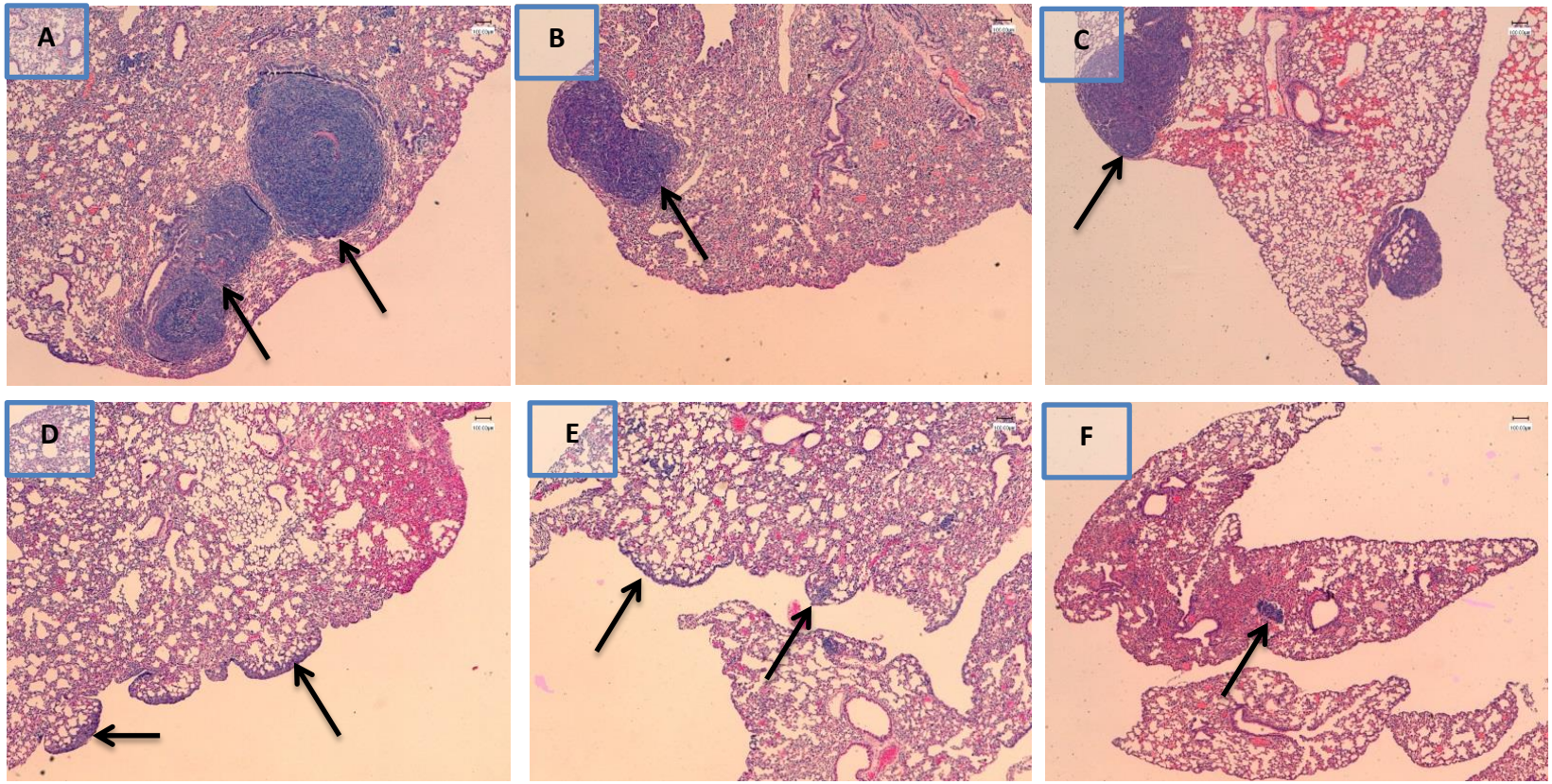


Figure 5.11: Histological analysis of tumour nodules in BALB/c mice inoculated with WT or siRNA treated CT26 cells. BALB/c mice were inoculated intravenously with CT26 WT cells expressing different levels of FliI and the numbers of microscopic nodules were counted in H&E-stained sections. Representative histological images of microscopic nodules in the lungs of mice inoculated with **(A-C)** CT26 WT and **(D-F)** siRNA FliI CT26 cells. Arrows indicate tumour nodules in the lung tissue (4X magnification).

5.2.4 Expression of Flil I in primary and lung tumours

Expression of FliI was determined in the lung sections by immunohostochemistry. As a positive control, FliI was detected in skeletal muscle sections, as shown above in Figure 5.4, and staining was then optimised and FliI expression detected in tumours (both primary and lung). Analysis of immunohistochemistry staining for FliI showed a lower expression of FliI in the primary tumours of mice inoculated with siRNA FliI-treated CT26 cells compared to tumours derived from CT26 WT cells (Figure 5.12).

Representative images of the immunohistochemical staining of FliI expression in primary and lung tumour nodules after inoculation with CT26 cells expressing different levels of FliI are shown in Figure 5.12.

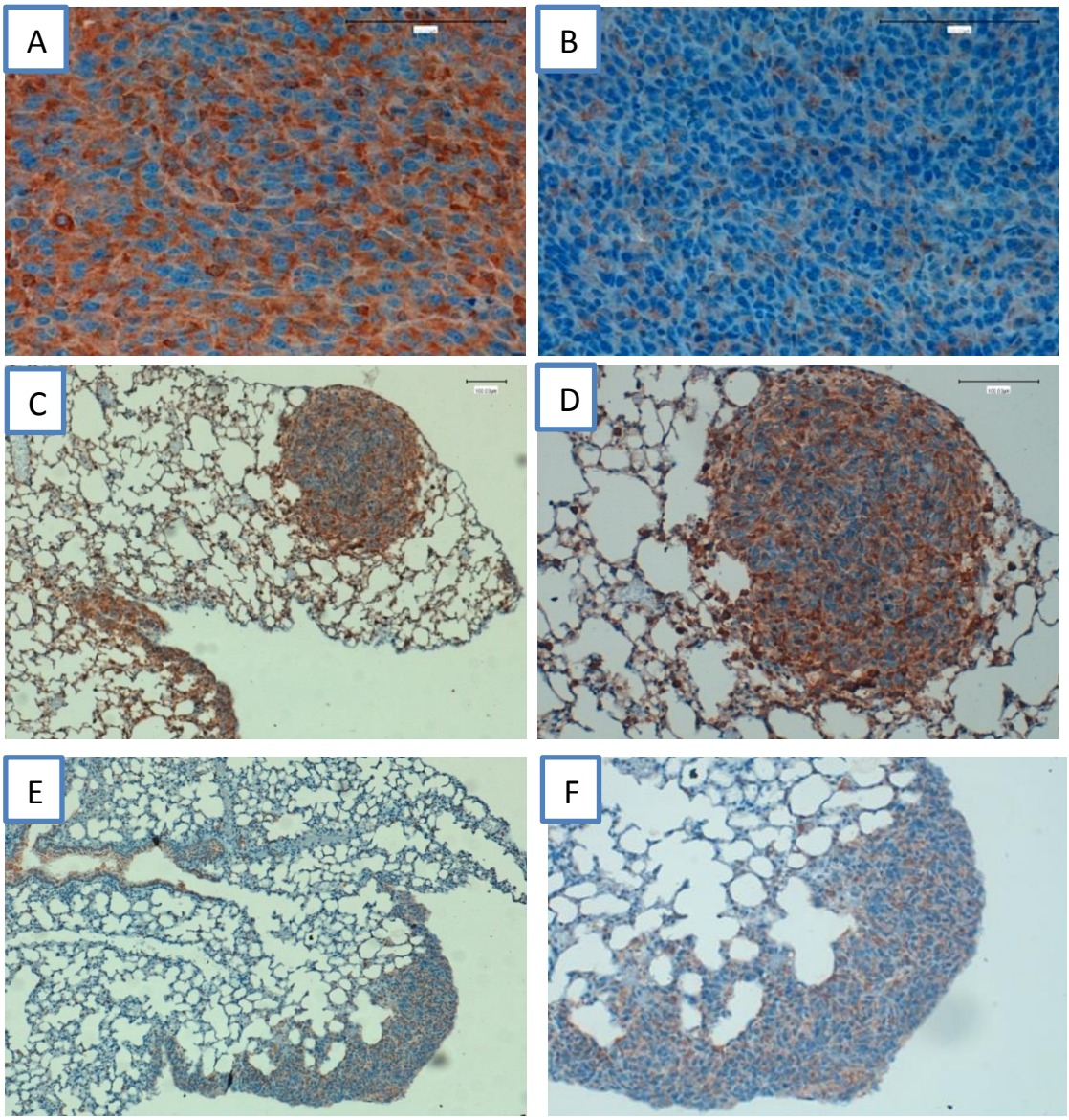


Figure 5.12: FliI expression in primary and lung tumours in BALB/c mice inoculated with CT26 colon cancer cells. Primary tumour immunostaining with FliI in **(A)** BALB/c mice injected with CT26 WT and **(B)** BALB/c mice injected with siRNA FliI-treated CT26 cells (40X magnification). Tumours in the lung immunostained with FliI showing FliI expression in **(C, D)** BALB/c mice injected with CT26 WT cells and **(E, F)** BALB/c mice injected with siRNA FliI CT26 cells which **(C)** and **(E)** show low magnification (10X) and **(D)** and **(F)** show high magnification (20X).

5.2.4.1 Histological analysis of FliI expression in primary tumours

Analysis of FliI expression in primary tumours was performed by scoring the sections immunostained with FliI from the tumours of BALB/c mice inoculated with CT26 WT or siRNA FliI CT26 cells. Immunoreactive scores for FliI expression were significantly lower for the expression of FliI in primary tumours in BALB/c mice inoculated with siRNA FliI CT26 cells, compared to the expression of FliI in primary tumours in BALB/c mice inoculated with CT26 WT cells ($p<0.05$) (Figure 5.13) post tumour inoculation on the right flank. This result suggests that tumour cells had different levels of FliI expression depending on the type of cells inoculated, with siRNA FliI CT26 cells successfully lowering FliI expression.

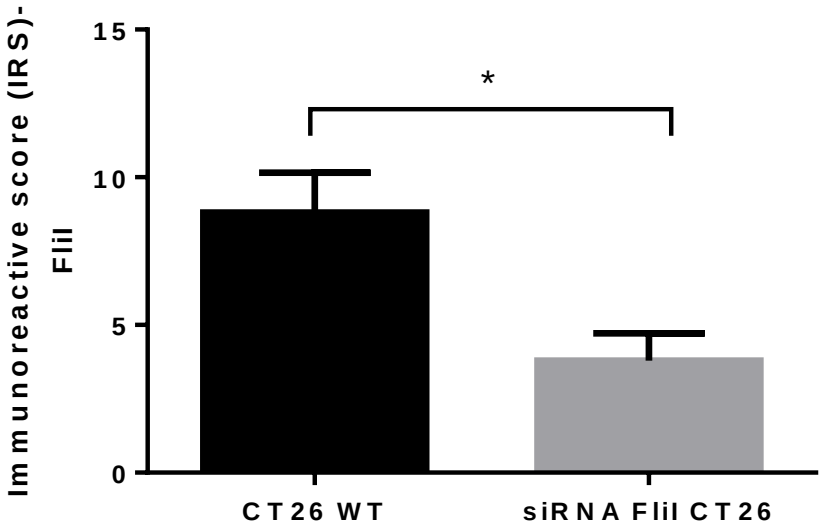


Figure 5.13: Quantification of FliI expression in primary tumours in BALB/c mice inoculated with colon CT26 cancer cells expressing different levels of FliI. BALB/c mice were inoculated with CT26 cells expressing different levels of FliI and primary tumours were collected and analysed by immunohistochemistry staining. Primary tumours were immunostained with FliI and the immunoreactive score of FliI expression determined in primary tumours in BALB/c mice inoculated with CT26 WT and siRNA FliI CT26 (n=5). Results are shown as mean score ± SEM. *$p<0.05$ shows significant difference from control group (Student's unpaired t-test).

5.2.4.2 Histological analysis of FliI expression in lung tumours

Analysis of FliI expression in lung tumours was performed by scoring the lung sections from BALB/c mice inoculated with either CT26 WT or siRNA FliI CT26 cells. The immunoreactive score for FliI expression was also significantly lower in tumours in the lungs of mice inoculated with siRNA FliI CT26 cells, compared to those of mice inoculated with CT26 WT cells ($p<0.001$) (Figure 5.14). This result also suggests that CT26 cells that formed tumours in the lung had a lower level of FliI expression after siRNA FliI treatment.

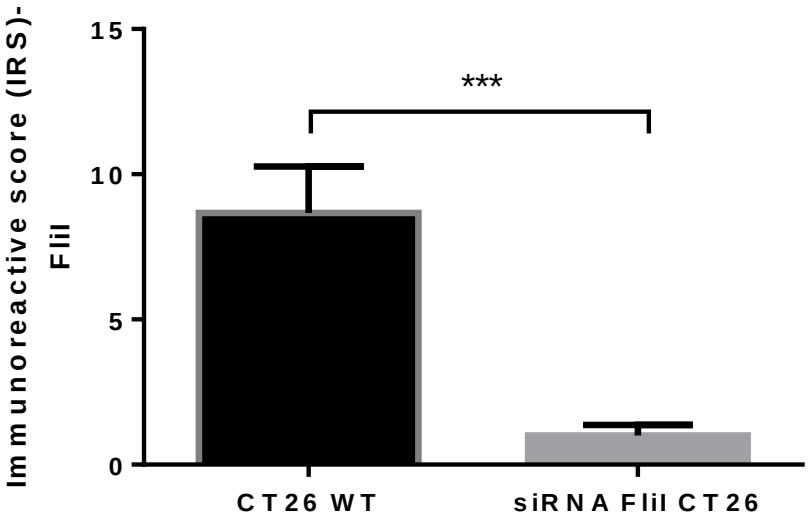


Figure 5.14: Quantification of FliI expression in lung tumours in BALB/c mice inoculated with colon CT26 cancer cells expressing different levels of FliI. BALB/c mice were inoculated intravenously with CT26 cells expressing different levels of FliI and the lungs were collected and analysed by immunohistochemical staining. Tumour nodules were immunostained with FliI and the immunoreactive score for FliI expression determined in tumours from BALB/c mice inoculated with CT26 WT and siRNA FliI CT26 (n=5). Results are shown as mean score ± SEM. *$p<0.05$ shows significant difference from control group (Student's unpaired t-test).

5.2.5 Expression of PCNA in primary and lung tumours

Primary and lung tumours were immunostained for the proliferation marker PCNA to determine the effect of FliI expression on cell proliferation. A lower proliferation rate was observed in the primary tumours of mice inoculated with siRNA FliI CT26 cells compared to those of mice inoculated with CT26 WT ($p<0.01$) (Figure 5.16). Increased PCNA positivity was also observed in tumour nodules in the lungs of BALB/c mice inoculated with CT26 WT cells compared to those inoculated with siRNA FliI CT26 cells ($p<0.05$) (Figure 5.17). It should be noted that the very low number of tumour nodules observed in the lungs of siRNA FliI CT26 injected cells made it more difficult to validly compare the proliferation rates in the lung tumour model.

Representative images of immunohistochemical staining with PCNA of primary tumours and lung nodules are shown in Figure 5.15. There was a greater number of proliferating cells which stained positively with PCNA (cellular staining) in BALB/c mice injected with CT26 WT cells compared to those of BALB/c mice injected with siRNA FliI CT26 cells in both primary and lung tumours.

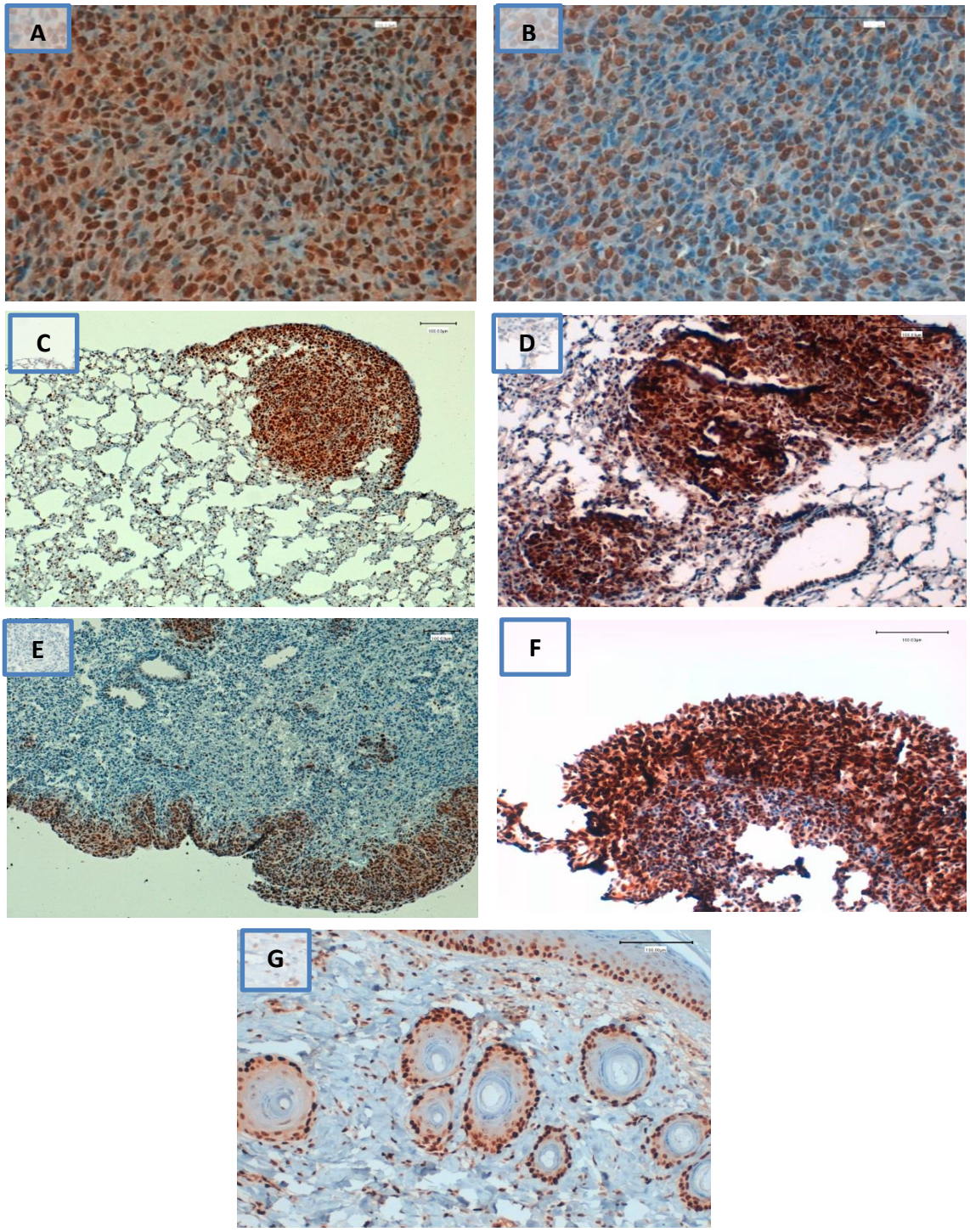


Figure 5.15: PCNA expression detected by immunohistochemical staining in primary and lung tumours in BALB/c mice. Primary tumour immunostaining with PCNA in **(A)** BALB/c mice inoculated with CT26 WT cells and **(B)** BALB/c mice inoculated with siRNA FliI CT26 cells (40X magnification). Tumour nodules in the lungs immunostained with PCNA showing PCNA expression in **(C,D)** mice injected with CT26 WT cells, **(E,F)** mice injected with siRNA FliI CT26 cells (10X and 20X magnification) and **(E)** positive control of normal low-proliferating skin tissue (20X magnification).

5.2.5.1 Proliferative index of PCNA in primary tumours

Analysis of PCNA expression in primary tumours was performed by scoring the immunostained sections from tumours in BALB/c mice inoculated with CT26 WT or siRNA FliI CT26 cells. A significantly lower proliferative index score was found in primary tumours of mice inoculated with siRNA FliI CT26 cells compared to those of mice inoculated with CT26 WT cells ($p<0.01$) (Figure 5.16). Lower cell proliferation staining in the primary tumours of BALB/c mice inoculated with siRNA FliI CT26 cells thus correlated with the reduced size of primary tumours in this experimental group.

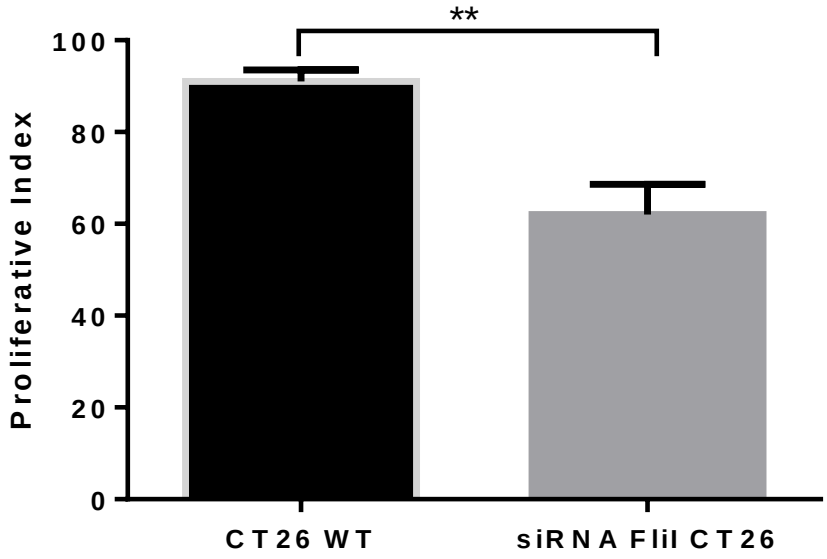


Figure 5.16: Quantification of PCNA expression in primary tumours in BALB/c mice inoculated with colon CT26 cancer cells expressing different levels of FliI expression. Primary tumours were immunostained with PCNA and the immunoreactive score for PCNA expression determined in primary tumours in BALB/c mice inoculated with CT26 WT or siRNA FliI CT26 cells (n=5). Results are shown as mean score ± SEM. *$p<0.05$ shows significant difference from control group (Student's unpaired t-test).

5.2.5.2 Proliferative index of PCNA in lung tumours

Analysis of PCNA expression in lung tumours was performed by scoring lung sections immunostained with PCNA. Fewer proliferating PCNA-positive cells were detected in nodules in the lungs of mice injected with siRNA FliI CT26 cells compared to those of mice injected with CT26 WT cells ($p<0.05$) (Figure 5.17). However, the high level of staining in both groups made it difficult to see negatively stained nuclei in the lung nodules.

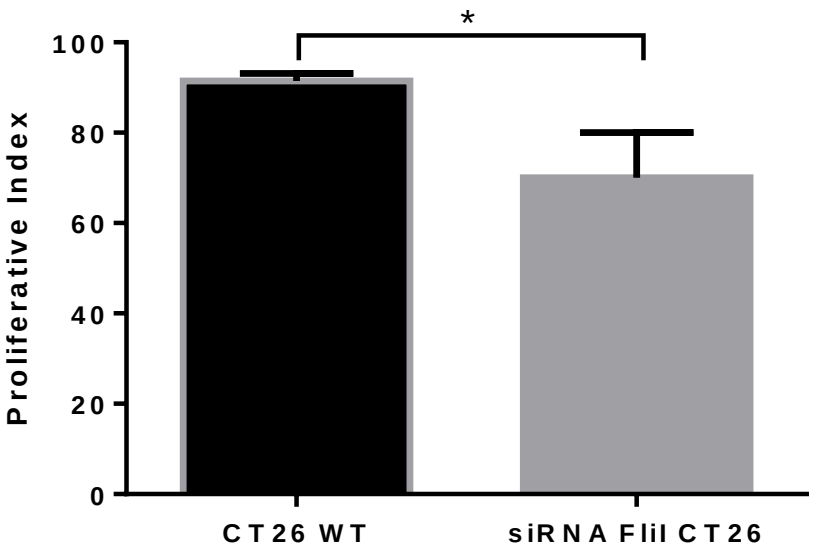


Figure 5.17: Quantification of PCNA expression in lung tumours in BALB/c mice inoculated with colon CT26 cancer cells expressing different levels of FliI. Tumour nodules were immunostained with PCNA and the immunoreactive score for PCNA expression determined in lung tumours in mice inoculated with CT26 WT and siRNA FliI CT26 (n=5). Results are shown as mean score ± SEM. *$p<0.05$, significant difference from control group (Student's unpaired t-test).

5.2.6 Expression of cortactin in primary and lung tumours

The expression of cortactin was studied as an invadopodia marker, and was evaluated in primary and lung tumours. Primary and lung tumours were immunostained with an anti-cortactin antibody to determine the effect of FliI expression on the expression of cotactin and thus potentially on the capacity for tumour cells to invade. Lower expression of cortactin was observed in mice inoculated with siRNA FliI CT26 cells compared to mice inoculated with CT26 WT cells (Figure 5.18).

Representative images of immunohistochemical staining for cortactin in primary and lung nodules are shown in Figure 5.18. There was a higher level of cortactin cytoplasmic expression observed in mice injected with CT26 WT cells compared to mice injected with siRNA FliI CT26 cells in both primary and lung tumours.

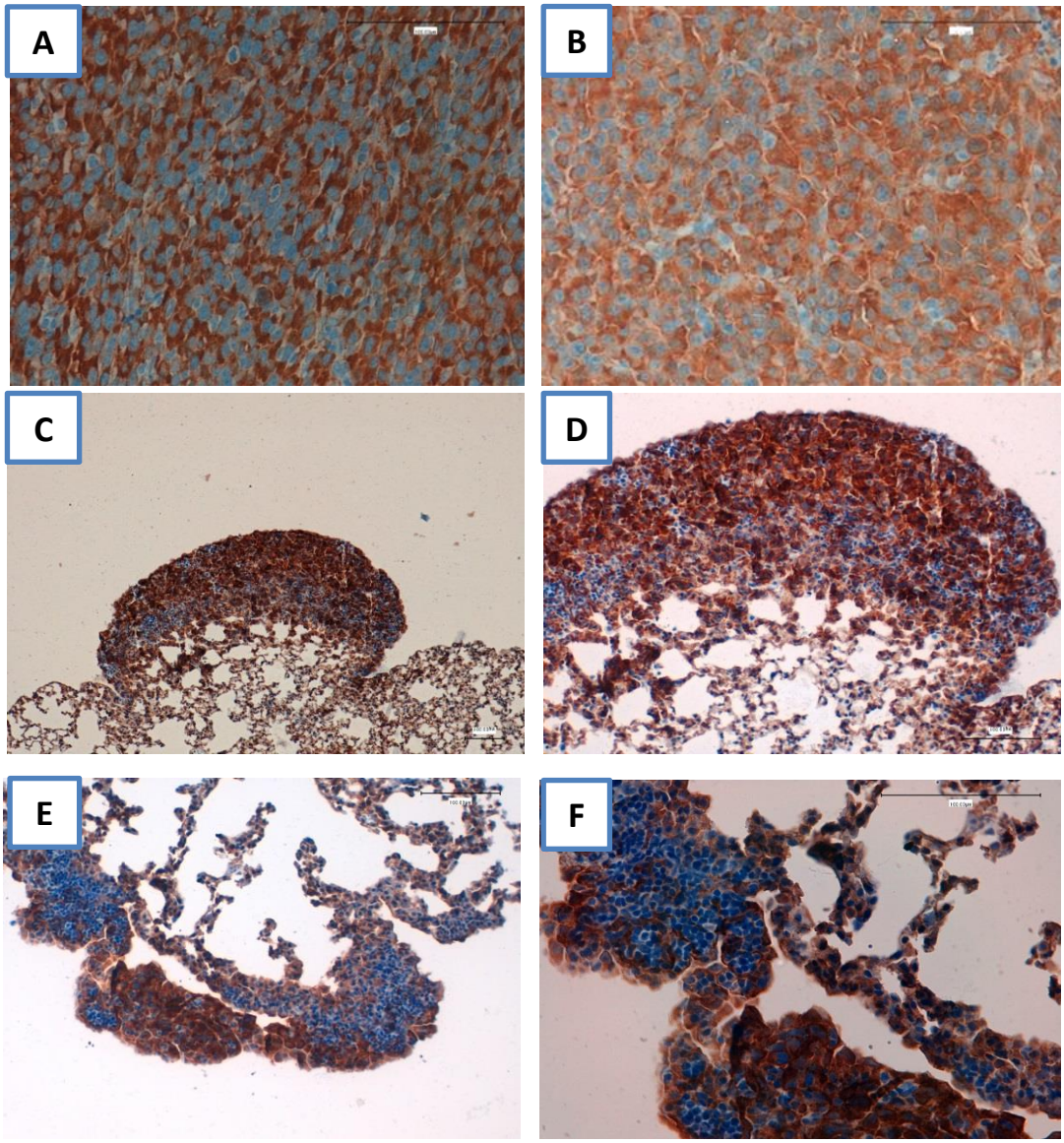


Figure 5.18: Cortactin expression detected by immunohistochemical staining in primary and lung tumours in BALB/c mice. Primary tumour immunostaining for cortactin in BALB/c mice injected with **(A)** CT26 WT cells and BALB/c mice injected with **(B)** siRNA FliI CT26 cells. Lung tumour immunostaining for cortactin showing expression of cortactin in **(C, D)** mice injected with CT26 WT cells and **(E, F)** mice injected with siRNA FliI CT26 cells which **(C)** and **(E)** show low magnification (10X and 20X respectively) and **(D)** and **(F)** show high magnification (20X and 40X respectively).

5.2.6.1 Quantitative analysis of cortactin staining in primary tumours

Analysis of cortactin expression in primary tumours was performed by scoring the tumour sections immunostained for cortactin from mice inoculated with CT26 WT or siRNA FliI CT26 cells. Immunoreactive scores for the invadopodia marker cortactin showed lower levels of expression in primary tumours in mice injected with siRNA FliI CT26 cells compared to mice injected with CT26 WT cells ($p<0.05$) (Figure 5.19).

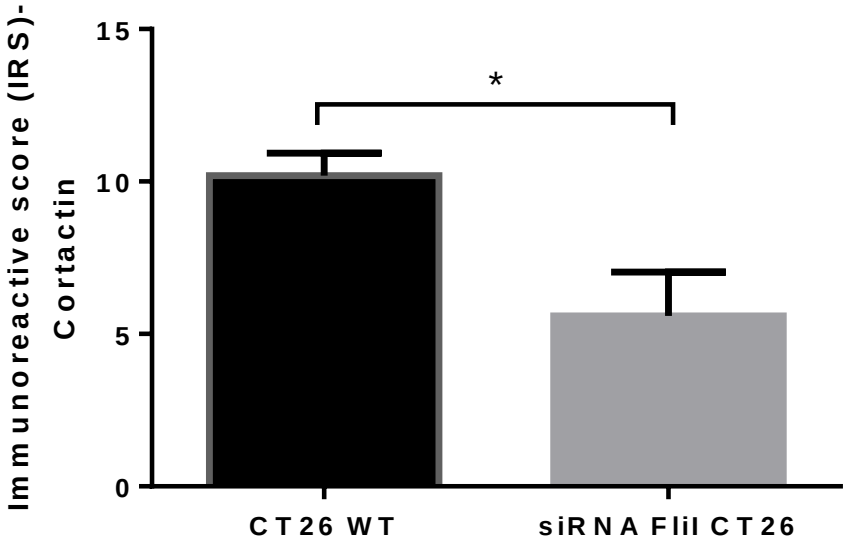


Figure 5.19: Quantification of cortactin expression in primary tumours in BALB/c mice. BALB/c mice were inoculated with CT26 cells expressing different levels of FliI and primary tumours were collected and analysed by immunohistochemistry staining. Primary tumours were immunostained with cortactin and the immunoreactive score of cortactin expression determined in primary tumours in BALB/c mice inoculated with CT26 WT and siRNA FliI CT26 (n=5). Results are shown as mean score ± SEM. *$p<0.05$ is significantly different from control group (Student's unpaired t-test).

5.2.6.2 Histological analysis of cortactin expression in lung tumours

Analysis of cortactin expression in lung tumours was performed by scoring the sections immunostained for cortactin in tumours in BALB/c mice inoculated with CT26 WT or siRNA FliI CT26 cells. There was also a significantly lower level of staining for cortactin found in lung tumours in mice inoculated with siRNA FliI CT26 cells compared to mice inoculated with CT26 WT cells ($p<0.05$) (Figure 5.20). Lower cortactin expression suggests less invadopodia formation was observed in the lung tumours of BALB/c mice inoculated with siRNA FliI CT26 cells.

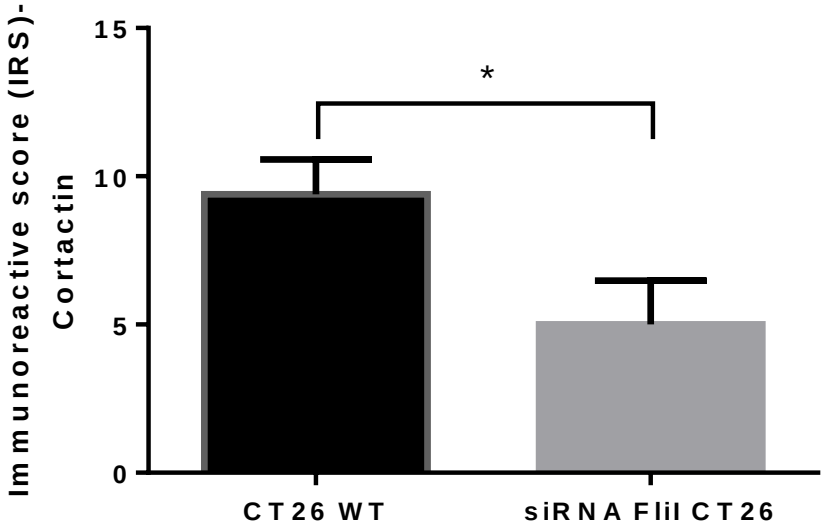


Figure 5.20: Quantification of cortactin expression in lung tumours in BALB/c mice inoculated with colon CT26 cancer cells expressing different levels of FliI. BALB/c mice were inoculated intravenously with CT26 cells expressing different levels of FliI and tumours present in the lungs were collected and analysed by immunohistochemistry. Tumour nodules were immunostained with cortactin and the immunoreactive score of cortactin expression determined in tumours from BALB/c mice inoculated with either CT26 WT or siRNA FliI CT26 (n=5). Results are shown as mean score ± SEM. *$p<0.05$ is significantly different from control group (Student's unpaired t-test).

5.2.7 Expression of α-smooth muscle actin (α-SMA) in primary and lung tumours

The expression of α-SMA was used as a myofibroblast marker, and was studied and evaluated in primary and lung tumours. Primary and lung tumours were immunostained with α-SMA to determine the effect of FliI expression on the possible activation of stromal fibroblasts as cancer-associated (myo) fibroblasts. The expression of α-SMA was observed in fibroblasts present in the primary tumours of mice inoculated with CT26 WT or siRNA FliI CT26 cells. No significant difference was observed in the staining between the two groups of mice. However, higher expression of α-SMA was observed in lung tumours in mice inoculated with CT26 WT compared to mice inoculated with siRNA FliI CT26 cells (Figure 5.21).

Representative images of immunohistochemical staining of α-SMA in primary and lung nodules are shown in Figure 5.21. There was greater expression of α-SMA in BALB/c mice injected with CT26 WT cells compared to BALB/c mice injected with siRNA FliI CT26 cells in lung tumours, while no significant difference was found in primary tumours.

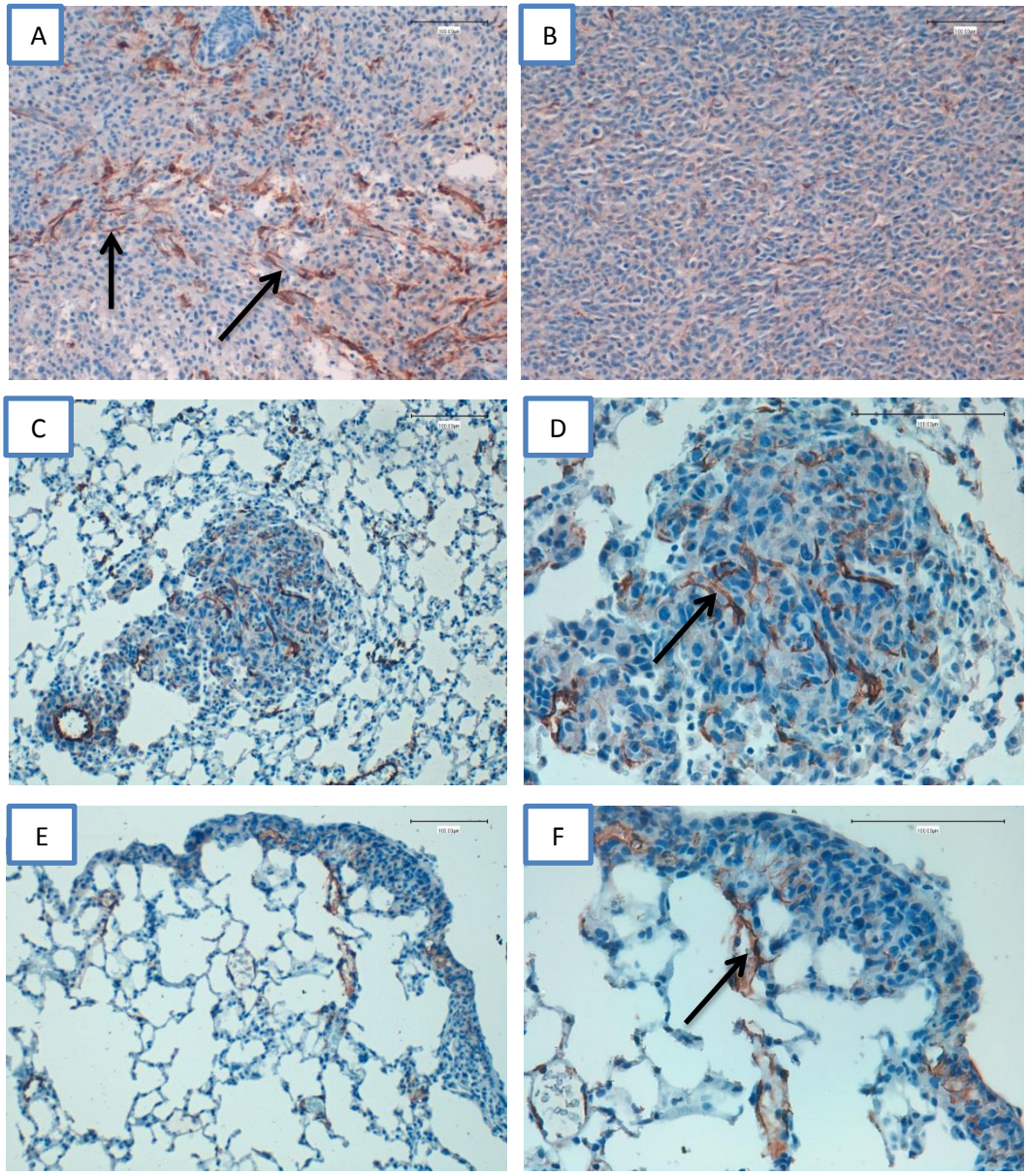


Figure 5.21: α-SMA expression in primary and lung tumours in BALB/c mice. Primary tumour immunostaining with α-SMA in **(A)** mice injected with CT26 WT cells and **(B)** mice injected with siRNA FliI CT26 cells (20X magnification). Lung tumours immunostained with α-SMA in **(C, D)** mice injected with CT26 WT cells and **(E, F)** mice injected with siRNA FliI CT26 cells; **(C)** and **(E)** show low magnification (20X) and **(D)** and **(F)** show high magnification (40X). Stromal staining of myofibroblasts (indicated by arrows) in tumours is characterised by long spindle-shaped cells commonly aligned in groups (indicated by arrows).

5.2.7.1 Histological analysis of α-SMA expression in primary tumours

Analysis of α-SMA expression in primary tumours was performed by scoring the sections immunostained for α-SMA in tumours in BALB/c mice inoculated with CT26 WT or siRNA FliI CT26 cells. No statistically significant difference was found in the expression of the myofibroblast marker α-SMA in the primary tumours of mice inoculated with siRNA FliI CT26 cells compared to the primary tumours from CT26 WT inoculation (Figure 5.22).

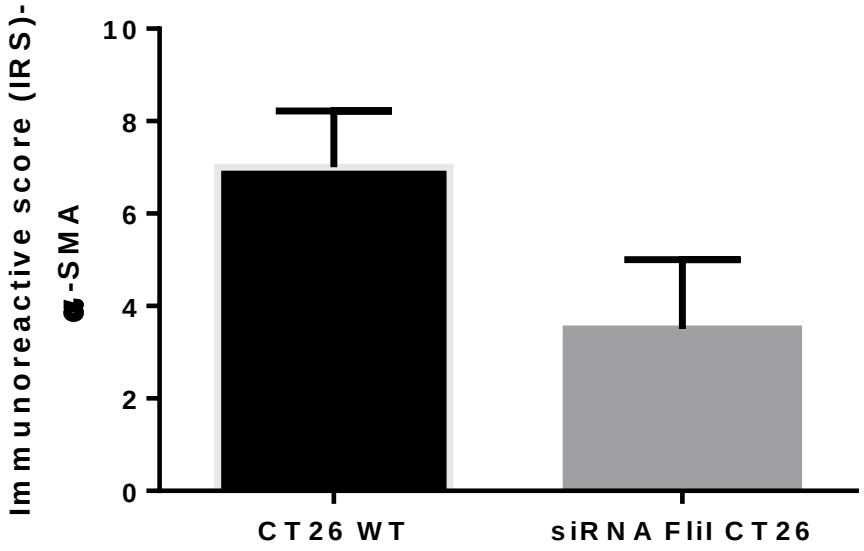


Figure 5.22: Quantification of α-SMA expression in primary tumours in BALB/c mice inoculated with colon CT26 cells expressing different levels of FliI. BALB/c mice were inoculated with CT26 cells expressing different levels of FliI and primary tumours were collected and analysed by immunohistochemistry. Primary tumours were immunostained for α-SMA and the immunoreactive score determined in BALB/c mice inoculated with CT26 WT and siRNA FliI CT26 (n=5). Results are shown as mean score ± SEM (Student's unpaired t-test).

5.2.7.2 Histological analysis of α-SMA expression in lung tumours

Analysis of α-SMA expression in lung tumours was performed by scoring sections immunostained for α-SMA from BALB/c mice inoculated with CT26 WT or siRNA FliI CT26 cells. The expression of α-SMA in lung tumours was higher in mice inoculated with CT26 WT cells as compared to mice inoculated with siRNA FliI CT26 cells ($p<0.05$) (Figure 5.23).

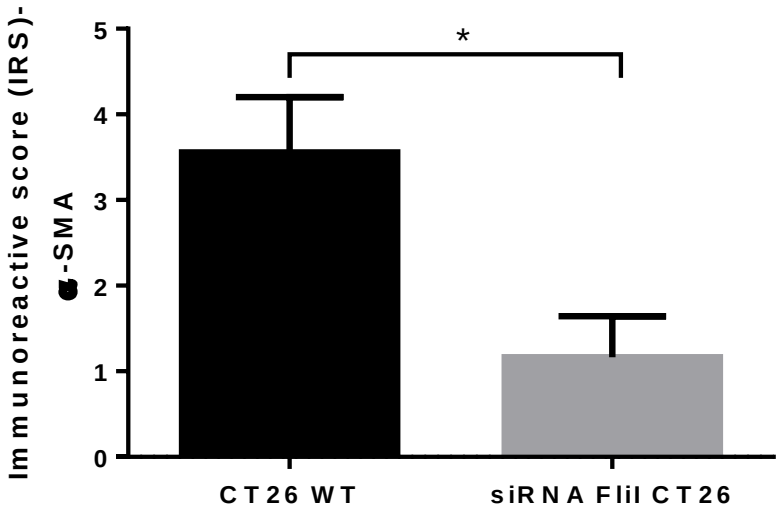


Figure 5.23: Quantification of α-SMA expression in lung tumours in BALB/c mice inoculated with colon cancer CT26 cells expressing different levels of FliI. BALB/c mice were inoculated intravenously with CT26 cells expressing different levels of FliI and tumours present in the lungs were collected and analysed by immunohistochemistry. Tumour nodules were immunostained with α-SMA and the immunoreactive score determined in lung tumours from mice inoculated with CT26 WT or siRNA FliI CT26 (n=5). Results are shown as mean score ± SEM. *$p<0.05$ shows significant difference from control group (Student's unpaired t-test).

5.3 Discussion

Dynamic interaction between cancer cells and their microenvironment is now thought to be extremely important in regulating cancer development (Tlsty & Coussens, 2006) and the tumour stroma has been found to be critically altered during neoplastic changes (Ronnov-Jessen, Petersen, & Bissell, 1996). During cancer development, stromal fibroblast cells acquire expression of α-sma, vimentin and in some cases smooth muscle myosin, tenascin and desmin, and the expression of altered ECM molecules in the stroma has also been found to be capable of directly or indirectly regulating cell proliferation, inflammation, angiogenesis and dissemination of cells to distant sites (Van den Hooff, 1988). FliI is believed to have a major role in rearrangement of the actin cytoskeleton, which is crucial in cell motility, adhesion and invasion, and may also serve as a linkage protein between actin filaments and various intracellular signalling proteins (Davy et al 2000).

Major components and functions in the local microenvironment that functionally regulate cancer development include inflammatory cells, the activation of fibroblasts as myofibroblasts, tumour angiogenesis, and the secretion and reorganisation of the ECM. In many cancers, activation of fibroblasts as a CAF phenotype has been found to be crucial, as these cells are primarily involved in reorganisation and deposition of the ECM, as well as altering its composition, and also importantly in regulating cell proliferation and survival by releasing paracrine growth factors (Tlsty & Coussens, 2006). The activation of fibroblasts as the myofibroblast phenotype in the tumour stroma has also been linked to poor prognosis (Tsujino et al., 2007).

Although the role of FliI may be mainly intracellular, it has also been reported that FliI was secreted from cells and was found to be present in the serum, especially on tissue injury or

wounding (Cowin et al., 2007). Since FliI may play both intracellular and secreted roles, understanding the regulation and role of FliI in cancer cells, in addition to its potential role in stromal cells, is important if FliI is to be a target for therapeutic use. Using mice with different genetic backgrounds, in particular FliI heterozygous knockout ($FliI^{+/-}$) mice, has enabled us to examine the effect of reducing FliI expression in the host (stromal) cells and the possible importance of their interaction with WT cancer cells in influencing tumour growth. By using siRNA FliI knockdown in tumour cells, we aimed to be able to see the effect of reducing FliI expression in the tumour cells themselves, and their interaction with a WT stroma. However, the limitation of this technique is that the siRNA knockdown of FliI is likely to be transient. Therefore, these experiments at best may tell us something about the effects of FliI expression during early events in tumour growth. Future experiments should look at the possibility of knocking down FliI *in vivo*, potentially by direct injection of FliI siRNA or antisense directly into primary tumours.

In this study, the inhibition of FliI promisingly showed a reduction in tumour formation in both primary and lung cancer models. Reduction of the expression level of FliI in FliI heterozygous mice ($FliI^{+/-}$) significantly reduced tumour nodules in the lung. Previously, preliminary studies in our laboratory showed that colon cancer cell inoculation resulted in increased tumour size and increased seeding of lung nodules in mice that had higher expression levels of FliI (FliI Tg^{+} mice), while mice with reduced FliI expression showed smaller primary tumours and fewer tumour nodules in the lung (Darby et al., unpublished data), which we have now confirmed in this study. This suggests that host FliI expression levels can regulate tumour growth in this model, possibly through an effect on the behaviour of tumour stromal cells leading to increased tumour development.

Interestingly, we also found that knockdown of FliI in tumour cells using siRNA FliI CT26 resulted in smaller primary tumours and fewer nodules in the lung, suggesting FliI expression in cancer cells may also influence tumour growth in this model. Smaller tumour size and reduced tumour formation were observed at days 10 and 14 after inoculation; however, tumours started to increase in size after day 14 and there was no longer a significant difference compared to WT cells, most likely because the knockdown of FliI using siRNA is a transient process and, based on our finding (not shown), the effectiveness of the siRNA FliI CT26 cells decreased *in vitro* after two weeks and was likely more transient *in vivo*. In addition, similar findings were demonstrated in an *in vitro* study where decreased cell invasion was observed with siRNA FliI CT26 cells, as described in the previous chapter.

FliI, from its pro-scarring activity discovered in skin wounds, may have the capability to cause remodelling of the ECM in a manner that favours tumour growth. FliI has also been found to regulate cytoskeletal remodelling where FliI co-localises with actin-associated molecules and other molecules to regulate the actin cytoskeleton and other molecules involved in the regulation of cell motility and movement, such as GTP binding proteins, Ras and RhoA. In addition, an association between FliI and membrane ruffles and actin arcs shows the potential importance of FliI in regulating cell migration, proliferation and remodelling of cytoskeletal proteins (Cowin et al., 2007; Davy et al., 2001). Previous studies have shown that the expression levels of actin-remodelling proteins may play a role in regulating tumour progression, and inhibition of those proteins may reduce tumour growth and metastasis (Kwang Won Jeong, 2014; Anske Van den Abbeele et al., 2007).

These findings suggest that FliI expression can promote tumour formation and establishment of lung nodules in a colon cancer model, possibly via a role in mediating cell

adhesion and increasing cellular invasion, based on our *in vitro* findings. Furthermore, FliI also may have effects on the tumour microenvironment that favour tumour growth, which is supported by the observation of alterations in tumour effector function markers, such as expression of the myofibroblast marker α-SMA and the invadopodia marker cortactin. Correlation with increased cancer cell proliferation using PCNA was also observed. A recent study by collaborators also showed similar findings where FliI was shown to exhibit tumour-promoting activity in an SCC model in mouse skin, and it was concluded that this was potentially due to its effects on cell adhesion and migration associated with modification of the tumour microenvironment (Z Kopecki et al., 2015). In the same study, FliI was confirmed to associate with cortaction in the MET-1 squamous carcinoma cell line at the invading edge of cancer cells when they were cultured on a Matrigel basement membrane. In this present study, elevated level of cortactin with the expression of FliI suggest that FliI is one of the proteins that could possibly mediate invadopodia by association with cortactin to form a complex and based on its ability to affect reorganisation of the actin cytoskeleton (Z Kopecki et al., 2015; Mohammad et al., 2012).

5.3.1 Effect of FliI expression on proliferation and tumour effector markers

It has been shown that the activation of cellular and/or extracellular elements of the local host microenvironment can modify the invasive and proliferative behaviour of tumour cells (Liotta & Kohn, 2001). In this study, we have shown data that suggest that the inhibition of FliI expression in either the host or the tumour cells may affect the expression of markers of proliferation and invasion.

FliI knockdown resulted in a decrease in the expression of the invadopodia marker cortactin and a reduced proliferative index at both primary and lung sites. The inhibition of FliI within

either host or cancer cells could potentially reduce interactions between cell-to-cell junctions and also with the surrounding ECM; these interactions have been found to be crucial for initiation of remodelling of the ECM and are also an essential step for local invasion and promotion of tumourigenesis (Hanahan & Weinberg, 2011). In addition, it has been described previously that host–tumour interdependence effects show an invasion process that is mainly regulated by host cells (Coussens & Werb, 2002). However, in this study we show that modifying the expression of FliI in invading cancer cells may also affect tumour growth, progression and spread.

Further experiments are needed to understand the relative importance of stromal versus tumour cell expression of FliI. These could involve either direct inhibition of FliI expression in tumour cells, possibly by injection of antisense or siRNA at the tumour site *in vivo*, or the production of colon tumours in mice overexpressing FliI or FliI-deficient. This would require chemical induction of cancer in mice of the FliI transgenic and heterozygous ($FliI^{+/-}$) background, for example using azoxymethane and SDS. Lastly, it would be important to know the effects of FliI expression on a truly metastatic model of cancer.

Changes in stromal cell behaviour have been shown to promote EMT, which can alter both cell–cell and cell–matrix signalling. In tumours, there is a significant contribution of epithelial cells to the pool of cancer-associated stromal myofibroblasts via EMT (I. A. Darby et al., 2016). This may act to allow tumour cells to migrate across tissue boundaries (Liotta & Kohn, 2001) by altering matrix-degrading enzyme expression and activation. Furthermore, changes in the stiffness of the ECM can also influence tumour growth, as increased ECM stiffness has been shown to correlate with highly invasive tumours (I. A. Darby et al., 2016; Wei et al., 2015). Increased FliI expression has been previously reported to result in

excessive scar formation, including increased collagen I deposition (Z. Kopecki & Cowin, 2008), suggesting the ability of FliI to cause remodelling and increased deposition of the ECM and thus influence the stiffness of the ECM.

Additionally, MMPs produced by stromal fibroblasts and endothelial cells can lead to subsequent matrix degradation, thus mediating tumour-cell invasion via invadopodia (Zigrino et al., 2009). FliI was recently found to co-localise with the invadopodia marker cortactin, showing a possible mechanism for FliI to interact with or influence invadopodia formation. In addition, the polymerisation of actin filaments and their associated actin-binding proteins in actin remodelling was found to be one of the important elements in the formation of invadopodia, which has been shown to mediate invasion and metastasis in cancer cells (Yamaguchi & Condeelis, 2007).

5.4 Conclusion

In conclusion, we have demonstrated that FliI expression, particularly in the host animal stroma, may regulate tumour growth and the establishment of lung tumours in a murine colon cancer model. Reducing the expression of FliI by using heterozygous knockout mice resulted in a reduced number of nodules in the lung. Promisingly, transient inhibition of FliI expression in cancer cells themselves also seemed to reduce primary tumour formation and lung seeding, possibly by altering the invasive capacity of tumour cells. Further experiments are needed to show whether this can be confirmed, using direct local inhibition of FliI expression. FliI inhibition may thus be associated with an improved tumour microenvironment that is unfavourable to cancer cell progression and metastasis when FliI is decreased in the host tissue or tumour microenvironment. We have also shown that FliI

expression levels in tumour cells may regulate cell invasion and metastasis by increasing invadopodia activity and thus remodelling of the ECM.

It remains unclear exactly how Flil influences tumour growth and spread and, furthermore, the relative importance of the Flil protein intracellularly and in its secreted form. The importance of Flil in the tumour stroma has been suggested by tumour models in animals expressing higher or lower levels of Flil. However, the manipulation of Flil in the tumour cells themselves would be an important step in examining the importance of its expression in the tumour cells themselves. Further experiments are required to address these issues in the future.

Chapter 6 Therapeutic Effects of Flightless I Monoclonal Antibody in Colon Cancer Models

6.1 Introduction

Recombinant proteins and humanised monoclonal antibodies have become important new tools in therapies including treatment of cancers; for example, the application of recombinant interferons (IFNs) for multiple sclerosis, hepatitis and cancer treatment, and established monoclonal antibody therapies that target growth factors or growth factor receptors, including VEGF (known as bevacizumab), which is currently used as a first-line therapy for metastatic colorectal cancer (Ferrara, Hillan, Gerber, & Novotny, 2004; Schellekens, 2002).

It is important to acknowledge the crucial interplay between cancer cells and their local microenvironment, which has been shown to be actively involved in cancer progression. The changes occurring within the surrounding tissue have an important influence on the fate of cancer cells, either favouring or inhibiting tumour growth, and the tumour microenvironment is now considered to be one of the important cancer hallmarks correlating with prognosis and the effectiveness of cancer therapy (Hanahan & Coussens, 2012). Thus therapies for targeting the tumour microenvironment are now definitely a

major target for discovering new treatments, but are potentially limited by the complexity and variety of the stromal cells (Sounni & Noel, 2013).

FliI has been reported to be a negative regulator of wound repair, and treatment with FliI-neutralising monoclonal antibody (FnAb) by local injection into the wound site was found to be effective in porcine and diabetic mouse wound-healing models. These studies showed FliI inhibition to have the potential to enhance wound repair and reduce scar formation (Jackson et al., 2012; Ruzehaji et al., 2014). In addition, topical application of FnAb in a cream onto established blisters showed similar results, with a reduction in the severity of skin blistering observed (Zlatko Kopecki et al., 2013). Intradermal injection of FnAb during SCC initiation and progression in a chemically induced model of SCC significantly reduced tumour size, and treatment using FnAb *in vitro* was shown to alter cell proliferation and migration and to block cellular invasion into basement membrane proteins (Z Kopecki et al., 2015). Thus FliI seems to play a potential role as a promoter of both scarring and tumour growth, at least in an SCC model.

This chapter discusses the effect of administration of a monoclonal antibody (FnAb) that blocks FliI function on primary and lung tumour growth, and the outcome on tumour progression in primary and secondary lung models of colon cancer.

6.2 Results

6.2.1 Therapeutic effects of FliI-neutralising monoclonal antibody on lung tumour growth

To ascertain the effect of FnAb on lung tumour growth, BALB/c mice were injected intravenously with CT26 WT cells. These mice showed no difference in bodyweight with or

without treatment with FnAb (Figure 6.1). Experimental mice in each group maintained their bodyweight within 10% of their original starting weight. The number of cancer nodules observed in the lungs was similar in mice treated with antibodies (either non-specific IgG or FnAb) throughout the 14 days of the experimental period. A slightly reduced number of macroscopic and microscopic metastatic nodules in the lungs were observed in mice treated with 50 µg FnAb compared to the control group treated with non-specific IgG, as shown in Figure 6.2, although this was not statistically significant.

Although the number of lung nodules was not different in mice injected with either FnAb or non-specific IgG antibody, when the metastatic nodules were measured for size the area of nodules in the lung showed a significant decrease in size in FnAb-treated mice compared to mice receiving the non-specific IgG control ($p<0.0001$) (Figure 6.3).

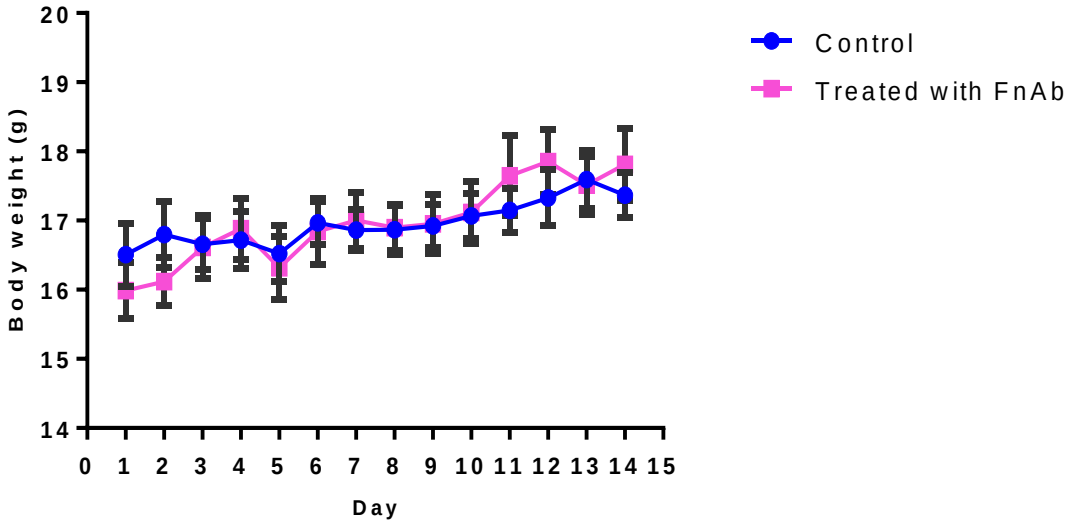


Figure 6.1: Monitoring of bodyweight in BALB/c mice after treatment with FnAb post-induction with colon cancer cells. BALB/c mice were injected intravenously with $2x10^5$ CT26 WT cells and at days 4 and 8 post tumour induction, mice were treated with control IgG (blue line with circle) and FnAb (pink line with square) (n=10). Bodyweight of mice was monitored on a daily basis for the duration of the experiment. Bodyweight is shown as mean weight (g) ± SEM.

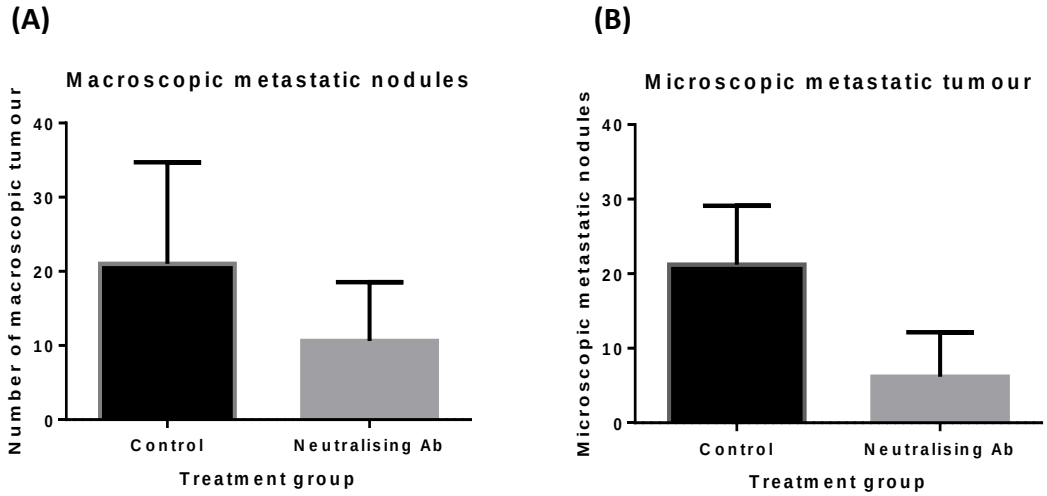


Figure 6.2: Number of macroscopic and microscopic lung nodules in BALB/c mice after treatment with FnAb post tumour induction. BALB/c mice were injected intravenously with CT26 WT cells and at days 4 and 8 post tumour induction, mice were treated with control IgG and FnAb, showing the number of **(A)** macroscopic and **(B)** microscopic tumour nodules present in the lungs of mice treated with FnAb as compared to control group (treated with non-specific IgG) (n=10). Results are shown as mean ± SEM. $p<0.05$ is significantly different from control group (Student's unpaired t-test).

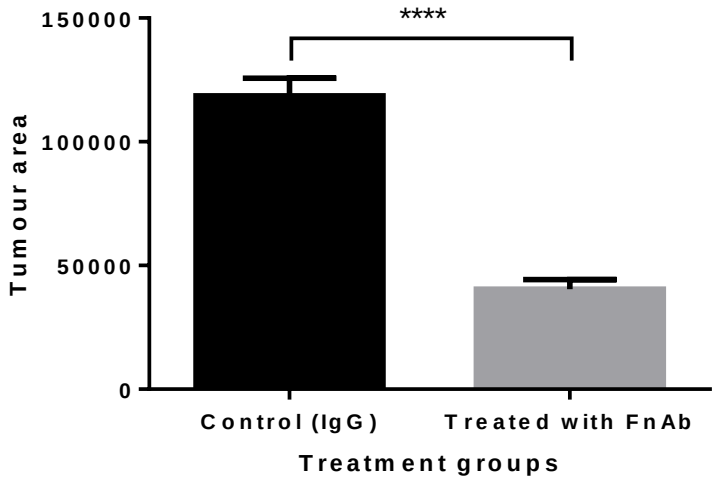


Figure 6.3: Quantification of tumour area of microscopic tumour nodules in BALB/c mice after treatment with FnAb. BALB/c mice were injected intravenously with CT26 WT cells and at days 4 and 8 post tumour induction, mice were treated with control IgG or FnAb (n=5). The size of tumour nodules in the lung was quantified using Image J analysis. Results are shown as mean ± SEM. ****$p<0.0001$ is significantly different from control group (Student's unpaired t-test).

Representative images of H&E-stained microscopic tumour nodules in mice treated with FnAb and the control group are shown in Figure 6.4. FnAb histological images show the reduced size of lung nodules in mice injected with FnAb compared to the control group.

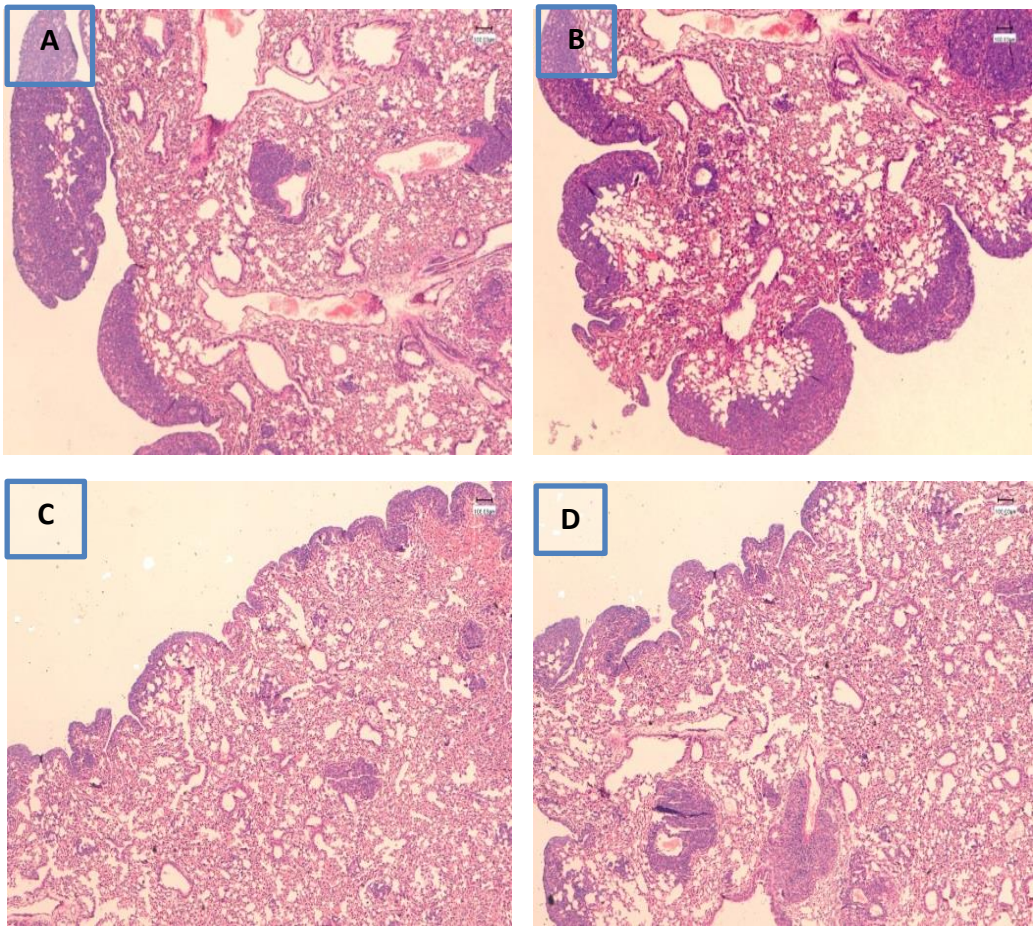


Figure 6.4: Histological analysis of lung tumours in BALB/c mice after treatment with FnAb post-induction with intravenous injection of CT26 cells. Representative histological images of lung sections stained with H&E showing microscopic lung nodules of **(A, B)** control groups (BALB/c mice treated with non-specific IgG) and **(C, D)** BALB/c mice treated with FnAb (4X magnification).

6.2.2 Effects of Flil-neutralising monoclonal antibody on expression of PCNA in lung tumour model

Immunohistochemical staining of the proliferation marker PCNA showed greater areas of expression of PCNA in the tumours in the lungs of the control mice (treated with non-specific IgG) compared to smaller areas showing expression in the tumours of mice treated with FnAb, as shown in representative images (Figure 6.5). Representative images of the immunohistochemical staining of nodules for PCNA are shown in Figure 6.5. Nodules in both control mice and FnAb-treated mice showed high levels of PCNA expression (positive nuclei) in the tumour cells.

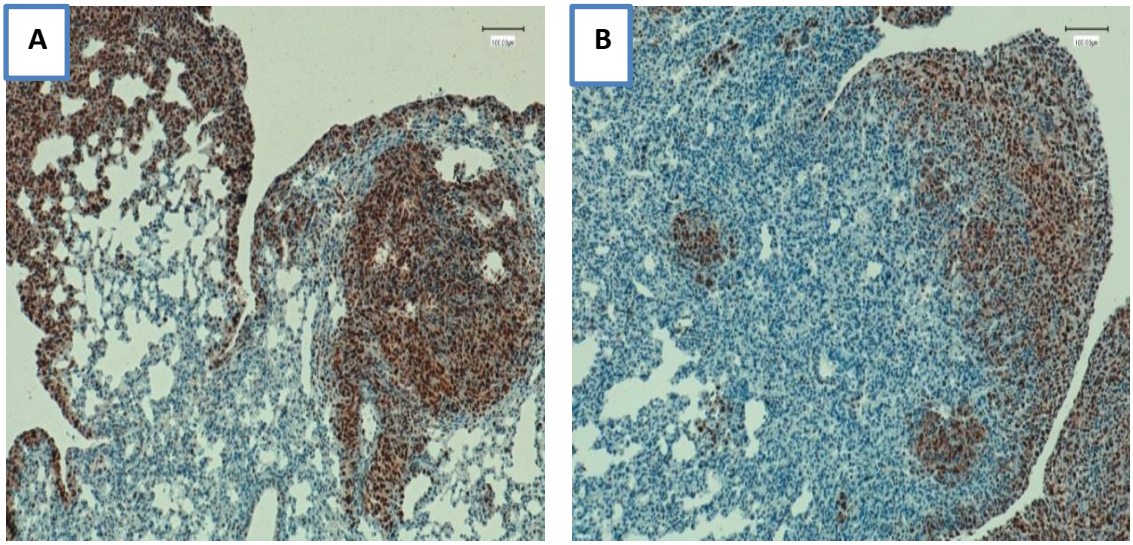


Figure 6.5: PCNA expression in lung tumours of BALB/c mice after treatment with FnAb post tumour induction. BALB/c mice were injected intravenously with CT26 WT cells and at days 4 and 8 post tumour induction, mice were treated with control IgG and FnAb and tumours present in the lungs were collected and analysed after 14 days. Tumour nodules immunostained for PCNA in **(A)** BALB/c mice treated with non-specific IgG (control) and **(B)** BALB/c mice treated with FnAb (20X magnification).

6.2.3 Proliferation index for lung tumours

Analysis of PCNA expression in the lung tumours was performed by scoring the lung sections immunostained for PCNA in the control BALB/c mice treated with non-specific IgG and the mice treated with FnAb (Figure 6.6). No difference was found in staining for the cell proliferation marker PCNA in tumours when comparing the control group treated with non-specific IgG and the mice treated with FnAb. Staining showed high levels of PCNA proliferation in both groups.

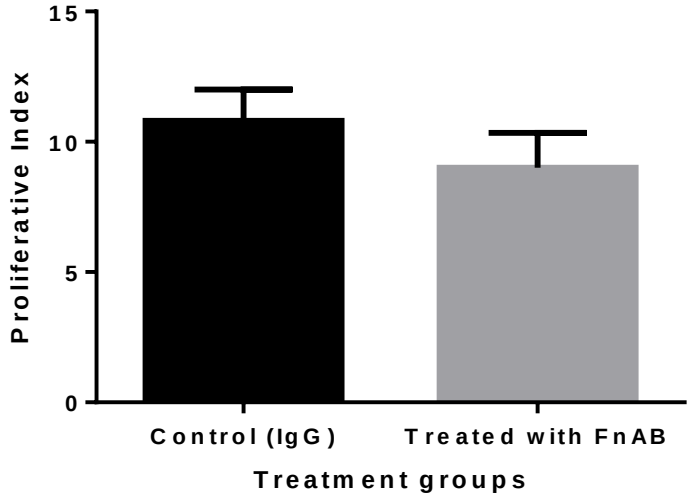


Figure 6.6: Analysis of PCNA expression in lung tumours in BALB/c mice after treatment with FnAb post tumour induction. BALB/c mice were injected intravenously with CT26 WT cells and at days 4 and 8 post tumour induction, mice were treated with control IgG and FnAb and metastatic tumours present in the lungs were collected and analysed after 14 days' duration of the experiment. Sections were immunostained for PCNA and the number of PCNA positive cells counted and represented as the proliferative index in mice treated with FnAb and the control group (mice treated with non-specific IgG) (n=6). Results are shown as mean proliferative index ± SEM. $p<0.05$ is significantly different from control group (Student's unpaired t-test).

6.2.4 Effects of Flil-neutralising monoclonal antibody on the expression of α-SMA in lung tumours

The expresssion of the myofibroblast marker α-SMA was determined using immunohistochemistry in lung tumour sections from the experimental mice. BALB/c mice injected with non-specific IgG showed higher expression of α-SMA in tumour nodules compared to BALB/c mice treated with FnAb (Figure 6.7).

Representative images of the immunohistochemical staining of lung sections showing α-SMA expression are shown in Figure 6.7.

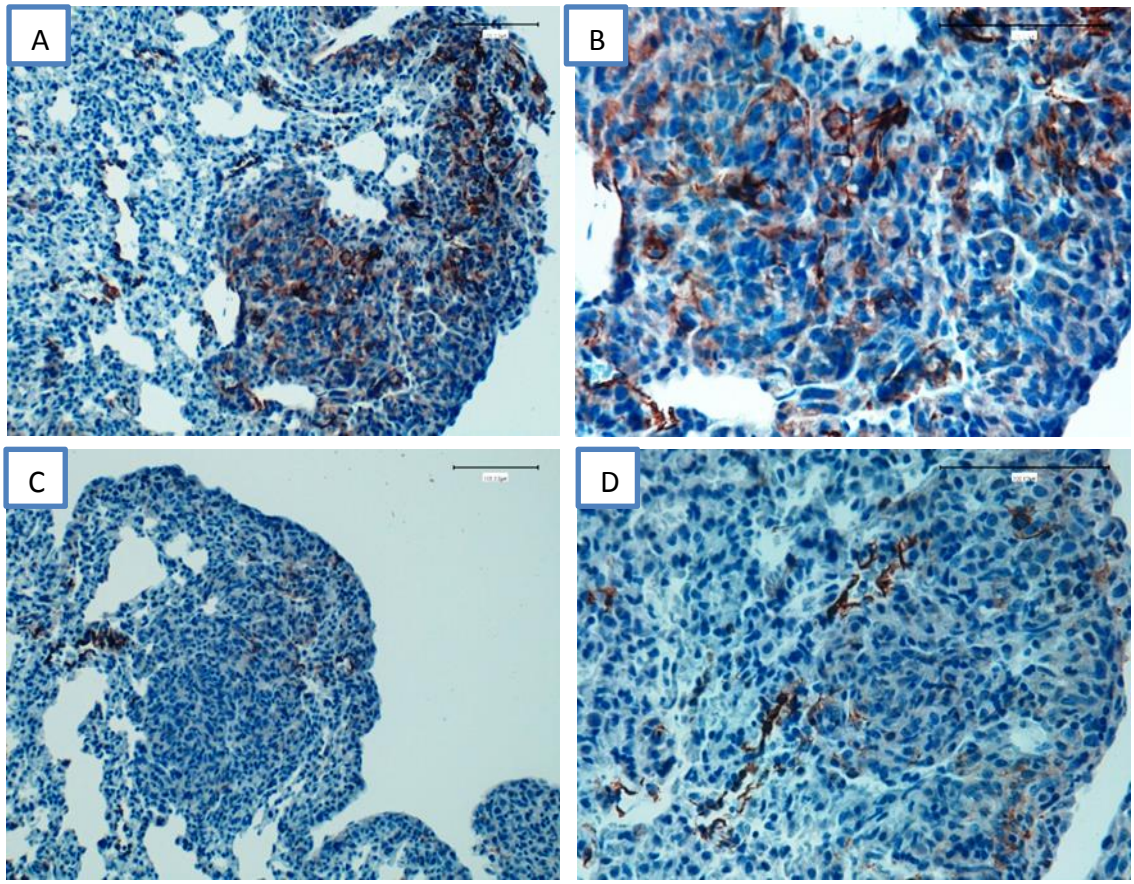


Figure 6.7: α-SMA expression in lung tumours of BALB/c mice after treatment with FnAb post tumour induction. BALB/c mice were injected intravenously with CT26 WT cells and at days 4 and 8 post tumour induction, mice were treated with control IgG and FnAb and tumours present in the lungs were collected and analysed after 14 days' duration of the experiment. Tumour nodules immunostained with α-SMA in **(A, B)** mice treated with non-specific IgG (control) and **(C, D)** mice treated with FnAb. **(A)** and **(C)** show histological images at low magnification (20X) and **(B, D)** show histological images at high magnification (40X).

6.2.5 Histological analysis of α-SMA expression in lung tumours

Analysis of α-SMA expression in lung tumours was performed by scoring the sections immunostained for α-SMA in control BALB/c mice treated with non-specific IgG and mice treated with FnAb (Figure 6.8). Tumour nodules were immunostained and scored using immunoreactive scores for quantitative analysis. The expression of the myofibroblast marker α-SMA was significantly lower ($p<0.05$) in lung tumours in BALB/c mice treated with FnAb at days 4 and 8 post-inoculation as compared to the control group administered non-specific IgG.

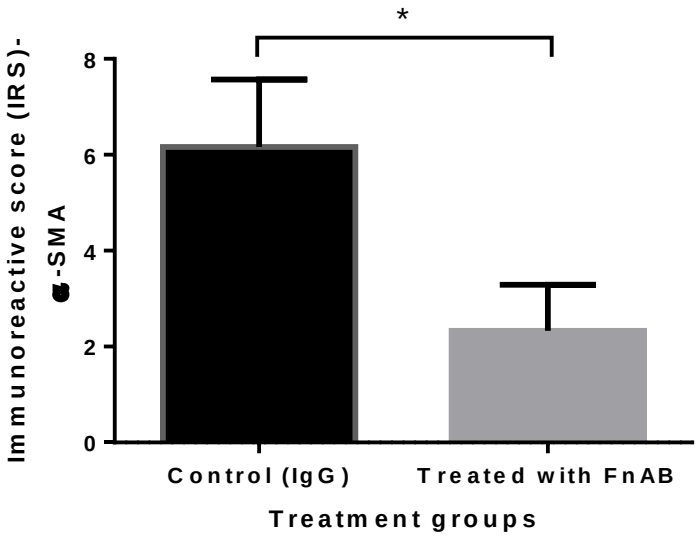


Figure 6.8: Quantitative analysis of α-SMA expression in lung tumours in BALB/c mice after treatment with FnAb post tumour induction. BALB/c mice were injected intravenously with CT26 WT cells and at days 4 and 8 post tumour induction, mice were treated with control IgG and FnAb and tumours present in the lungs were collected and analysed after 14 days. The immunoreactive score for α-SMA expression was determined in tumours in mice treated with FnAb and the control group (mice treated with non-specific IgG) (n=6). Results are shown as mean immunoreactive score ± SEM. $p<0.05$ is significantly different from control group (Student's unpaired t-test).

6.2.6 Effects of FliI-neutralising monoclonal antibody on expression of cortactin in lung tumours

The expression of the invadopodia marker cortactin was evaluated in the lung tumours in mice, comparing a control group (BALB/c mice treated with non-specific IgG) and a treatment group (BALB/c mice treated with FnAb) (Figure 6.9).

Representative images of the immunohistochemical staining of lung nodules showing cortactin expression are shown in Figure 6.8. Immunostaining showed slightly lower cortactin staining in the nodules of mice treated with FnAb compared to the control mice; however, the immnohistochemical scoring showed no statistically significant difference (Figure 6.10).

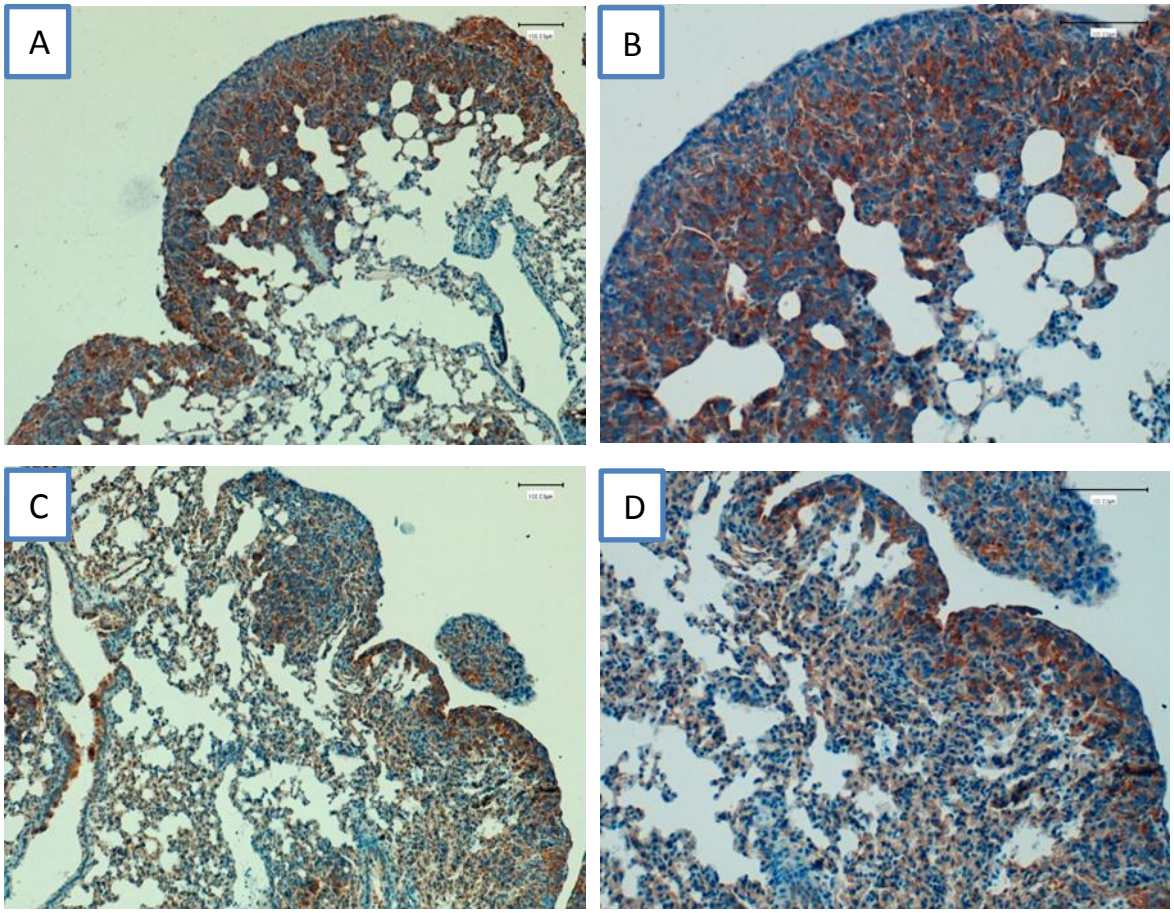


Figure 6.9: Cortactin expression in lung tumours of BALB/c mice after treatment with FnAb. Lung tumour nodules immunostained with anti-cortactin antibody in **(A)** mice treated with non-specific IgG (control) and **(B)** mice treated with FnAb. **(A)** and **(C)** show histological images at low magnification (10X) and **(B)** and **(D)** show histological images at higher magnification (20X).

6.2.7 Histological analysis of cortactin expression in lung tumours

Analysis of cortactin expression in metastatic tumours was performed by scoring the immunohistological lung sections immunostained with anti-cortactin in control BALB/c mice administered non-specific IgG and mice treated with FnAb. Tumour nodules were immunostained with anti-cortactin and scored using immunoreactive scores for quantitative analysis. There was slightly lower ($p>0.05$) staining intensity of cortactin in the lungs of mice treated with FnAb; however, no significant difference was found in the expression levels of cortactin between the treatment groups (Figure 6.10).

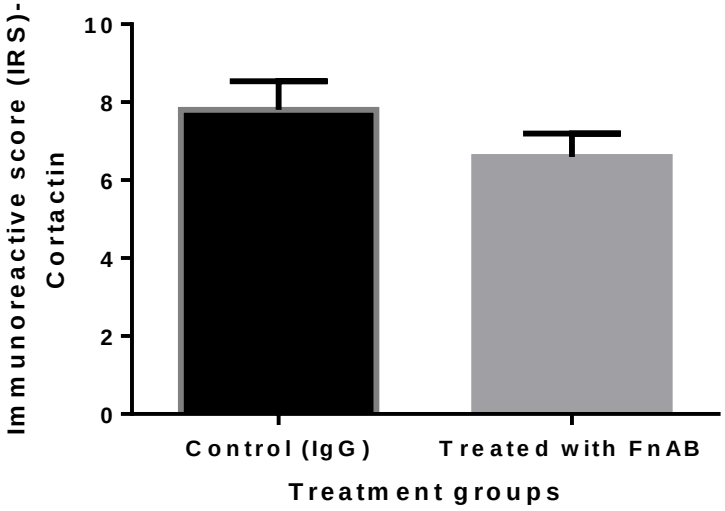


Figure 6.10: Quantitative analysis of cortactin expression in lung tumours in BALB/c mice after treatment with FnAb post tumour induction. BALB/c mice were injected intravenously with CT26 WT cells and at days 4 and 8 post tumour induction, mice were treated with control IgG and FnAb and the lungs were collected and analysed after 14 days. Tumour nodules were immunostained with cortactin and the immunoreactive score of cortactin expression determined in tumours in mice treated with FnAb and the control group (mice treated with non-specific IgG) (n=6). Results are shown as mean immunoreactive score ± SEM. $p<0.05$ is significantly different from control group (Student's unpaired t-test).

6.2.8 Inhibition of FliI reduced tumour sphere formation in tumour spheroid invasion assay

Tumour spheroid invasion assays were performed and established using low-adherent cell culture plates to generate spheroid formations in CT26 cells. Cells expressing different levels of FliI with and without treatment with FnAb were used to generate tumour spheroids. The spheroids were photographed and their diameter was measured at days 2, 5 and 10. The size of the spheroids produced by siRNA FliI-treated CT26 cells was significantly smaller compared to the spheroids of CT26 WT cells and CT26 WT cells treated with FnAb at days 2 and 5 (Figure 6.11). At day 2, there were smaller diameters of spheroids in both CT26 WT and siRNA FliI CT26 cells treated with FnAb, respectively; however, this was not statistically significant. Promisingly, treatment of FnAb on CT26 WT cells significantly reduced the spheroid size at day 5; however, adding FnAb to siRNA FliI-treated CT26 cells did not result in an additional reduction in diameter. At day 10, all tumour spheroids were observed to have similar diameters and were starting to dissociate, with prominent necrotic cells at the centre.

Representative images of tumour spheroids for CT26 WT and siRNA FliI CT26 cells treated with either non-specific IgG or FnAb at days 2, 5 and 10 are shown in Figure 6.12. Smaller spheroids were formed using siRNA FliI-treated CT26 cells compared to CT26 WT cells, and both cells also showed a reduction in spheroid diameter after treatment with FnAb at days 2 and 5.

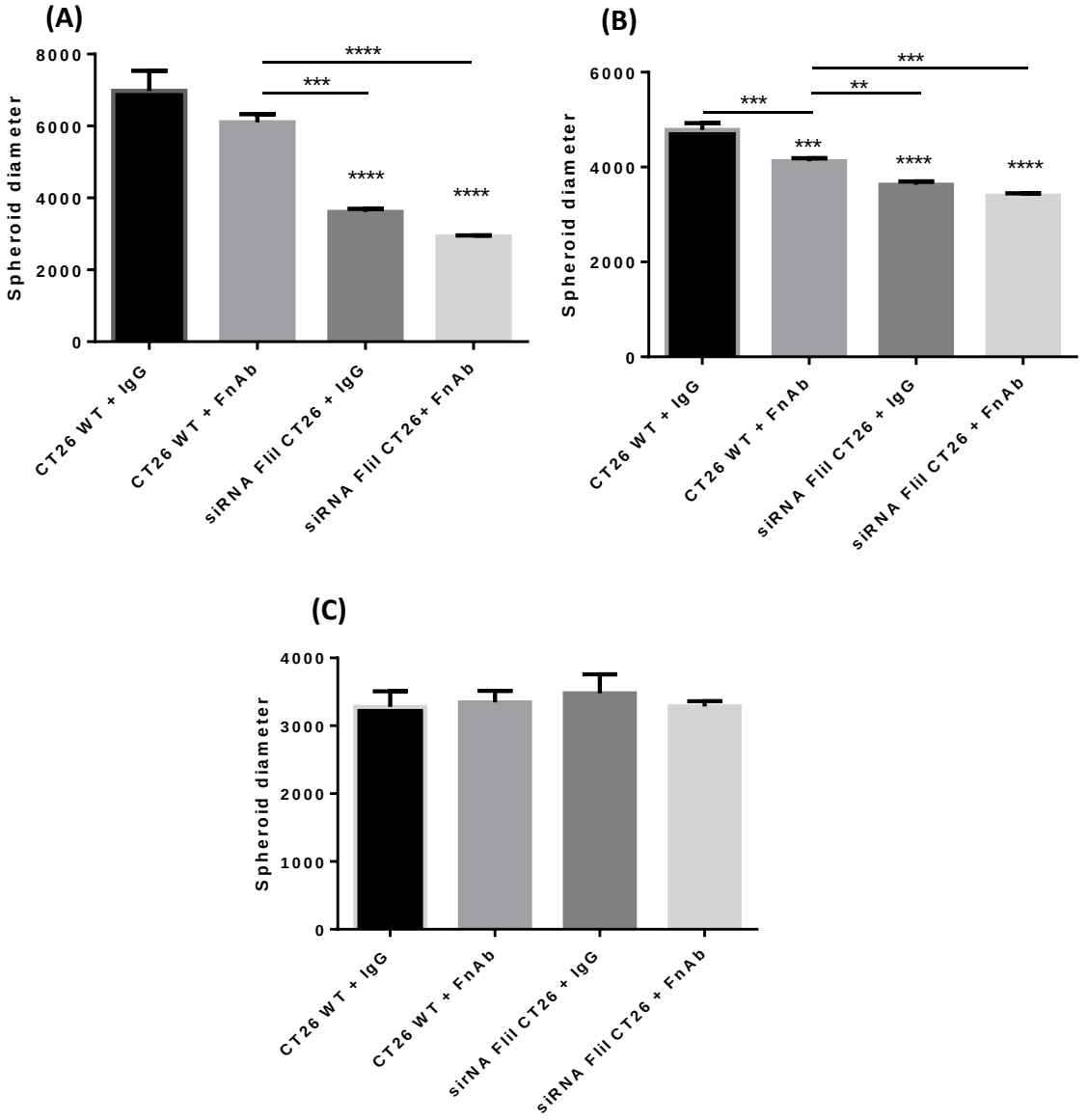


Figure 6.11: Analysis of diameter of spheroids in CT26 cells expressing different levels of FliI, with and without treatment with FnAb. Tumour spheroids of CT26 WT and siRNA FliI CT26 cells were grown in growth media containing 50 µg/ml of non-specific IgG or 50 µg/ml of FnAb for 10 days and the diameter of the spheroids were measured at days 2, 5 and 10. Tumour spheroid growth assays show the diameters of the spheroids at **(A)** day 2, **(B)** day 5 and **(C)** day 10 of CT26 WT cells and siRNA FliI CT26 cells treated with either non-specific IgG (control) or FnAb. Results are shown as mean ± SEM (n=3).****p<0.0001, ***p<0.001 and **p< 0.01 are significantly different to control cells CT26 WT (Tukey's multiple comparison test).

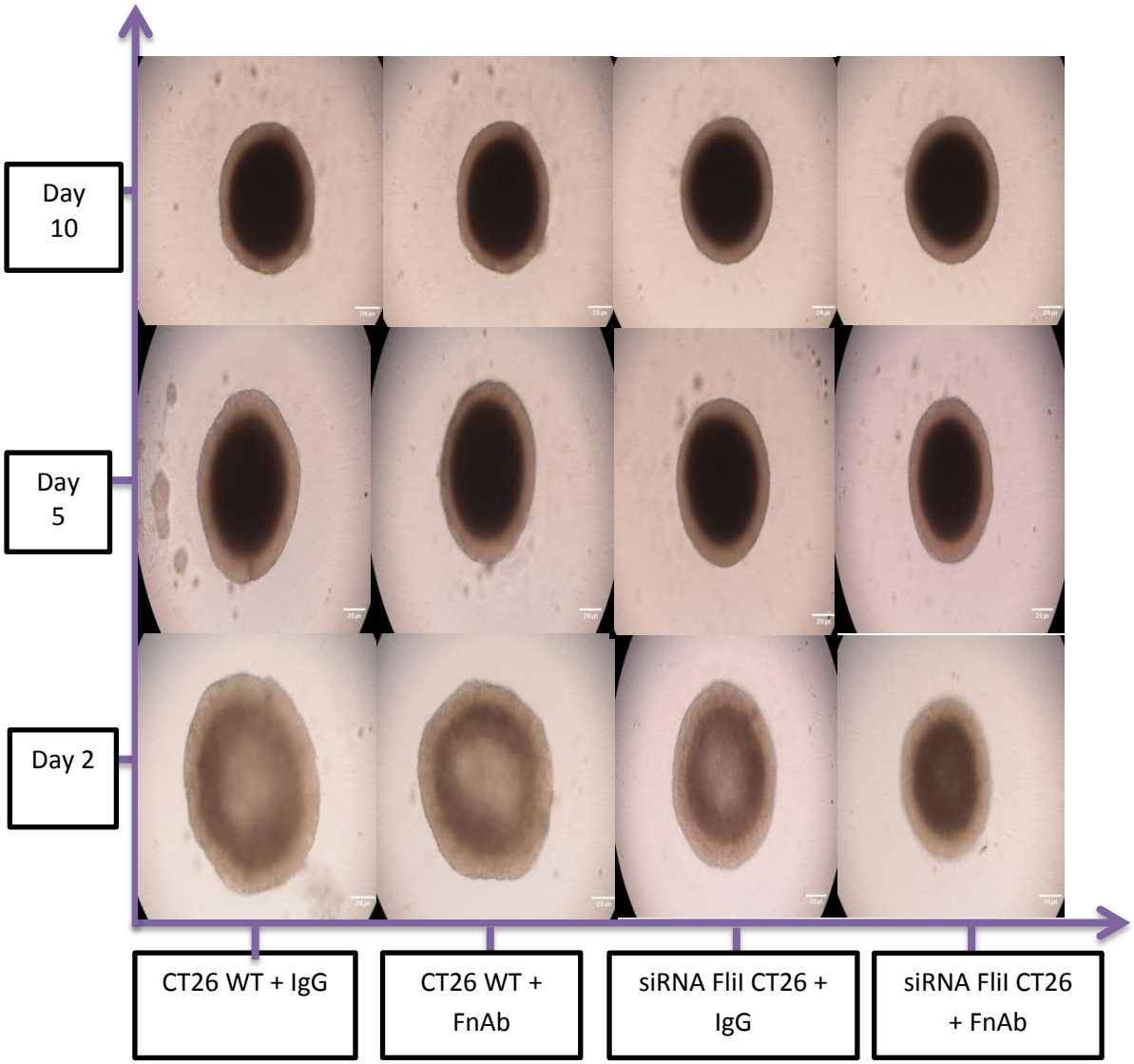


Figure 6.12: Tumour spheroid formation assays in CT26 cells expressing different levels of FliI and after treatment with FnAb. Inhibition of FliI using siRNA reduced the size of tumour spheroids in CT26 cells. Addition of FnAb had no additional effect on spheroid size. Tumour spheroids of CT26 WT and siRNA FliI CT26 were grown in growth medium containing 50 µg/ml of non-specific IgG or 50 µg/ml of FnAb for 10 days in cells and were photographed at days 2, 5 and 10. Representative sphere formation images show spheroids at days 2, 5 and 10 of CT26 WT and siRNA FliI CT26 cells. Both cell types were treated with either non-specific IgG as the control or FnAb.

6.2.8.1 Spheroid live/dead staining

Live/dead staining of the tumour spheroids was performed using calcein AM (live cells) and propidium iodide (dead cells). Stacked 3D images of the spheroids were visualised using confocal microscopy at day 5 (Figure 6.13) and day 10 (Figure 6.15). Representative fluorescence images of the spheroids at day 5 (Figure 6.14) and day 10 (Figure 6.16) are also shown. As stated above, the inhibition of FliI using siRNA FliI CT26 cells reduced the size of tumour spheroids at day 5, and blocking FliI using FnAb also reduced the size of tumour spheroids in CT26 WT cells at day 5. Tumour depth of spheroids in CT26 WT control cells treated with non-specific IgG was approximately 200 µm (Figure 6.13(A)) as compared to the CT26 WT cells treated with FnAb and siRNA FliI CT26, which was approximately 60 µm. At day 10, there was no difference in the size of spheroids, possibly as most cells had died and the spheroids had developed necrotic centres, as shown in 3D and 2D images at day 10 (Figures 6.15 and 6.16).

The number of live and dead cells in the spheroids was quantified using NIS Element software (Nikon) at days 5 and 10 (Figure 6.17). At day 5, there was a small decrease in the number of live and dead cells in siRNA FliI CT26 cells treated with either non-specific IgG or FnAb as compared to CT26 WT cells (both control and treated with FnAb); however, these changes were not statistically significant. The number of live cells also decreased at day 10 compared to day 5, likely indicating more cell death. At day 10, a significant reduction of live cells in siRNA FliI CT26 cells treated with FnAb as compared to the number of live cells in CT26 WT cells treated with FnAb (Figure 6.17 (B)) was observed.

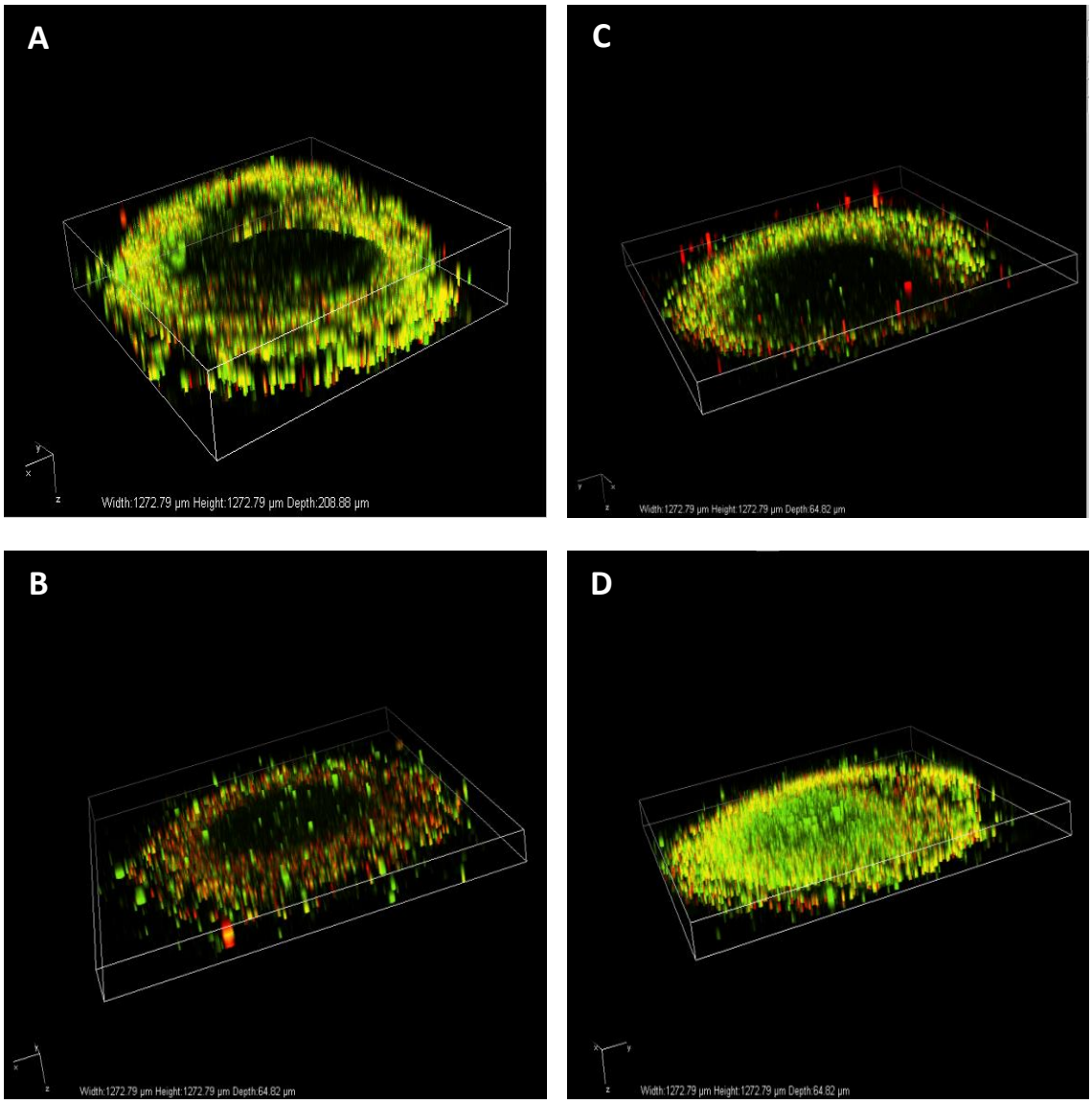


Figure 6.13: 3D stacked confocal images of tumour spheroids in CT26 cells expressing different levels of FliI and after treatment with FnAb. Representative live/dead staining of spheroids shown as stacked 3D images from confocal microscopy at day 5 of **(A)** CT26 WT and **(B)** siRNA FliI CT26 cells treated with non-specific IgG as a control, and **(C)** CT26 WT treated with FnAb and **(D)** siRNA FliI CT26 cell treated with FnAb. Live cells were stained with calcein AM (green) and dead cells were stained with propidium iodide (red). Tumour spheroids were viewed by an A1 confocal microscope and analysed using NIS Elements software (Nikon) at 10X magnification.

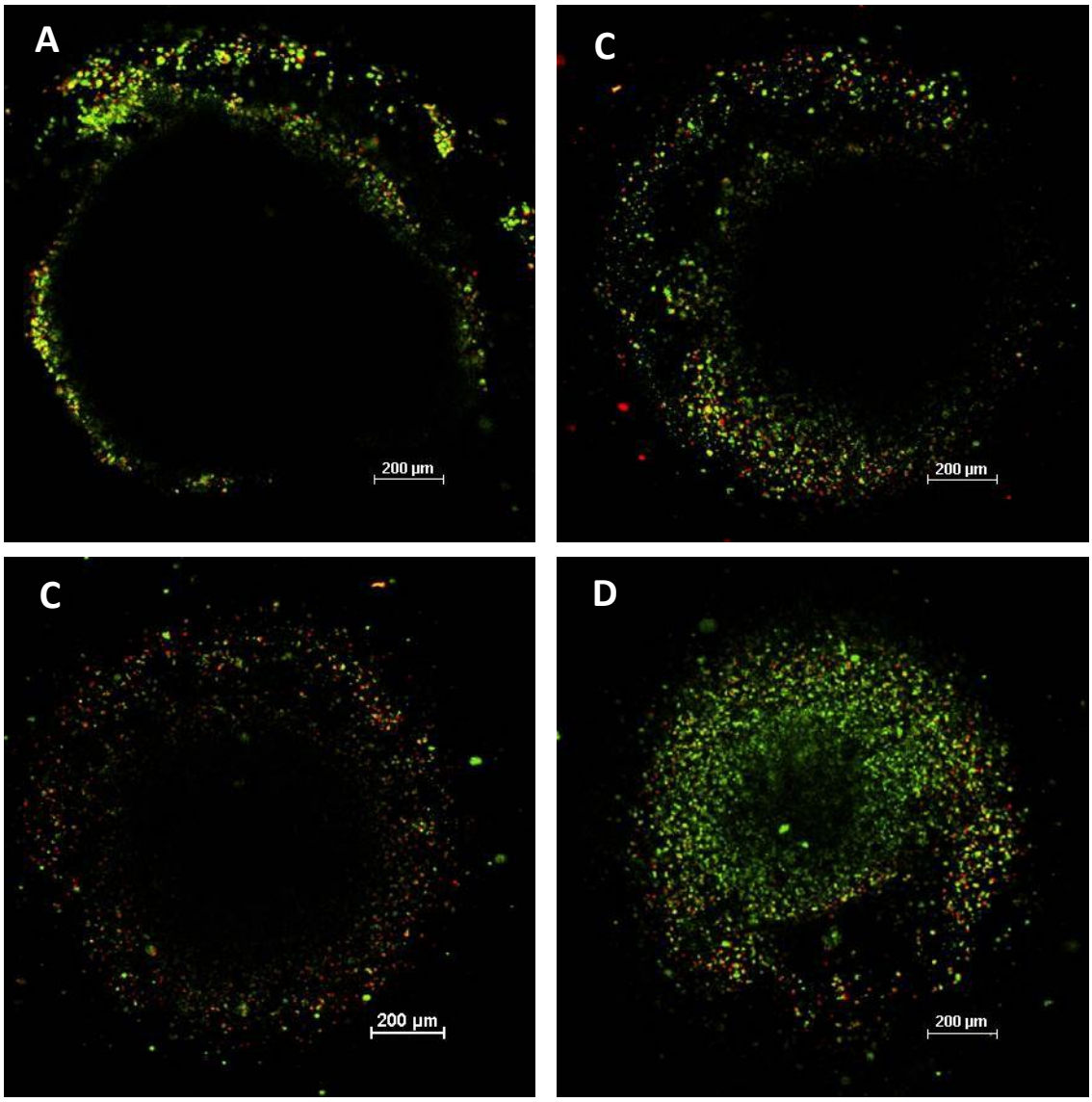


Figure 6.14: 2D confocal images of tumour spheroids in CT26 cells expressing different levels of FliI and after treatment with FnAb. Representative fluorescent 2D images of the spheroids at day 5. Live/dead staining of spheroids from confocal microscopy of **(A)** CT26 WT and **(B)** siRNA FliI CT26 cells treated with non-specific IgG as a control, and **(C)** CT26 WT cells treated with FnAb and **(D)** siRNA FliI CT26 cells treated with FnAb. Calcein AM (green) stained live cells and dead cells were stained with propidium iodide (red). Spheroids were viewed by an A1 confocal microscope and analysed using NIS Elements software (Nikon) at 10X magnification.

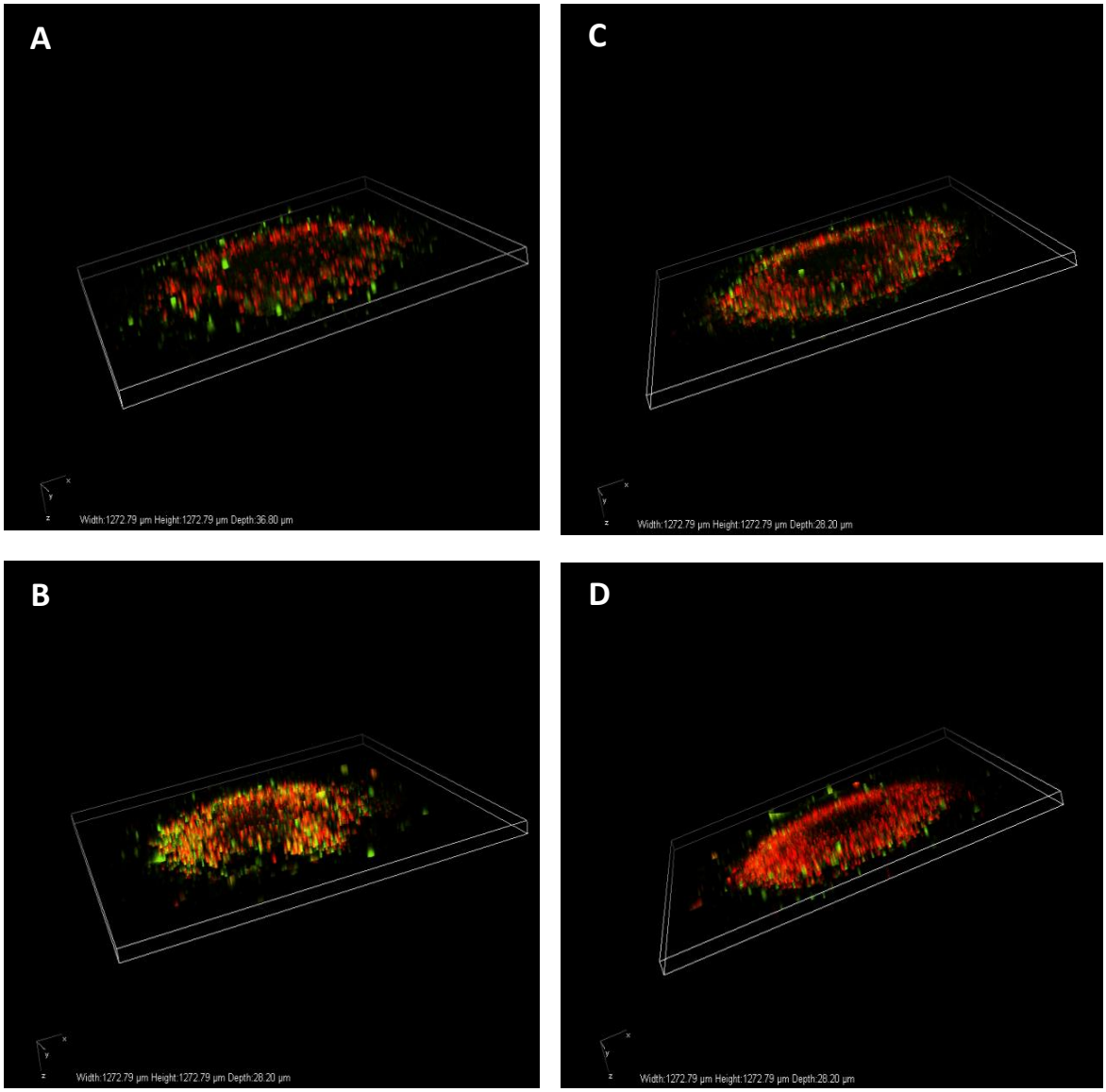


Figure 6.15: 3D stacked confocal images of tumour spheroids in CT26 cells expressing different levels of FliI and after treatment with FnAb. Representative live/dead staining of spheroids shown by stacking 3D images from confocal microscopy at day 10 of **(A)** CT26 WT and **(B)** siRNA FliI CT26 cells treated with non-specific IgG as a control, and **(C)** CT26 WT cells treated with FnAb and **(D)** siRNA FliI CT26 cells treated with FnAb. Calcein AM (green) stained live cells and dead cells were stained with propidium iodide (red). Spheroids were viewed by an A1 confocal microscope and analysed using NIS Elements software (Nikon) at 10X magnification.

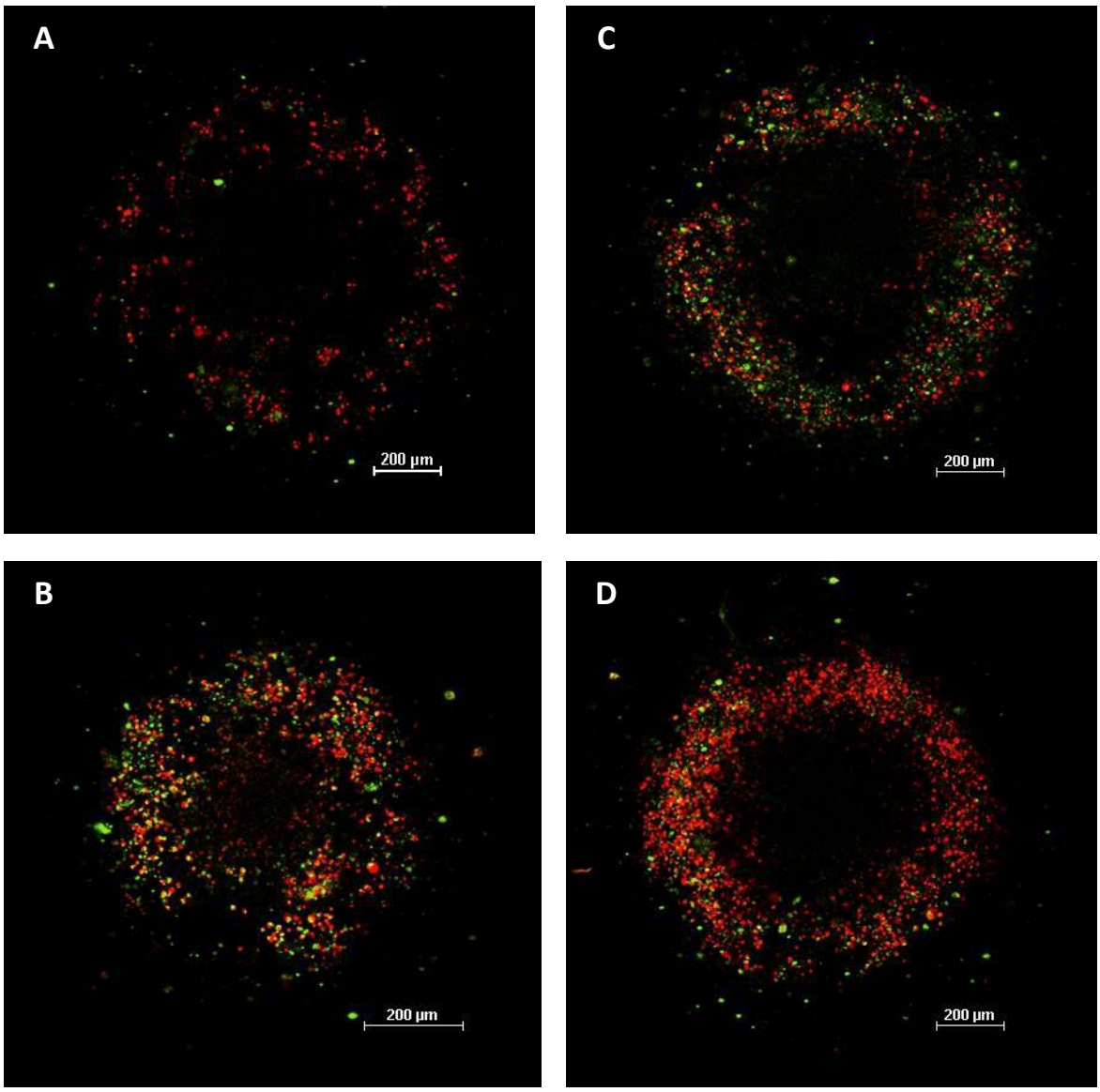


Figure 6.16: 2D confocal images of tumour spheroids in CT26 cells expressing different levels of FliI and after treatment with FnAb. Representative fluorescent 2D images of the spheroids at day 10. Live/dead staining of spheroids from confocal microscopy of **(A)** CT26 WT and **(B)** siRNA FliI CT26 cells treated with non-specific IgG as a control, and **(C)** CT26 WT cells treated with FnAb and **(D)** siRNA FliI CT26 cells treated with FnAb. Calcein AM (green) shows live cells and dead cells were stained with propidium iodide (red). Spheroids were viewed by an A1 confocal microscope and analysed using NIS Elements software (Nikon) at 10X magnification.

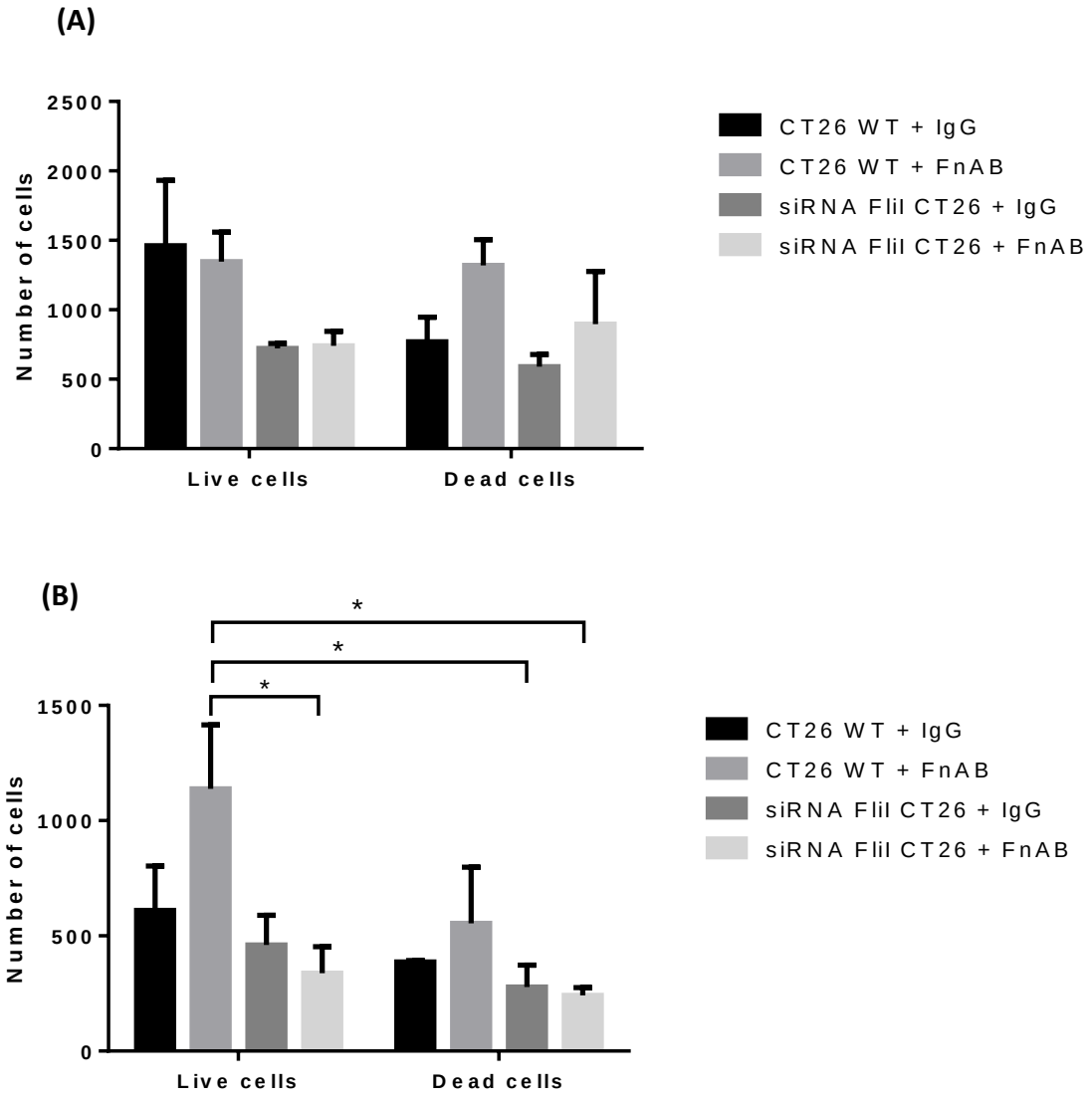


Figure 6.17: Quantification of live and dead cells stained with calcein AM (live cells) and propidium iodide (dead cells) in CT26 cells expressing different levels of FliI and after treatment with FnAb. Number of live and dead cells in tumour spheroids at **(A)** day 5 and **(B)** day 10 of CT26 WT cells and siRNA FliI CT26 cells treated with either non-specific IgG (control) or FnAb. Number of fluorescent cells was analysed using NIS Elements software (Nikon). Results are shown as mean ± SEM (n=3). *p<0.05 significantly different to control cells CT26 WT (Tukey's multiple comparison test).

6.3 Discussion

Though there have been improvements in cancer therapies in recent years, there is still a great need to discover new and more effective therapies to treat many cancers. A novel approach is to target both cancer cells and the tumour microenvironment or stroma, since the tumour stroma is now known to provide important properties and functions that are crucial for tumour growth and metastatic dissemination (Sounni & Noel, 2013). It is known that a variety of stromal cells, including infiltrating immune cells, CAFs, angiogenic vascular cells and lymphatic cells, as well as the non-cellular component of the metastatic niche, that is, the ECM, contribute to cancer progression, not only at the primary sites, but also at the premetastatic and metastatic niche in distant organs (Hanahan & Weinberg, 2011; Lu, Weaver, & Werb, 2012).

Promising approaches to targeting the cellular microenvironment in cancer have been established in different types of human cancer, such as the use of anti-angiogenic and anti-inflammatory therapies (Ebos & Kerbel, 2011). Previously, there were few therapeutic approaches that have been designed and successfully undergone clinical trials to target different types of stromal cells within the tumour microenvironment (Joyce, 2005). Currently, various therapeutic monoclonal antibodies have become established ways of targeting angiogenesis. These include bevacizumab (anti-VEGF), volociximab (anti-integrin α-5β1), antibodies targeting growth and differentiation signalling such as cetuximab (anti-EFGR) and mapatumumab (anti-TRAILR1), and antibodies targeting stromal and extracellular matrix such as sibrotuzumab (anti-FAP) and 816C (anti-Tenascin).

FliI has been shown to regulate cell migration and proliferation, and to have effects on the remodelling of the ECM (Cowin et al., 2007). It has also been shown to mediate cell

adhesion (Zlatko Kopecki et al., 2009) and recently has been shown to increase tumour cell invasion in an SCC model in mice (Z Kopecki et al., 2015). Since cell migration, proliferation and invasion and also remodelling of the ECM have been shown to be very crucial in cancer progression, this suggests FliI could be a possible target for cancer therapy, since it has been shown to play a role in all of the processes mentioned. However, to date very little is known about its possible role in cancer progression or metastasis.

FnAb has been previously found to neutralise the secreted form of FliI, and FliI was observed to be secreted in response to wounding, suggesting a potential extracellular role for this actin-remodelling protein (Cowin et al., 2007). FnAb cream applied on blistered skin was shown to be absorbed into the epidermis and papillary dermis, could be detected on the skin for up to three days and was found to effectively reduce endogenous FliI protein levels (Jackson et al., 2012). However, FnAb was not detected in serum or other organs post topical application of FnAb, suggesting that the application of FnAb cream to the skin remains localised to the skin (Zlatko Kopecki et al., 2013). Intradermal injection of FnAb in porcine models of wound healing has been shown to accelerate wound re-epithelialisation and reduce scar formation (Jackson et al., 2012). The systemic effects of FnAb were also evaluated in relation to changes in immunological reaction, specifically on the cellular infiltration of neutrophils and macrophages; however, no difference was found (Zlatko Kopecki et al., 2013).

In the abovementioned studies, mouse monoclonal antibodies were generated against the leucine-rich repeat (LRR) domain of FliI protein, which has been found to mediate protein–protein interactions, and then the antibody was used *in vivo* at a concentration of 50 µg/ml in mouse models (Jackson et al., 2012). This optimisation study demonstrated that at the

concentration of 50 µg/ml, FnAb was optimum to improved porcine incisional wound repair (Jackson et al., 2012). Administration of antibodies against these LRR domains was also found to improve wound repair in a murine wound-healing model (Cowin et al., 2007). In addition, another study showed that intradermal injection of FnAb significantly improved healing of burn wounds in WT mice in a burn-injury repair study (D. H. Adams et al., 2009). Since there are similarities at the cellular level between wound healing and tumour progression, particularly in the activation of the stroma, we have investigated the therapeutic effects of the FliI-neutralising antibody (FnAb) in a murine colon tumour model.

In the present study, a slight reduction in the number of tumour nodules in the lung was observed in mice treated with FnAb; however, this did not reach significance as compared to the control group treated with non-specific IgG. Interestingly, we did observe a smaller size in the tumours present in the lungs of mice treated with FnAb compared to the control group treated with non-specific IgG, based on the tumour area. Measurement of this nodule size using image analysis confirmed that FnAb appears to reduce the size of tumour nodules in the lung, compared to treatment with non-specific IgG.

In our experiments, FnAb was administered at days 4 and 8 post-inoculation and this could be a potential reason why treatment did not show a significant reduction in the number of nodules in the lungs; however, promisingly it may have inhibited tumour growth and progression of the nodules, resulting in a reduction of the tumour size. Further experiments are needed to see whether increased dosage, pre-treatment prior to inoculation or a different mode of delivery might result in a greater effect. In the present model, we also demonstrated that activated CAFs decreased with a reduction in the expression of the myofibroblast or CAF marker α-SMA in mice treated with FnAb. In addition, there appeared

to be slight reductions in the expression of PCNA and cortactin, although neither of these reached significance. This suggests that treatment with FnAb may inhibit tumour growth, possibly by inhibition of the activation of stromal cells, which can lead to subsequent cell invasion and angiogenesis.

Our data suggesting a decrease in fibroblast activation is in alignment with previous studies on fibroblasts in wound healing. It has been shown previously that the expression of α-SMA decreased in FliI $^{+/-}$ heterozygous knockout mice after injury, suggesting a decrease in contractile myofibroblasts, which then resulted in less scar formation in a murine model of burn-injury repair (D. H. Adams et al., 2009). Increased α-SMA correlates with activated myofibrolasts or CAFs in the stroma, which has commonly been linked to poor prognosis in cancer metastasis (Castelino & Varga, 2014; Pickup et al., 2014). Future experiments that would aid in elucidating the role of FliI could include daily treatment or more frequent administration of FnAb. Also, it would be worthwhile to repeat this experiment using a higher dose of FnAb than was used in our study.

A tumour spheroid assay was used as a 3D model to mimic tumour growth and determine the effects of FliI expression on tumour cell aggregation and growth. Interestingly, *in vitro* tumour spheroid assays showed that reduced FliI expression using FnAb resulted in a decrease in size of the tumour spheroids at day 5 in CT26 WT cells. In addition, the inhibition of FliI expression intracellularly using siRNA FliI CT26 cells (knockdown of FliI in CT26 cells) greatly decreased the size of tumour spheroids at days 2 and 5 as compared to control CT26 WT cells. A combination of FnAb and siRNA treatment had no additional effect on either day. However, there was no difference observed in any treatment by day 10, possibly due to necrosis and the development of necrotic centres in the spheroids. This finding suggests

that the administration of FnAb and the inhibition of intracellular FliI could inhibit tumour cell invasion, possibly by neutralisation of intracellular FliI and its secreted form. However, the exact mechanism of this inhibition needs to be confirmed. It is not known whether it is taken into the cell and the functions that it might affect inside the cell.

Similar findings were shown previously using MET-1-SCC cell lines treated with FnAb, which also reduced tumour spheroid area compared to control antibody-treated cells (Z Kopecki et al., 2015). Uptake of FnAb by cells has been shown in a previous study (Zlatko Kopecki et al., 2013), which showed that the FnAb exhibited neutralisation activity in both cultured cells and animal models, with effects on cell migration and on inflammation and repair processes (Cowin et al., 2007; Jackson et al., 2012). Uptake of the antibody suggests that administration of the neutralising antibody could affect both extracellular (secreted) FliI and intracellular FliI.

Reduced FliI expression was also shown to reduce tumour cell invasion *in vitro* and potentially decreased the establishment or seeding of tumour cells in the lung. FliI's role in the cell may allow it to mediate tumour cell invasion, possibly by facilitating invadopodia formation, based on its roles in actin skeleton reorganisation (Mohammad et al., 2012) and its coprecipitation with cortactin, an invadopodia marker (Z Kopecki et al., 2015). Furthermore, recent findings showed that FliI acts as a P-Rex-1 binding partner, which has been shown to increase cell migration in 3D migration assays. This contradicts previous findings that FliI negatively regulates cell migration, which was shown in 2D culture systems (Marei et al., 2016), suggesting the role of FliI in cells in the tumour microenvironment may be to mediate cell migration as an initial step to promotion of cell invasion.

6.4 Conclusion

This present study has demonstrated that the inhibition of FliI expression both intracellularly (via siRNA) and potentially in its secreted form using a FliI-neutralising antibody reduced tumour cell invasion *in vitro*, but did not have a significant effect on the number of tumour nodules established in the lungs in our mouse model. When the sizes of tumour nodules were compared, there was a significant effect on nodule size, with a reduction seen in mice that were administered the neutralising antibody. Therefore, this finding suggests that it may be beneficial to target and block FliI within the tumour microenvironment to potentially inhibit cells from penetrating tissue barriers such as the basement membrane or the ECM. FliI inhibition may thus inhibit the metastatic spread of tumour cells to other organs, although this requires further experiment using better animal models of metastasis. Further experiments looking at varying doses and times of delivery of the neutralising antibody are also needed to provide stronger evidence that FliI can reduce the number and/or the size of tumour nodules in the lungs.

Chapter 7 General Discussion

7.1 Flil Expression: Role in *in vitro* Cellular Functions

In the last decade, most cancer studies have focused on detecting cancer cell mutations, such as those that are critically involved in cell cycle control. Tumour suppressor genes, or oncogenes, and cell-signalling pathways regulate cell cycle control and thus determine cell proliferation and/or cell death. To date, less attention has been paid to the impact of surrounding cells/tissue and the stromal microenvironment. However, the fact that the tumour stroma is a dynamic microenvironment which consists of heterogeneous cellular components and shows reciprocal communication with cancer cells suggests that tumour stroma modification in cancer is worthy of research. It is hoped that treatments will be discovered which may revert tumour-promoting stroma to normal stroma and therefore provide a less favourable tumour-promoting microenvironment, reducing cancer progression and metastasis to secondary sites.

Crosstalk between cancer cells and their local environment is crucial for tumour survival, and the ability to modify their surroundings is important for cancer cells to promote both tumour progression and dissemination (Sounni & Noel, 2013). Therefore, targeting the tumour microenvironment may be beneficial in providing new therapeutic targets to limit the growth of cancer cells. The composition of the ECM is now recognised as an important regulator of cell behaviour, and changes in the ECM could also alter cancer cell proliferation, survival and migration. Reorganisation of the ECM can also stress ECM molecules and

potentially release or activate molecules such as latent or bound growth factors known to regulate cell migration, proliferation and angiogenesis (Noël, 2012).

As a member of the actin-remodelling family of gelsolin-like proteins, FliI is involved in cell motility, adhesion and contraction (Davy et al., 2000). FliI has also been reported to have interactions with several other proteins, including association with β-actin. It is likely, therefore, that FliI plays a role in the actin remodelling and polymerisation process (H. D. Campbell et al., 2002). FliI has also been shown to regulate cell adhesion to the ECM and thus can be associated with changes in cell arrangement and motility (Zlatko Kopecki et al., 2009). Specifically, FliI has been shown to mediate actin filament reorganisation, as FliI is expressed in motility-associated structures such as actin arcs and membrane ruffles, and in the migratory structures lamellipodia and filopodia (Davy et al., 2001). The unique structure of FliI compared to other actin-remodelling proteins may lead to it mediating signalling pathways in actin remodelling (Zlatko Kopecki et al., 2013).

FliI has been identified as a tumour promoter in various cancers such as colorectal, breast and hepatocellular carcinomas (Kwang Won Jeong, 2014; Lifang Wu et al., 2013). Recent findings have shown that FliI promotes the progression of SCC in mouse models of SCC, with FliI regulating enhanced cell invasion (Z Kopecki et al., 2015). In the current study, we have demonstrated that FliI expression both within the host stromal cells (using either WT or heterozygous +/− mice) and in cancer cells (by siRNA knockdown of FliI in colon carcinoma cells) can influence tumour growth and the establishment of lung nodules in mouse colon cancer models. Additionally, the inhibition of FliI *in vitro* has been found to increase cell adhesion and decrease cell invasion capacity, suggesting a possible mechanism by which FliI inhibition could limit tumour progression and cancer cell invasion.

In vitro findings in this study (Chapter 2) show that FliI expression has effects at the cellular level on cell migration, apoptosis and proliferation. Knockdown of FliI in mouse (CT26) and human (HT29) colon carcinoma cell lines showed an increase in cell migration in two-dimensional models, as well as increased proliferation and early apoptosis. This data concurs with what has been reported in wound-healing models and suggests that FliI plays a possible dual role in either tumour promotion or inhibition, possibly depending on cell type. The inhibition of FliI expression in cancer cells also reduced the level of cytokine expression, specifically TNF-α, VEGF and 1L-6. In addition, the administration of exogenous recombinant FliI (rFliI) to CT29 and HT29 cell cultures was shown to induce apoptosis, while showing no effect on other cellular functions such as migration and proliferation.

The effects of recombinant FliI need to be viewed with some caution, as only a truncated form of FliI is available presently, and ours and collaborators' laboratories have had difficulty expressing the full-length form of the protein. The availability of a full-length recombinant Flightless 1 protein in the future may help to clarify exactly what exogenous FliI induces in tumour cells, giving a better indication of the role of secreted or released FliI.

The deregulation of cell migration and in particular cell invasion contributes to pathological conditions such as tumour angiogenesis and metastasis (Ridley et al., 2003). Aberrant regulation of cell motility is commonly linked to increased migration and invasion of cancer cells, which is a key step in metastasis (Marei et al., 2016). Studies done previously (Cowin et al., 2007) showed that FliI deficiency (using siRNA knockdown) in fibroblast monolayers derived from mouse skin increased the rate of wound closure in scratch assays compared to WT and FliI overexpressing fibroblasts. In addition, the same study showed that FliI-deficient ($FliI^{+/-}$) mice improved *in vivo* wound healing by increasing epithelial migration and

proliferation. However, FliI has also been found to promote cell migration/invasion in 3D cell migration assays via a Rac-1-dependent pathway, which contradicts previous findings on the migratory properties influenced by FliI in 2D models (Marei et al., 2016) and provides support for our finding that FliI increased 3D cell invasion and decreased cell adhesion (Chapter 4).

FliI expression correlated with reduced adhesiveness and increased invasiveness of cancer cells (Chapter 4). We have showed that the inhibition of FliI greatly reduced cell invasion in a 3D invasion model using Matrigel-coated chambers. Interestingly, the addition of exogenous recombinant FliI (rFliI) increased the invasiveness of cancer cells. This finding suggests that intracellular and secreted forms of FliI may both play a role in driving cell invasion in a 3D model. FliI has been shown to mediate cell migration via activation of P-Rex1-Rac1, where FliI has been recognised as a novel Rac1 effector protein (Marei et al., 2016). This points to a possible pathway for the promotion of cellular invasion by FliI. Invasive cells have generally been found to be less adherent, and this has been associated with high motility and higher metabolic activity (Turner & Sherratt, 2002). Upregulation of the invadopodia marker cortactin with increased FliI expression suggests FliI may act via driving invadopodia formation, and thus may lead to increased cancer cell invasion and spread. In addition, FliI appears to have effects on stromal cells (such as fibroblasts) and cancer-associated (myo) fibroblasts, through upregulating differentiation to the myofibroblast phenotype in the tumour microenvironment, and the presence of these cells has been shown to favour tumour progression.

Several studies have shown that the expression levels of actin-remodelling proteins are altered during tumour progression, and research aimed at development of therapies

targeting those proteins may lead to treatments that will inhibit cell motility and invasion (Baig et al., 2013; Dong et al., 2002; K. W. Jeong, 2014; A. Van den Abbeele et al., 2007). As FliI has been identified as a tumour promoter (K. W. Jeong, 2014; L. Wu et al., 2013), it could be a potential target for reducing invasion and subsequent metastasis. During invasion, reorganisation of the cytoskeleton results in actin protrusions and the production of lamellipodia and invadopodia, as well as influencing EMT, which has been widely implicated in cancer cell dissemination (Grünert et al., 2003). On wounding, it has been shown that FliI is involved in lamellipodia formation in keratinocytes and also in remodelling of the ECM at the wound site (Cowin et al., 2007). Invasive tumour cells have been demonstrated to present dysregulated cell motility in response to extracellular signals from growth factors and cytokines (Wells, 1999). Furthermore, invasive cells were found to be less adherent, and this was also associated with higher motility and higher metabolic activity (Turner & Sherratt, 2002).

Significantly, this study finds that FliI expression increased cell invasion, suggesting FliI may play a role in tumour progression via effects on cell adhesion and invasion, potentially via effects on invadopodia formation. These experiments point to the difficulty in interpreting 2D culture models and the importance of using 3D systems to examine cell invasion. Experiments using CT26 WT and siRNA FliI CT26 cells have showed that knockdown of FliI significantly downregulated the expression of cortactin. Cortactin is involved in cell protrusions such as lamellipodia and invadopodia, as it is present at dynamic sites of actin assembly (Yamaguchi & Condeelis, 2007). In fact, several studies have demonstrated that cortactin drives the invasion process and allows cells to penetrate through ECM barriers such as the basement membrane (Bryce et al., 2005; van Rossum, Moolenaar, & Schuuring,

2006). Invadopodia also appear to correlate with invasion via ECM degradation (Weaver, 2008).

Specifically, FliI has been shown to be co-localised with the invadopodia marker cortactin at the invading site of SCC cells, indicating enhanced cancer cell invasion into the basement membrane via this process (Z Kopecki et al., 2015). Therefore, cortactin is both an important regulator and a specific marker for invadopodia, based on its capability to promote protease secretion at the active adhesion site (Clark & Weaver, 2008). Overexpression of cortactin was shown to increase ECM degradation and also correlated with elevated secretion of MMP-2 and MMP-9 (Clark & Weaver, 2008; Clark et al., 2007). Increased secretion of MMPs in tumour cells is closely related to invasive capability, increased metastasis and poor prognosis in several types of cancers (Buday & Downward, 2007; Stetler-Stevenson et al., 1996). A previous study (Bowden et al., 2006) found that cortactin and phosphotyrosine signalling is crucial for the activation of invadopodia proteases, leading to degradation of ECM and mediating cellular invasion.

In the present study, the inhibition of FliI expression increased cell adhesion, with upregulation of the FA proteins paxillin and vinculin (Chapter 4). FliI has been shown to regulate cell adhesion, actin remodelling and intracellular signalling via co-localisation with actin and microtubule-based structures (H. D. Campbell et al., 2002; Davy et al., 2001; Z. Kopecki & Cowin, 2008). Knockdown of FliI can regulate cell adhesion via upregulation of the FA proteins paxillin and vinculin. Previous studies have found that FliI was co-localised with talin, paxillin and vinculin, allowing it to play a role in regulating cell adhesion (Mohammad et al., 2012).

It has also been shown that cell adhesion was increased in heterozygous $Flil^{+/-}$ while significantly impaired in transgenic $Flil^{Tg/-}$ keratinocytes and fibroblasts (which overexpress FliI) on different substrates (Zlatko Kopecki et al., 2009). During wound healing, FliI was observed to regulate adhesion via hemidesmosome formation and integrin-mediated cellular adhesion molecules (Zlatko Kopecki et al., 2009; Thiery, 2003). Reduced paxillin activity was also found in FliI-overexpressing fibroblasts via inhibition of the paxillin–tyrosine–phosphorylation signalling pathway mediated by the adhesion molecules Src and p130cas (Z. Kopecki et al., 2011). During the adhesion process, protein complexes bind to the ECM, then trigger the linkage signal between integrin receptors and the actin cytoskeleton to form an active FA site (Hehlgans et al., 2007).

This result indicates that FliI may have the ability to reorganise the cytoskeleton, which creates a protrusive actin meshwork, suggesting invadopodia formation in cancer cells; however, this needs to be investigated in the future. Understanding the mode of cancer cell invasion is crucial in designing strategies to target evolving cancer cells and block their movement through tissues and organs (Kramer et al., 2013). Therefore, this finding suggests that targeting and blocking FliI is potentially a means of limiting cell penetration of the barrier matrix (basement membrane) and thus serving to inhibit metastasis.

7.2 FliI Expression in Primary Growth and Lung Seeding Murine Colon Tumour Model

Primary and metastatic murine colon tumour models have been used in this study in order to determine the effects of FliI on tumour growth and the seeding of tumour nodules via the circulation. Tumour effector functions, including proliferation, invasion and myofibroblast expression within the tumour microenvironment, have also been evaluated (Chapter 5). FliI

expression levels in mice correlated with an increased size of primary tumours and increased number of tumour nodules in the lungs in mouse colon cancer models. In contrast, the inhibition of FliI reduced tumour nodule numbers, suggesting that host FliI expression levels can regulate tumour growth in this model, possibly through an effect on the behaviour of tumour stromal cells.

Changes in stromal behaviour have been shown to promote epithelial transformation that could alter cell–cell and cell–matrix signals, allowing tumour cells to migrate across tissue boundaries (Liotta & Kohn, 2001) and promote tumour cell invasion and seeding at distant sites. The stromal cells that are abundant and active at the invasive edge of the tumour are TAMs and CAFs (Condeelis & Pollard, 2006). These cells actively promote the invasion of tumour cells (Van Zijl et al., 2009). Interestingly, the inhibition of FliI expression in cancer cells using siRNA FliI CT26 cells in the current study resulted in smaller tumours in mice, delay in onset of tumour formation and slower cancer progression. This finding needs to be confirmed using more stable methods for lowering FliI expression, since it is likely that the FliI knockdown is only transient once cells are in the *in vivo* environment. Local injection of FliI siRNA or antisense oligonucleotides would be a useful approach to this in the future.

This suggests that FliI expression within cancer cells is linked with tumour growth in this model. Similar findings were demonstrated in the SCC model, where there was reduced tumour volume in FliI heterozygous (FliI $^{+/-}$) mice, while overexpression of FliI in FliI transgenic mice (Tg^{+}) resulted in larger tumours and more severe SCC progression (Z Kopecki et al., 2015). The tumour-promoting activity of FliI is possibly due to its effects on cell adhesion and invasion, as well as potential modification of the tumour microenvironment. Reduced FliI expression was shown to reduce tumour cell invasion *in vitro* and potentially

decreased the tumour seeding in the lung. This demonstrates a potential role of FliI in mediating tumour invasion. A possible mechanism associated with FliI that leads to tumour invasion is the facilitation of invadopodia formation. As FliI expression is evident in the invadopodia complex and has been shown to play a role in actin cytoskeleton reorganisation, it is possible that, via these mechanisms, FliI assists tumour invasion (Z Kopecki et al., 2015; Mohammad et al., 2012).

Activation of intracellular and/or extracellular elements of the local host microenvironment could modify the invasive and proliferative behaviour of tumour cells (Liotta & Kohn, 2001; Vaccariello et al., 1999). FliI may modify the tumour microenvironment so as to favour tumour growth, which is evidenced by the observation that the expression of the myofibroblast marker cortactin and greater cancer cell proliferation were seen in both primary and lung models (Chapter 5). In contrast, we have demonstrated that the inhibition of FliI could affect the tumour microenvironment to make it less favourable to cancer cells, leading to reduced invasion and seeding of lung tumours in the mouse model. Several studies have shown that the re-education of stromal cells within the tumour microenvironment can potentially normalise tumour cell behaviour (Quail & Joyce, 2013). In fact, many studies show that a deregulated microenvironment commonly exhibited higher cancer incidence (Grivennikov et al., 2010).

In Chapter 6, we have also demonstrated the potential therapeutic effects of a FliI-neutralising antibody (FnAB) in reducing tumour growth and spread, using *in vivo* tumour models and spheroid growth assays *in vitro*. Even though only a slight reduction in the number of tumour nodules in the lungs was observed in mice treated with FnAB, we have also observed significantly reduced tumour size, where smaller nodules were present in the

lungs of mice treated with FnAB compared to the controls. Treatment with FnAB had no significant effect on the number of tumours in the lung; however, it has been showed to inhibit tumour growth. This may be due to the administration of FnAB on days 4 and 8 post-inoculation after the seeding event occurred within the lung, or to limitations in the delivery of sufficient antibody.

Therefore, there are several modifications that could be tried in the future, particularly with dosage, which needs to be optimised in this animal model. In addition, the treatment regimen and duration of the cancer model may need to be longer in order to observe an effect. Pre-treatment of the microenvironment with the neutralising antibody may also show a greater effect than treatment being applied after tumour cells have had a chance to become established. Promisingly, FnAB treatment reduced the size of tumour spheroids in 3D spheroid invasion assays, indicating a possible action in the microenvironment that reduces cell invasion and tumour growth. Similar findings showed that administration of FnAB in SCC cell line (MET-1-SCC) spheroids reduced sphere formation and invasion (Z Kopecki et al., 2015). Intradermal injection of FnAB was also found to reduce tumour size in an SCC mouse model in the same study.

Targeting of the tumour microenvironment is now a very fruitful new target; however, the approach is complicated due to the complexity and variety of the stromal cells found in the microenvironment (Sounni & Noel, 2013). As with other monoclonal antibodies that have become important new tools in cancer therapy, the FliI-neutralising antibody could be a new approach to targeting cancer cells and, more importantly, the tumour microenvironment in order to reduce tumour growth and metastasis.

7.3 Future Recommendations

Further experiments are needed to provide a better understanding of the effects of the expression of FliI in *in vitro* and animal models of tumour growth and metastasis. Ideally, we would use FliI-overexpressing cancer cells and transgenic mice (that overexpress FliI). In our study, we encountered difficulties in expressing the FliI plasmid because it did not produce the expected level of FliI expression in CT26 cells. Other groups have reported similar difficulties with overexpression studies *in vitro* (A/Prof. Allison Cowin, UniSA, personal communication).

The mechanism of FliI regulation of invadopodia formation also needs further investigation. The exact nature of the association between FliI and the invadopodia complex is also worthy of more detailed study. In terms of the influence of intracellular FliI on cellular invasion, the role of FliI, if any, in protease secretion and expression of MMPs is another possible subject for investigation. In addition, to further strengthen the understanding of how regulation of FliI affects cellular adhesion, paxillin localisation would be very beneficial to show how the regulation of FliI affects paxillin-rich focal adhesions.

To deepen our understanding of the potential use of the FliI-neutralising antibody, more experiments need to be carried out in animal models of primary tumours and in better models of metastatic tumours. Possible beneficial effects of FnAB could be studied in cancer models that have a longer duration of initiation and progression, such as chemically induced models of colon cancer (for example azoxymethane), which would give a longer and slower induction process, as well as a larger window within which to treat animals with the FnAB. All of the mode of administration of the FnAB, the time course of administration and the dose should also be considered in experiments in order to optimise FnAB treatment.

Chapter 8 Conclusion

Taken together, both *in vitro* and *in vivo* studies in this present thesis have contributed to a better understanding of the role of FliI in promoting tumour growth and the establishment of nodules in distant organs. FliI has been shown to potentially regulate important cellular functions in cancer, including cell migration, apoptosis, proliferation, adhesion and invasion, all of which can contribute to tumour cell invasion and dissemination. Importantly, both intracellular FliI and the secreted form of FliI may play significant roles in contributing to this process. FliI expression by either stromal cells or tumour cells may affect the tumour cells and the microenvironment, favouring tumour growth, while reducing or inhibiting FliI may modify tumour cell behaviour and the tumour stroma, to reduce the severity and potentially the spread of cancer.

Therefore, FliI is a potential therapeutic target such that either intracellular inhibition or inhibition of the secreted form via molecular inhibitors or monoclonal antibodies might result in a reduction in tumour growth and spread. The administration of a FliI-neutralising antibody targeting FliI activity could thus be a strategy in reducing cancer severity, and could also inhibit the invasion and establishment of secondary tumours, as well as helping to normalise the tumour microenvironment.

References

Adams, D. H., Ruzehaji, N., Strudwick, X. L., Greenwood, J. E., Campbell, H. D., Arkell, R., & Cowin, A. J. (2009). Attenuation of Flightless I, an actin-remodelling protein, improves burn injury repair via modulation of transforming growth factor (TGF)-β1 and TGF-β3. *British Journal of Dermatology, 161*(2), 326-336.

Adams, D. O., & Hamilton, T. A. (1984). The cell biology of macrophage activation. *Annual review of immunology, 2*(1), 283-318.

Adams, J., & Cory, S. (2007). The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene, 26*(9), 1324-1337.

Ai, K.-X., Lu, L.-Y., Huang, X.-Y., Chen, W., & Zhang, H.-Z. (2008). Prognostic significance of S100A4 and vascular endothelial growth factor expression in pancreatic cancer. *World journal of gastroenterology: WJG, 14*(12), 1931.

Allavena, P., Sica, A., Garlanda, C., & Mantovani, A. (2008). The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. *Immunological reviews, 222*(1), 155-161.

Ammer, A. G., & Weed, S. A. (2008). Cortactin branches out: roles in regulating protrusive actin dynamics. *Cell motility and the cytoskeleton, 65*(9), 687-707.

Arends, M. J., McGregor, A. H., & Wyllie, A. H. (1994). Apoptosis is inversely related to necrosis and determines net growth in tumors bearing constitutively expressed myc, ras, and HPV oncogenes. *The American journal of pathology, 144*(5), 1045.

Artym, V. V., Matsumoto, K., Mueller, S. C., & Yamada, K. M. (2011). Dynamic membrane remodeling at invadopodia differentiates invadopodia from podosomes. *European journal of cell biology, 90*(2), 172-180.

Artym, V. V., Yamada, K. M., & Mueller, S. C. (2009). ECM degradation assays for analyzing local cell invasion *Extracellular Matrix Protocols* (pp. 211-219): Springer.

Arwert, E. N., Hoste, E., & Watt, F. M. (2012). Epithelial stem cells, wound healing and cancer. *Nature reviews cancer, 12*(3), 170-180.

Bachem, M. G., Schünemann, M., Ramadani, M., Siech, M., Beger, H., Buck, A., . . . Adler, G. (2005). Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells. *Gastroenterology, 128*(4), 907-921.

Badowski, C., Pawlak, G., Grichine, A., Chabadel, A., Oddou, C., Jurdic, P., . . . Block, M. R. (2008). Paxillin phosphorylation controls invadopodia/podosomes spatiotemporal organization. *Molecular biology of the cell, 19*(2), 633-645.

Baig, R. M., Mahjabeen, I., Sabir, M., Masood, N., Ali, K., Malik, F. A., & Kayani, M. A. (2013). Mutational spectrum of Gelsolin and its down regulation is associated with breast cancer. *Dis Markers, 34*(2), 71-80. doi: 10.3233/DMA-120952

Bakhtyar, N., Wong, N., Kapoor, A., Cutz, J.-C., Hill, B., Ghert, M., & Tang, D. (2013). Clear cell renal cell carcinoma induces fibroblast-mediated production of stromal periostin. *European journal of cancer, 49*(16), 3537-3546.

Baldassarre, M., Ayala, I., Beznoussenko, G., Giacchetti, G., Machesky, L. M., Luini, A., & Buccione, R. (2006). Actin dynamics at sites of extracellular matrix degradation. *European journal of cell biology, 85*(12), 1217-1231.

Balkwill, F. (2004). Cancer and the chemokine network. *Nature reviews cancer, 4*(7), 540-550.

Balkwill, F. (2005). Immunology for the next generation. *Nature Reviews Immunology, 5*(6), 509-512.

Balkwill, F., Charles, K. A., & Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer cell, 7*(3), 211-217.

Barcellos-Hoff, M. H., Lyden, D., & Wang, T. C. (2013). The evolution of the cancer niche during multistage carcinogenesis. *Nature reviews cancer, 13*(7), 511-518.

Beaty, B. T., & Condeelis, J. (2014). Digging a little deeper: the stages of invadopodium formation and maturation. *European journal of cell biology, 93*(10), 438-444.

Ben-Ze'ev, A. (1997). Cytoskeletal and adhesion proteins as tumor suppressors. *Current opinion in cell biology, 9*(1), 99-108.

Bhowmick, N. A., Neilson, E. G., & Moses, H. L. (2004). Stromal fibroblasts in cancer initiation and progression. *Nature, 432*(7015), 332-337.

Bingle, L., Brown, N., & Lewis, C. (2002). The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *The Journal of pathology, 196*(3), 254-265.

Bissell, M. J., & Radisky, D. (2001). Putting tumours in context. *Nature reviews cancer, 1*(1), 46-54.

Blaser, M. J., Chyou, P., & Nomura, A. (1995). Age at establishment of Helicobacter pylori infection and gastric carcinoma, gastric ulcer, and duodenal ulcer risk. *Cancer research, 55*(3), 562-565.

Bobba, R. K., Holly, J. S., Loy, T., & Perry, M. C. (2011). Scar carcinoma of the lung: a historical perspective. *Clinical lung cancer, 12*(3), 148-154.

Bommert, K., Bargou, R. C., & Stühmer, T. (2006). Signalling and survival pathways in multiple myeloma. *European journal of cancer, 42*(11), 1574-1580.

Bonifacino, J. S., & Glick, B. S. (2004). The mechanisms of vesicle budding and fusion. *Cell, 116*(2), 153-166.

Borrello, M. G., Alberti, L., Fischer, A., Degl'Innocenti, D., Ferrario, C., Gariboldi, M., . . . Collini, P. (2005). Induction of a proinflammatory program in normal human thyrocytes by the RET/PTC1 oncogene. *Proceedings of the National Academy of Sciences of the United States of America, 102*(41), 14825-14830.

Bourboulia, D., & Stetler-Stevenson, W. G. (2010). *Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion.* Paper presented at the Seminars in cancer biology.

Bowden, E. T., Barth, M., Thomas, D., Glazer, R. I., & Mueller, S. C. (1999). An invasion-related complex of cortactin, paxillin and PKCmu associates with invadopodia at sites of extracellular matrix degradation. *Oncogene, 18*(31), 4440-4449.

Bowden, E. T., Onikoyi, E., Slack, R., Myoui, A., Yoneda, T., Yamada, K. M., & Mueller, S. C. (2006). Co-localization of cortactin and phosphotyrosine identifies active invadopodia in human breast cancer cells. *Experimental cell research, 312*(8), 1240-1253.

Boyle, P., & Leon, M. E. (2002). Epidemiology of colorectal cancer. *British medical bulletin, 64*(1), 1-25.

Branch, K. M., Hoshino, D., & Weaver, A. M. (2012). Adhesion rings surround invadopodia and promote maturation. *Biology open, 1*(8), 711-722.

Braun, S., Vogl, F. D., Naume, B., Janni, W., Osborne, M. P., Coombes, R. C., . . . Gebauer, G. (2005). A pooled analysis of bone marrow micrometastasis in breast cancer. *New England Journal of Medicine, 353*(8), 793-802.

Breckenridge, D. G., & Xue, D. (2004). Regulation of mitochondrial membrane permeabilization by BCL-2 family proteins and caspases. *Current opinion in cell biology, 16*(6), 647-652.

Bremer, E., van Dam, G., Kroesen, B. J., de Leij, L., & Helfrich, W. (2006). Targeted induction of apoptosis for cancer therapy: current progress and prospects. *Trends in molecular medicine, 12*(8), 382-393.

Brooks, P. C., Klemke, R. L., Schon, S., Lewis, J. M., Schwartz, M. A., & Cheresh, D. A. (1997). Insulin-like growth factor receptor cooperates with integrin alpha v beta 5 to promote tumor cell dissemination in vivo. *Journal of Clinical Investigation, 99*(6), 1390.

Broughton 2nd, G., Janis, J. E., & Attinger, C. E. (2006). The basic science of wound healing. *Plastic and reconstructive surgery, 117*(7 Suppl), 12S-34S.

Brouty-Boye, D. (2005). Developmental biology of fibroblasts and neoplastic disease *Developmental Biology of Neoplastic Growth* (pp. 55-77): Springer.

Brown, M. C., & Turner, C. E. (2004). Paxillin: adapting to change. *Physiological reviews, 84*(4), 1315-1339.

Bryce, N. S., Clark, E. S., Ja'Mes, L. L., Currie, J. D., Webb, D. J., & Weaver, A. M. (2005). Cortactin promotes cell motility by enhancing lamellipodial persistence. *Current Biology, 15*(14), 1276-1285.

Buckley, C. D., Pilling, D., Lord, J. M., Akbar, A. N., Scheel-Toellner, D., & Salmon, M. (2001). Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends in immunology, 22*(4), 199-204.

Buday, L., & Downward, J. (2007). Roles of cortactin in tumor pathogenesis. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1775*(2), 263-273.

Bukholm, I., Bukholm, G., Holm, R., & Nesland, J. (2003). Association between histology grade, expression of HsMCM2, and cyclin A in human invasive breast carcinomas. *Journal of clinical pathology, 56*(5), 368-373.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., & Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annual review of cell biology, 4*(1), 487-525.

Campbell, H., Schimansky, T., Claudianos, C., Ozsarac, N., Kasprzak, A., Cotsell, J., . . . Miklos, G. (1993). The Drosophila melanogaster flightless-I gene involved in gastrulation and muscle degeneration encodes gelsolin-like and leucine-rich repeat domains and is conserved in Caenorhabditis elegans and humans. *Proceedings of the National Academy of Sciences, 90*(23), 11386-11390.

Campbell, H. D., Fountain, S., McLennan, I. S., Berven, L. A., Crouch, M. F., Davy, D. A., . . . Lupski, J. R. (2002). Fliih, a gelsolin-related cytoskeletal regulator essential for early mammalian embryonic development. *Molecular and cellular biology, 22*(10), 3518-3526.

Campbell, H. D., Fountain, S., Young, I. G., Claudianos, C., Hoheisel, J. D., Chen, K.-S., & Lupski, J. R. (1997). Genomic Structure, Evolution, and Expression of Human< i> FLII,</i> a Gelsolin and Leucine-Rich-Repeat Family Member: Overlap with< i> LLGL</i>. *Genomics, 42*(1), 46-54.

Candido, J., & Hagemann, T. (2013). Cancer-related inflammation. *Journal of clinical immunology, 33*(1), 79-84.

Carisey, A., & Ballestrem, C. (2011). Vinculin, an adapter protein in control of cell adhesion signalling. *European journal of cell biology, 90*(2), 157-163.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. *Nature medicine, 6*(4), 389-395.

Castelino, F. V., & Varga, J. (2014). Emerging cellular and molecular targets in fibrosis: implications for scleroderma pathogenesis and targeted therapy. *Current opinion in rheumatology, 26*(6), 607-614.

Cavallaro, U., & Christofori, G. (2004). Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nature reviews cancer, 4*(2), 118-132.

Chaffer, C. L., & Weinberg, R. A. (2011). A perspective on cancer cell metastasis. *Science, 331*(6024), 1559-1564.

Chang, H. Y., Sneddon, J. B., Alizadeh, A. A., Sood, R., West, R. B., Montgomery, K., . . . Brown, P. O. (2004). Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. *PLoS biology, 2*(2), e7.

Charles, K. A., Kulbe, H., Soper, R., Escorcio-Correia, M., Lawrence, T., Schultheis, A., . . . Smyth, J. F. (2009). The tumor-promoting actions of TNF-α involve TNFR1 and IL-17 in ovarian cancer in mice and humans. *The Journal of clinical investigation, 119*(10), 3011-3023.

Chiang, Y., Chou, C. Y., Hsu, K. F., Huang, Y. F., & Shen, M. R. (2008). EGF upregulates Na+/H+ exchanger NHE1 by post-translational regulation that is important for cervical cancer cell invasiveness. *Journal of cellular physiology, 214*(3), 810-819.

Chuma, M., Sakamoto, M., Yasuda, J., Fujii, G., Nakanishi, K., Tsuchiya, A., . . . Hirohashi, S. (2004). Overexpression of cortactin is involved in motility and metastasis of hepatocellular carcinoma. *Journal of hepatology, 41*(4), 629-636.

Cirri, P., & Chiarugi, P. (2011). Cancer associated fibroblasts: the dark side of the coin. *Am J Cancer Res, 1*(4), 482-497.

Clark, E. S., Brown, B., Whigham, A. S., Kochaishvili, A., Yarbrough, W. G., & Weaver, A. M. (2009). Aggressiveness of HNSCC tumors depends on expression levels of cortactin, a gene in the 11q13 amplicon. *Oncogene, 28*(3), 431-444.

Clark, E. S., & Weaver, A. M. (2008). A new role for cortactin in invadopodia: regulation of protease secretion. *European journal of cell biology, 87*(8), 581-590.

Clark, E. S., Whigham, A. S., Yarbrough, W. G., & Weaver, A. M. (2007). Cortactin is an essential regulator of matrix metalloproteinase secretion and extracellular matrix degradation in invadopodia. *Cancer research, 67*(9), 4227-4235.

Collado, M., & Serrano, M. (2010). Senescence in tumours: evidence from mice and humans. *Nature reviews cancer, 10*(1), 51-57.

Condeelis, J., & Pollard, J. W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell, 124*(2), 263-266.

Coniglio, S. J., Eugenin, E., Dobrenis, K., Stanley, E. R., West, B. L., Symons, M. H., & Segall, J. E. (2012). Microglial stimulation of glioblastoma invasion involves epidermal growth factor receptor (EGFR) and colony stimulating factor 1 receptor (CSF-1R) signaling. *Molecular medicine, 18*(3), 519.

Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Nature, 420*(6917), 860-867.

Cowin, A. J., Adams, D. H., Strudwick, X., Chan, H., Hooper, J., Sander, G. R., . . . Campbell, H. D. (2007). Flightless I deficiency enhances wound repair by increasing cell migration and proliferation. *The Journal of pathology, 211*(5), 572-581.

Croucher, D. R., Rickwood, D., Tactacan, C. M., Musgrove, E. A., & Daly, R. J. (2010). Cortactin modulates RhoA activation and expression of Cip/Kip cyclin-dependent kinase inhibitors to promote cell cycle progression in 11q13-amplified head and neck squamous cell carcinoma cells. *Molecular and cellular biology, 30*(21), 5057-5070.

Crowther, M., Brown, N., Bishop, E., & Lewis, C. (2001). Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. *Journal of leukocyte biology, 70*(4), 478-490.

Curran, S., & Murray, G. (2000). Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. *European journal of cancer, 36*(13), 1621-1630.

Dai, P., Jeong, S. Y., Yu, Y., Leng, T., Wu, W., Xie, L., & Chen, X. (2009). Modulation of TLR signaling by multiple MyD88-interacting partners including leucine-rich repeat Fli-I-interacting proteins. *The Journal of Immunology, 182*(6), 3450-3460.

Dakhova, O., Ozen, M., Creighton, C. J., Li, R., Ayala, G., Rowley, D., & Ittmann, M. (2009). Global gene expression analysis of reactive stroma in prostate cancer. *Clinical Cancer Research, 15*(12), 3979-3989.

Darby, I., Skalli, O., & Gabbiani, G. (1990). α-Smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Laboratory Investigation, 63*(1), 21-29.

Darby, I. A., & Hewitson, T. D. (2016). Hypoxia in tissue repair and fibrosis. *Cell and Tissue Research, 365*(3), 553-562.

Darby, I. A., Zakuan, N., Billet, F., & Desmoulière, A. (2016). The myofibroblast, a key cell in normal and pathological tissue repair. *Cellular and Molecular Life Sciences, 73*(6), 1145-1157.

Davy, D. A., Ball, E. E., Matthaei, K. I., Campbell, H. D., & Crouch, M. F. (2000). The flightless I protein localizes to actin-based structures during embryonic development. *Immunology and cell biology, 78*(4), 423-429.

Davy, D. A., Campbell, H. D., Fountain, S., de Jong, D., & Crouch, M. F. (2001). The flightless I protein colocalizes with actin-and microtubule-based structures in motile Swiss 3T3 fibroblasts: evidence for the involvement of PI 3-kinase and Ras-related small GTPases. *Journal of cell science, 114*(3), 549-562.

de Bruin, E. C., & Medema, J. P. (2008). Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer treatment reviews, 34*(8), 737-749.

de Visser, K. E., Eichten, A., & Coussens, L. M. (2006). Paradoxical roles of the immune system during cancer development. *Nature reviews cancer, 6*(1), 24-37.

Deakin, N. O., Pignatelli, J., & Turner, C. E. (2012). Diverse roles for the paxillin family of proteins in cancer. *Genes & cancer, 3*(5-6), 362-370.

Deakin, N. O., & Turner, C. E. (2008). Paxillin comes of age. *Journal of cell science, 121*(15), 2435-2444.

Deakin, N. O., & Turner, C. E. (2011). Distinct roles for paxillin and Hic-5 in regulating breast cancer cell morphology, invasion, and metastasis. *Molecular biology of the cell, 22*(3), 327-341.

DeMali, K. A. (2004). Vinculin–a dynamic regulator of cell adhesion. *Trends in biochemical sciences, 29*(11), 565-567.

Desmoulière, A., Redard, M., Darby, I., & Gabbiani, G. (1995). Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *The American journal of pathology, 146*(1), 56.

Detchokul, S., Williams, E., Parker, M., & Frauman, A. (2013). Tetraspanins as regulators of the tumour microenvironment: implications for metastasis and therapeutic strategies. *British journal of pharmacology*.

Dong, Y., Asch, H. L., Ying, A., & Asch, B. B. (2002). Molecular mechanism of transcriptional repression of gelsolin in human breast cancer cells. *Exp Cell Res, 276*(2), 328-336. doi: 10.1006/excr.2002.5534

Donnem, T., Al-Saad, S., Al-Shibli, K., Andersen, S., Busund, L.-T., & Bremnes, R. M. (2008). Prognostic impact of platelet-derived growth factors in non-small cell lung cancer tumor and stromal cells. *Journal of Thoracic Oncology, 3*(9), 963-970.

Duell, E., Lucenteforte, E., Olson, S., Bracci, P., Li, D., Risch, H., . . . Holly, E. (2012). Pancreatitis and pancreatic cancer risk: a pooled analysis in the International Pancreatic Cancer Case-Control Consortium (PanC4). *Annals of Oncology*, mds140.

Dvorak, H. F. (1986). Tumors: wounds that do not heal: similarities between tumor stroma generation and wound healing. *The New England journal of medicine, 315*(26), 1650-1659.

Ebina, M., Steinberg, S. M., Mulshine, J. L., & Linnoila, R. I. (1994). Relationship of p53 overexpression and up-regulation of proliferating cell nuclear antigen with the clinical course of non-small cell lung cancer. *Cancer research, 54*(9), 2496-2503.

Ebos, J. M., & Kerbel, R. S. (2011). Antiangiogenic therapy: impact on invasion, disease progression, and metastasis. *Nature reviews Clinical oncology, 8*(4), 210-221.

Eddy, R. J., Weidmann, M. D., Sharma, V. P., & Condeelis, J. S. (2017). Tumor Cell Invadopodia: Invasive Protrusions that Orchestrate Metastasis. *Trends in cell biology*.

Elpek, G. Ö., Gelen, T., Aksoy, N. H., Karpuzoğlu, T., & Keles, N. (2000). Microvessel count, proliferating cell nuclear antigen and Ki-67 indices in gastric adenocarcinoma. *Pathology oncology research, 6*(1), 59-64.

Erkan, M., Michalski, C. W., Rieder, S., Reiser–Erkan, C., Abiatari, I., Kolb, A., . . . Kleeff, J. (2008). The activated stroma index is a novel and independent prognostic marker in pancreatic ductal adenocarcinoma. *Clinical Gastroenterology and Hepatology, 6*(10), 1155-1161.

Erler, J. T., & Weaver, V. M. (2009). Three-dimensional context regulation of metastasis. *Clinical & experimental metastasis, 26*(1), 35-49.

Etienne-Manneville, S., & Hall, A. (2003). Cdc42 regulates GSK-3β and adenomatous polyposis coli to control cell polarity. *Nature, 421*(6924), 753-756.

Evan, G. I., & Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature, 411*(6835), 342-348.

Eyden, B., Banerjee, S. S., Shenjere, P., & Fisher, C. (2009). The myofibroblast and its tumours. *Journal of clinical pathology, 62*(3), 236-249.

Fadeel, B., & Orrenius, S. (2005). Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *Journal of internal medicine, 258*(6), 479-517.

Fearon, E. R., & Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell, 61*(5), 759-767.

Fedchenko, N., & Reifenrath, J. (2014). Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue: a review. *Diagn Pathol, 9*, 221.

Ferrara, N., Hillan, K. J., Gerber, H.-P., & Novotny, W. (2004). Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nature reviews Drug discovery, 3*(5), 391-400.

Folkman, J. (2002). *Role of angiogenesis in tumor growth and metastasis.* Paper presented at the Seminars in oncology.

Fox, S., Turner, G., Gatter, K., & Harris, A. (1995). The increased expression of adhesion molecules ICAM-3, E-and P-selectins on breast cancer endothelium. *The Journal of pathology, 177*(4), 369-376.

Fraley, S. I., Feng, Y., Krishnamurthy, R., Kim, D.-H., Celedon, A., Longmore, G. D., & Wirtz, D. (2010). A distinctive role for focal adhesion proteins in three-dimensional cell motility. *Nature cell biology, 12*(6), 598-604.

Friedl, P., & Bröcker, E.-B. (2000). The biology of cell locomotion within three-dimensional extracellular matrix. *Cellular and Molecular Life Sciences CMLS, 57*(1), 41-64.

Friedl, P., Sahai, E., Weiss, S., & Yamada, K. M. (2012). New dimensions in cell migration. *Nature reviews Molecular cell biology, 13*(11), 743-747.

Friedl, P., & Weigelin, B. (2008). Interstitial leukocyte migration and immune function. *Nature immunology, 9*(9), 960-969.

Friedl, P., & Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nature reviews cancer, 3*(5), 362-374.

Friedl, P., & Wolf, K. (2010). Plasticity of cell migration: a multiscale tuning model. *The Journal of cell biology, 188*(1), 11-19.

Gabbiani, G., Ryan, G., & Majno, G. (1971). Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia, 27*(5), 549-550.

Gaggioli, C., Hooper, S., Hidalgo-Carcedo, C., Grosse, R., Marshall, J. F., Harrington, K., & Sahai, E. (2007). Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nature cell biology, 9*(12), 1392-1400.

Galluzzi, L., & Kroemer, G. (2008). Necroptosis: a specialized pathway of programmed necrosis. *Cell, 135*(7), 1161-1163.

Gardel, M. L., Schneider, I. C., Aratyn-Schaus, Y., & Waterman, C. M. (2010). Mechanical integration of actin and adhesion dynamics in cell migration. *Annual review of cell and developmental biology, 26*, 315-333.

Goetz, J. G., Minguet, S., Navarro-Lérida, I., Lazcano, J. J., Samaniego, R., Calvo, E., . . . Echarri, A. (2011). Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. *Cell, 146*(1), 148-163.

Goswami, S., Sahai, E., Wyckoff, J. B., Cammer, M., Cox, D., Pixley, F. J., . . . Condeelis, J. S. (2005). Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. *Cancer research, 65*(12), 5278-5283.

Greenburg, G. B., & Hunt, T. K. (1978). The proliferative response in vitro of vascular endothelial and smooth muscle cells exposed to wound fluids and macrophages. *Journal of cellular physiology, 97*(3), 353-360.

Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, inflammation, and cancer. *Cell, 140*(6), 883-899.

Grossi, F., Loprevite, M., Chiaramondia, M., Ceppa, P., Pera, C., Ratto, G., . . . Boni, L. (2003). Prognostic significance of K-< i> ras</i>, p53, bcl-2, PCNA, CD34 in radically resected non-small cell lung cancers. *European journal of cancer, 39*(9), 1242-1250.

Grünert, S., Jechlinger, M., & Beug, H. (2003). Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. *Nature reviews Molecular cell biology, 4*(8), 657-665.

Gullberg, D., Kletsas, D., & Pihlajaniemi, T. (2016). Editorial: Wound healing and fibrosis—two sides of the same coin: Springer.

Gupta, V., Bassi, D. E., Simons, J. D., Devarajan, K., Al-Saleem, T., Uzzo, R. G., & Cukierman, E. (2011). Elevated expression of stromal palladin predicts poor clinical outcome in renal cell carcinoma. *PloS one, 6*(6), e21494.

Haddow, A. (1972). Molecular repair, wound healing, and carcinogenesis: tumor production a possible overhealing. *Adv Cancer Res, 16*, 181-234.

Haerslev, T., Jacobsen, G. K., & Zedeler, K. (1996). Correlation of growth fraction by Ki-67 and proliferating cell nuclear antigen (PCNA) immunohistochemistry with histopathological parameters and prognosis in primary breast carcinomas. *Breast cancer research and treatment, 37*(2), 101-113.

Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., & Weinberg, R. A. (1999). Creation of human tumour cells with defined genetic elements. *Nature, 400*(6743), 464-468.

Hale, M. D., Hayden, J. D., & Grabsch, H. I. (2013). Tumour-microenvironment interactions: role of tumour stroma and proteins produced by cancer-associated fibroblasts in chemotherapy response. *Cellular Oncology*, 1-18.

Hall, A. (2009). The cytoskeleton and cancer. *Cancer and Metastasis Reviews, 28*(1-2), 5-14.

Hanahan, D., & Coussens, L. M. (2012). Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer cell, 21*(3), 309-322.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Hawinkels, L., Paauwe, M., Verspaget, H., Wiercinska, E., Van Der Zon, J., Van Der Ploeg, K., . . . Ten Dijke, P. (2014). Interaction with colon cancer cells hyperactivates TGF-β signaling in cancer-associated fibroblasts. *Oncogene, 33*(1), 97-107.

Hehlgans, S., Haase, M., & Cordes, N. (2007). Signalling via integrins: implications for cell survival and anticancer strategies. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1775*(1), 163-180.

Heldin, C.-H., & Westermark, B. (1999). Mechanism of action and in vivo role of platelet-derived growth factor. *Physiological reviews, 79*(4), 1283-1316.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature, 407*(6805), 770-776.

Hinz, B. (2010). The myofibroblast: paradigm for a mechanically active cell. *Journal of biomechanics, 43*(1), 146-155.

Hood, J. D., Bednarski, M., Frausto, R., Guccione, S., Reisfeld, R. A., Xiang, R., & Cheresh, D. A. (2002). Tumor regression by targeted gene delivery to the neovasculature. *Science, 296*(5577), 2404-2407.

Hood, J. D., & Cheresh, D. A. (2002). Role of integrins in cell invasion and migration. *Nature reviews cancer, 2*(2), 91-100.

Hoon, D. S., Kitago, M., Kim, J., Mori, T., Piris, A., Szyfelbein, K., . . . Chambers, A. F. (2006). Molecular mechanisms of metastasis. *Cancer and Metastasis Reviews, 25*(2), 203-220.

Huang, R. Y.-J., Guilford, P., & Thiery, J. P. (2012). Early events in cell adhesion and polarity during epithelial-mesenchymal transition: The Company of Biologists Ltd.

Humphries, J. D., Wang, P., Streuli, C., Geiger, B., Humphries, M. J., & Ballestrem, C. (2007). Vinculin controls focal adhesion formation by direct interactions with talin and actin. *The Journal of cell biology, 179*(5), 1043-1057.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell, 110*(6), 673-687.

Iwasaki, T., Nakata, A., Mukai, M., Shinkai, K., Yano, H., Sabe, H., . . . Terada, N. (2002). Involvement of phosphorylation of tyr-31 and tyr-118 of paxillin in MM1 cancer cell migration. *International journal of cancer, 97*(3), 330-335.

Jackson, J. E., Kopecki, Z., Adams, D. H., & Cowin, A. J. (2012). Flii neutralizing antibodies improve wound healing in porcine preclinical studies. *Wound Repair and Regeneration, 20*(4), 523-536.

Jacobson, M. D., Weil, M., & Raff, M. C. (1997). Programmed cell death in animal development. *Cell, 88*(3), 347-354.

Jeong, K. W. (2014). Flightless I (Drosophila) homolog facilitates chromatin accessibility of the estrogen receptor alpha target genes in MCF-7 breast cancer cells. *Biochem Biophys Res Commun, 446*(2), 608-613. doi: 10.1016/j.bbrc.2014.03.011

Jeong, K. W. (2014). Flightless I (Drosophila) homolog facilitates chromatin accessibility of the estrogen receptor α target genes in MCF-7 breast cancer cells. *Biochem Biophys Res Commun, 446*(2), 608-613.

Jianxin, A. Y., Deakin, N. O., & Turner, C. E. (2009). Paxillin-kinase-linker tyrosine phosphorylation regulates directional cell migration. *Molecular biology of the cell, 20*(22), 4706-4719.

Jin, G., Sah, R. L., Li, Y. S., Lotz, M., Shyy, J. Y. J., & Chien, S. (2000). Biomechanical regulation of matrix metalloproteinase-9 in cultured chondrocytes. *Journal of Orthopaedic Research, 18*(6), 899-908.

Jin, J., Yu, Q., Han, C., Hu, X., Xu, S., Wang, Q., . . . Cao, X. (2013). LRRFIP2 negatively regulates NLRP3 inflammasome activation in macrophages by promoting Flightless-I-mediated caspase-1 inhibition. *Nature communications, 4*.

Josson, S., Matsuoka, Y., Chung, L. W., Zhau, H. E., & Wang, R. (2010). *Tumor–stroma co-evolution in prostate cancer progression and metastasis.* Paper presented at the Seminars in cell & developmental biology.

Joyce, J. A. (2005). Therapeutic targeting of the tumor microenvironment. *Cancer cell, 7*(6), 513-520.

Joyce, J. A., & Pollard, J. W. (2009). Microenvironmental regulation of metastasis. *Nature reviews cancer, 9*(4), 239-252.

Jung, D. W., Che, Z. M., Kim, J., Kim, K., Kim, K. Y., Williams, D., & Kim, J. (2010). Tumor-stromal crosstalk in invasion of oral squamous cell carcinoma: a pivotal role of CCL7. *International journal of cancer, 127*(2), 332-344.

Junttila, M. R., & de Sauvage, F. J. (2013). Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature, 501*(7467), 346-354.

Kalluri, R., & Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation, 119*(6), 1420.

Kalluri, R., & Zeisberg, M. (2006). Fibroblasts in cancer. *Nature reviews cancer, 6*(5), 392-401.

Kassis, J., Lauffenburger, D. A., Turner, T., & Wells, A. (2001). *Tumor invasion as dysregulated cell motility.* Paper presented at the Seminars in cancer biology.

Kawai, T., Suzuki, M., Kono, S., Shinomiya, N., Rokutanda, M., Takagi, K., . . . Tamai, S. (1994). Proliferating cell nuclear antigen and Ki-67 in lung carcinoma. Correlation with DNA flow cytometric analysis. *Cancer, 74*(9), 2468-2475.

Keller, R. (2005). Cell migration during gastrulation. *Current opinion in cell biology, 17*(5), 533-541.

Kessenbrock, K., Plaks, V., & Werb, Z. (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell, 141*(1), 52-67.

Kienast, Y., von Baumgarten, L., Fuhrmann, M., Klinkert, W. E., Goldbrunner, R., Herms, J., & Winkler, F. (2010). Real-time imaging reveals the single steps of brain metastasis formation. *Nature medicine, 16*(1), 116-122.

Kiessling, R., Wasserman, K., Horiguchi, S., Kono, K., Sjöberg, J., Pisa, P., & Petersson, M. (1999). Tumor-induced immune dysfunction. *Cancer immunology, immunotherapy, 48*(7), 353-362.

Kitisin, K., Saha, T., Blake, T., Golestaneh, N., Deng, M., Kim, C., . . . Mishra, L. (2007). Tgf-Beta signaling in development. *Science Signaling, 2007*(399), cm1.

Klein, C. A. (2008). The metastasis cascade. *Science, 321*(5897), 1785-1787.

Kopecki, Z., Arkell, R., Powell, B. C., & Cowin, A. J. (2009). Flightless I regulates hemidesmosome formation and integrin-mediated cellular adhesion and migration during wound repair. *Journal of Investigative Dermatology, 129*(8), 2031-2045.

Kopecki, Z., Arkell, R., Powell, B. C., & Cowin, A. J. (2009). Flightless I regulates hemidesmosome formation and integrin-mediated cellular adhesion and migration during wound repair. *J Invest Dermatol, 129*(8), 2031-2045.

Kopecki, Z., & Cowin, A. J. (2008). Flightless I: an actin-remodelling protein and an important negative regulator of wound repair. *The international journal of biochemistry & cell biology, 40*(8), 1415-1419.

Kopecki, Z., O'Neill, G. M., Arkell, R., & Cowin, A. J. (2011). Regulation of focal adhesions by Flightless I involves inhibition of paxillin phosphorylation via a Rac1-dependent pathway. *J Invest Dermatol, 131*(7), 1450-1459.

Kopecki, Z., Ruzehaji, N., Turner, C., Iwata, H., Ludwig, R. J., Zillikens, D., . . . Cowin, A. J. (2013). Topically applied flightless I neutralizing antibodies improve healing of blistered skin in a murine model of epidermolysis bullosa acquisita. *Journal of Investigative Dermatology, 133*(4), 1008-1016.

Kopecki, Z., Yang, G., Jackson, J., Melville, E., Calley, M., Murrell, D., . . . Cowin, A. (2015). Cytoskeletal protein Flightless I inhibits apoptosis, enhances tumor cell invasion and promotes cutaneous squamous cell carcinoma progression. *Oncotarget*.

Kopecki, Z., Yang, G., Jackson, J., Melville, E., Martin, V., Matthew, C., . . . Darby, I. (2014). *TREATMENT OF SCC WITH FLIGHTLESS NEUTRALIZING ANTIBODY BLOCKS CELL INVASION AND REDUCES TUMOR GROWTH IN MICE.* Paper presented at the Wound Repair and Regeneration.

Kopecki, Z., Yang, G. N., Arkell, R. M., Jackson, J. E., Melville, E., Iwata, H., . . . Cowin, A. J. (2014). Flightless I over-expression impairs skin barrier development, function and recovery following skin blistering. *The Journal of pathology, 232*(5), 541-552.

Kramer, N., Walzl, A., Unger, C., Rosner, M., Krupitza, G., Hengstschläger, M., & Dolznig, H. (2013). < i> In vitro</i> cell migration and invasion assays. *Mutation Research/Reviews in Mutation Research, 752*(1), 10-24.

Kraning-Rush, C. M., Carey, S. P., Califano, J. P., Smith, B. N., & Reinhart-King, C. A. (2011). The role of the cytoskeleton in cellular force generation in 2D and 3D environments. *Physical biology, 8*(1), 015009.

Kreiseder, B., Orel, L., Bujnow, C., Buschek, S., Pflueger, M., Schuett, W., . . . Wiesner, C. (2013). α-Catulin downregulates E-cadherin and promotes melanoma progression and invasion. *International journal of cancer, 132*(3), 521-530.

Kwiatkowski, D. J. (1999). Functions of gelsolin: motility, signaling, apoptosis, cancer. *Current opinion in cell biology, 11*(1), 103-108.

Lauffenburger, D. A., & Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell, 84*(3), 359-369.

Lazarus, G. S., Cooper, D. M., Knighton, D. R., Margolis, D. J., Percoraro, R. E., Rodeheaver, G., & Robson, M. C. (1994). Definitions and guidelines for assessment of wounds and evaluation of healing. *Wound Repair and Regeneration, 2*(3), 165-170.

Lech-Maranda, E., Bienvenu, J., Michallet, A.-S., Houot, R., Robak, T., Coiffier, B., & Salles, G. (2006). Elevated IL-10 plasma levels correlate with poor prognosis in diffuse large B-cell lymphoma. *European cytokine network, 17*(1), 60-66.

Lee, J. M., Dedhar, S., Kalluri, R., & Thompson, E. W. (2006). The epithelial–mesenchymal transition: new insights in signaling, development, and disease. *J Cell biol, 172*(7), 973-981.

Lee, Y.-H., Campbell, H. D., & Stallcup, M. R. (2004). Developmentally essential protein flightless I is a nuclear receptor coactivator with actin binding activity. *Molecular and cellular biology, 24*(5), 2103-2117.

Leek, R., Harris, A., & Lewis, C. (1994). Cytokine networks in solid human tumors: regulation of angiogenesis. *Journal of leukocyte biology, 56*(4), 423-435.

Lei, N., Franken, L., Ruzehaji, N., Offenhäuser, C., Cowin, A. J., & Murray, R. Z. (2012). Flightless, secreted through a late endosome/lysosome pathway, binds LPS and dampens cytokine secretion. *Journal of cell science, 125*(18), 4288-4296.

Leight, J. L., Wozniak, M. A., Chen, S., Lynch, M. L., & Chen, C. S. (2012). Matrix rigidity regulates a switch between TGF-β1–induced apoptosis and epithelial–mesenchymal transition. *Molecular biology of the cell, 23*(5), 781-791.

Lemmon, C. A., Chen, C. S., & Romer, L. H. (2009). Cell traction forces direct fibronectin matrix assembly. *Biophysical journal, 96*(2), 729-738.

Lesina, M., Kurkowski, M. U., Ludes, K., Rose-John, S., Treiber, M., Klöppel, G., . . . Akira, S. (2011). Stat3/Socs3 activation by IL-6 transsignaling promotes progression of pancreatic intraepithelial neoplasia and development of pancreatic cancer. *Cancer cell, 19*(4), 456-469.

Levental, K. R., Yu, H., Kass, L., Lakins, J. N., Egeblad, M., Erler, J. T., . . . Weninger, W. (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell, 139*(5), 891-906.

Li, J., Yin, H. L., & Yuan, J. (2008). Flightless-I regulates proinflammatory caspases by selectively modulating intracellular localization and caspase activity. *The Journal of cell biology, 181*(2), 321-333.

Li, Y., Tondravi, M., Liu, J., Smith, E., Haudenschild, C. C., Kaczmarek, M., & Zhan, X. (2001). Cortactin potentiates bone metastasis of breast cancer cells. *Cancer research, 61*(18), 6906-6911.

Libby, P., Ridker, P. M., & Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation, 105*(9), 1135-1143.

Lin, E. Y., Li, J.-F., Gnatovskiy, L., Deng, Y., Zhu, L., Grzesik, D. A., . . . Pollard, J. W. (2006). Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer research, 66*(23), 11238-11246.

Linder, S., & Aepfelbacher, M. (2003). Podosomes: adhesion hot-spots of invasive cells. *Trends in cell biology, 13*(7), 376-385.

Lindgren, G., Kjellén, E., Wennerberg, J., & Ekblad, L. (2016). Wound-healing factors can prime head and neck cancer cells to increase their tumor-forming capacity. *The Laryngoscope*.

Liotta, L. A., & Kohn, E. C. (2001). The microenvironment of the tumour–host interface. *Nature, 411*(6835), 375-379.

Liu, Y.-T., & Yin, H. L. (1998). Identification of the binding partners for flightless I, A novel protein bridging the leucine-rich repeat and the gelsolin superfamilies. *Journal of Biological Chemistry, 273*(14), 7920-7927.

Liu, Y. (2011). Cellular and molecular mechanisms of renal fibrosis. *Nature Reviews Nephrology, 7*(12), 684-696.

Lohela, M., Bry, M., Tammela, T., & Alitalo, K. (2009). VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. *Current opinion in cell biology, 21*(2), 154-165.

Lorusso, G., & Rüegg, C. (2008). The tumor microenvironment and its contribution to tumor evolution toward metastasis. *Histochemistry and cell biology, 130*(6), 1091-1103.

Lowe, S. W., Cepero, E., & Evan, G. (2004). Intrinsic tumour suppression. *Nature, 432*(7015), 307-315.

Lu, P., Weaver, V. M., & Werb, Z. (2012). The extracellular matrix: a dynamic niche in cancer progression. *The Journal of cell biology, 196*(4), 395-406.

Mabuchi, S., Altomare, D. A., Cheung, M., Zhang, L., Poulikakos, P. I., Hensley, H. H., . . . Testa, J. R. (2007). RAD001 inhibits human ovarian cancer cell proliferation, enhances cisplatin-induced apoptosis, and prolongs survival in an ovarian cancer model. *Clinical Cancer Research, 13*(14), 4261-4270.

Maeshima, A. M., Niki, T., Maeshima, A., Yamada, T., Kondo, H., & Matsuno, Y. (2002). Modified scar grade. *Cancer, 95*(12), 2546-2554.

Maniati, E., Bossard, M., Cook, N., Candido, J. B., Emami-Shahri, N., Nedospasov, S. A., . . . Hagemann, T. (2011). Crosstalk between the canonical NF-κB and Notch signaling pathways inhibits Pparγ expression and promotes pancreatic cancer progression in mice. *The Journal of clinical investigation, 121*(121 (12)), 4685-4699.

Mantovani, A., Allavena, P., Sica, A., & Balkwill, F. (2008). Cancer-related inflammation. *Nature, 454*(7203), 436-444.

Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., & Ruco, L. (1992). The origin and function of tumor-associated macrophages. *Immunology today, 13*(7), 265-270.

Mantovani, A., Sozzani, S., Locati, M., Allavena, P., & Sica, A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in immunology, 23*(11), 549-555.

Marei, H., Carpy, A., Woroniuk, A., Vennin, C., White, G., Timpson, P., . . . Malliri, A. (2016). Differential Rac1 signalling by guanine nucleotide exchange factors implicates FLII in regulating Rac1-driven cell migration. *Nature communications, 7*.

Massagué, J. (2008). TGFβ in cancer. *Cell, 134*(2), 215-230.

Massagué, J., & Obenauf, A. C. (2016). Metastatic colonization by circulating tumour cells. *Nature, 529*(7586), 298-306.

Mattila, P. K., & Lappalainen, P. (2008). Filopodia: molecular architecture and cellular functions. *Nature reviews Molecular cell biology, 9*(6), 446-454.

Mayer, A., Takimoto, M., Fritz, E., Schellander, G., Kofler, K., & Ludwig, H. (1993). The prognostic significance of proliferating cell nuclear antigen, epidermal growth factor receptor, and mdr gene expression in colorectal cancer. *Cancer, 71*(8), 2454-2460.

McCloy, R. A., Rogers, S., Caldon, C. E., Lorca, T., Castro, A., & Burgess, A. (2014). Partial inhibition of Cdk1 in G2 phase overrides the SAC and decouples mitotic events. *Cell Cycle, 13*(9), 1400-1412.

Mendelsohn, J., & Baselga, J. (2000). The EGF receptor family as targets for cancer therapy. *Oncogene, 19*(56), 6550-6565.

Mohammad, I., Arora, P. D., Naghibzadeh, Y., Wang, Y., Li, J., Mascarenhas, W., . . . McCulloch, C. A. (2012). Flightless I is a focal adhesion-associated actin-capping protein that regulates cell migration. *The FASEB Journal, 26*(8), 3260-3272.

Moore, R. J., Owens, D. M., Stamp, G., Arnott, C., Burke, F., East, N., . . . Pasparakis, M. (1999). Mice deficient in tumor necrosis factor-α are resistant to skin carcinogenesis. *Nature medicine, 5*(7), 828-831.

Mowers, E. E., Sharifi, M. N., & Macleod, K. F. (2016). Novel insights into how autophagy regulates tumor cell motility. *Autophagy, 12*(9), 1679-1680.

Mudter, J., Amoussina, L., Schenk, M., Yu, J., Brüstle, A., Weigmann, B., . . . Hoffman, A. (2008). The transcription factor IFN regulatory factor–4 controls experimental colitis in mice via T cell–derived IL-6. *The Journal of clinical investigation, 118*(118 (7)), 2415-2426.

Mueller-Huebenthal, B., Azemar, M., Lorenzen, D., Huber, M., Freudenberg, M. A., Galanos, C., . . . Hildenbrand, B. (2009). Tumour Biology: tumour-associated inflammation versus antitumor immunity. *Anticancer research, 29*(11), 4795-4805.

Mumm, J., & Oft, M. (2008). Cytokine-based transformation of immune surveillance into tumor-promoting inflammation. *Oncogene, 27*(45), 5913-5919.

Murugaiyan, G., & Saha, B. (2009). Protumor vs antitumor functions of IL-17. *The Journal of Immunology, 183*(7), 4169-4175.

Nanji, A. A., & Hiller-Sturmhöfel, S. (1997). Apoptosis and necrosis. *Alcohol Health Res World, 21*, 325-330.

Nathan, C. F., & Hibbs Jr, J. B. (1991). Role of nitric oxide synthesis in macrophage antimicrobial activity. *Current opinion in immunology, 3*(1), 65-70.

Nayal, A., Webb, D. J., & Horwitz, A. F. (2004). Talin: an emerging focal point of adhesion dynamics. *Current opinion in cell biology, 16*(1), 94-98.

Nobes, C. D., & Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *The Journal of cell biology, 144*(6), 1235-1244.

Noble, P. W., Barkauskas, C. E., & Jiang, D. (2012). Pulmonary fibrosis: patterns and perpetrators. *The Journal of clinical investigation, 122*(8), 2756-2762.

Noël, A. (2012). New and paradoxical roles of matrix metalloproteinases in the tumor microenvironment. *Frontiers in pharmacology, 3*, 140.

Numasaki, M., Fukushi, J.-i., Ono, M., Narula, S. K., Zavodny, P. J., Kudo, T., . . . Lotze, M. T. (2003). Interleukin-17 promotes angiogenesis and tumor growth. *Blood, 101*(7), 2620-2627.

O'Brate, A., & Giannakakou, P. (2003). The importance of p53 location: nuclear or cytoplasmic zip code? *Drug resistance updates, 6*(6), 313-322.

Öhlund, D., Elyada, E., & Tuveson, D. (2014). Fibroblast heterogeneity in the cancer wound. *The Journal of experimental medicine, 211*(8), 1503-1523.

Ola, M. S., Nawaz, M., & Ahsan, H. (2011). Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. *Molecular and cellular biochemistry, 351*(1-2), 41-58.

Orimo, A., Gupta, P. B., Sgroi, D. C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., . . . Weinberg, R. A. (2005). Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell, 121*(3), 335-348.

Orimo, A., & Weinberg, R. A. (2006). Stromal fibroblasts in cancer: a novel tumor-promoting cell type. *Cell Cycle, 5*(15), 1597-1601.

Orimo, A., & Weinberg, R. A. (2007). Heterogeneity of stromal fibroblasts in tumors. *CANCER BIOLOGY AND THERAPY, 6*(4), 618.

Osuský, R., Malik, P., & Ryan, S. (1997). Retinal pigment epithelium cells promote the maturation of monocytes to macrophages in vitro. *Ophthalmic research, 29*(1), 31-36.

Panetti, T. S. (2002). Tyrosine phosphorylation of paxillin, FAK, and p130CAS: effects on cell spreading and migration. *Front Biosci, 7*(January), d143-d150.

Partridge, M. A., & Marcantonio, E. E. (2006). Initiation of attachment and generation of mature focal adhesions by integrin-containing filopodia in cell spreading. *Molecular biology of the cell, 17*(10), 4237-4248.

Philip, M., Rowley, D. A., & Schreiber, H. (2004). *Inflammation as a tumor promoter in cancer induction.* Paper presented at the Seminars in cancer biology.

Pickup, M. W., Mouw, J. K., & Weaver, V. M. (2014). The extracellular matrix modulates the hallmarks of cancer. *EMBO reports*, e201439246.

Pietras, K., & Östman, A. (2010). Hallmarks of cancer: interactions with the tumor stroma. *Experimental cell research, 316*(8), 1324-1331.

Pollard, J. W. (2009). Trophic macrophages in development and disease. *Nature Reviews Immunology, 9*(4), 259-270.

Polyak, K., Haviv, I., & Campbell, I. G. (2009). Co-evolution of tumor cells and their microenvironment. *Trends in Genetics, 25*(1), 30-38.

Provenzano, P. P., Eliceiri, K. W., & Keely, P. J. (2009). Shining new light on 3D cell motility and the metastatic process. *Trends in cell biology, 19*(11), 638-648.

Quail, D. F., & Joyce, J. A. (2013). Microenvironmental regulation of tumor progression and metastasis. *Nature medicine, 19*(11), 1423-1437.

Quinn, C. M., & Wright, N. A. (1990). The clinical assessment of proliferation and growth in human tumours: evaluation of methods and applications as prognostic variables. *The Journal of pathology, 160*(2), 93-102.

Ren, Y., Cao, B., Law, S., Xie, Y., Lee, P. Y., Cheung, L., . . . Zhao, P. (2005). Hepatocyte growth factor promotes cancer cell migration and angiogenic factors expression: a prognostic marker of human esophageal squamous cell carcinomas. *Clinical Cancer Research, 11*(17), 6190-6197.

Rhee, S., Jiang, H., Ho, C.-H., & Grinnell, F. (2007). Microtubule function in fibroblast spreading is modulated according to the tension state of cell–matrix interactions. *Proceedings of the National Academy of Sciences, 104*(13), 5425-5430.

Rich, T., Watson, C. J., & Wyllie, A. (1999). Apoptosis: the germs of death. *Nature cell biology, 1*(3), E69-E71.

Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., . . . Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. *Science, 302*(5651), 1704-1709.

Roncucci, L., Stamp, D., Medline, A., Cullen, J. B., & Robert Bruce, W. (1991). Identification and quantification of aberrant crypt foci and microadenomas in the human colon. *Human pathology, 22*(3), 287-294.

Ronnov-Jessen, L., Petersen, O. W., & Bissell, M. J. (1996). Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. *Physiological reviews, 76*(1), 69-125.

Ross, J., & Auger, M. (2002). The biology of the macrophage. *The macrophage, 2*, 3-47.

Rudas, M., Gnant, M., Mittlböck, M., Neumayer, R., Kummer, A., Jakesz, R., . . . Reiner, A. (1994). Thymidine labeling index and Ki-67 growth fraction in breast cancer: comparison and correlation with prognosis. *Breast cancer research and treatment, 32*(2), 165-175.

Ruzehaji, N., Kopecki, Z., Melville, E., Appleby, S. L., Bonder, C. S., Arkell, R. M., . . . Cowin, A. J. (2014). Attenuation of flightless I improves wound healing and enhances angiogenesis in a murine model of type 1 diabetes. *Diabetologia, 57*(2), 402-412.

Rybinski, B., Franco-Barraza, J., & Cukierman, E. (2014). The wound healing, chronic fibrosis, and cancer progression triad. *Physiological genomics, 46*(7), 223-244.

Saito, T., Kuss, I., Dworacki, G., Gooding, W., Johnson, J. T., & Whiteside, T. L. (1999). Spontaneous ex vivo apoptosis of peripheral blood mononuclear cells in patients with head and neck cancer. *Clinical Cancer Research, 5*(6), 1263-1273.

Salgia, R., Li, J.-L., Ewaniuk, D. S., Wang, Y.-B., Sattler, M., Chen, W.-C., . . . Rollins, B. J. (1999). Expression of the focal adhesion protein paxillin in lung cancer and its relation to cell motility. *Oncogene, 18*(1), 67-77.

Salvesen, H. B., & Akslen, L. A. (1999). Significance of tumour-associated macrophages, vascular endothelial growth factor and thrombospondin-1 expression for tumour angiogenesis and prognosis in endometrial carcinomas. *International journal of cancer, 84*(5), 538-543.

Sandbo, N., & Dulin, N. (2011). Actin cytoskeleton in myofibroblast differentiation: ultrastructure defining form and driving function. *Translational Research, 158*(4), 181-196.

Saunders, R. M., Holt, M. R., Jennings, L., Sutton, D. H., Barsukov, I. L., Bobkov, A., . . . Critchley, D. R. (2006). Role of vinculin in regulating focal adhesion turnover. *European journal of cell biology, 85*(6), 487-500.

Schäfer, M., & Werner, S. (2008). Cancer as an overhealing wound: an old hypothesis revisited. *Nature reviews Molecular cell biology, 9*(8), 628-638.

Schellekens, H. (2002). Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clinical therapeutics, 24*(11), 1720-1740.

Schoumacher, M., Goldman, R. D., Louvard, D., & Vignjevic, D. M. (2010). Actin, microtubules, and vimentin intermediate filaments cooperate for elongation of invadopodia. *The Journal of cell biology, 189*(3), 541-556.

Scibelli, A., d'Angelo, D., Pelagalli, A., Tafuri, S., Avallone, L., Della Morte, R., & Staiano, N. (2003). Expression levels of the focal adhesion-associated proteins paxillin and p130 $^{{\ rm CAS}} $ in canine and feline mammary tumors. *Veterinary research, 34*(2), 193-202.

Seluanov, A., Vaidya, A., & Gorbunova, V. (2010). Establishing primary adult fibroblast cultures from rodents. *Journal of visualized experiments: JoVE*(44).

Seward, M. E., Easley, C. A. t., McLeod, J. J., Myers, A. L., & Tombes, R. M. (2008). Flightless-I, a gelsolin family member and transcriptional regulator, preferentially binds directly to activated cytosolic CaMK-II. *FEBS Lett, 582*(17), 2489-2495. doi: S0014-5793(08)00548-6 [pii]

10.1016/j.febslet.2008.06.037

Shacter, E., & Weitzman, S. A. (2002). Chronic inflammation and cancer. *Oncology, 16*(2), 217-230.

Sheibani, N., Tang, Y., & Sorenson, C. M. (2008). Paxillin's LD4 motif interacts with bcl-2. *Journal of cellular physiology, 214*(3), 655-661.

Short, S. M., Yoder, B. J., Tarr, S. M., Prescott, N. L., Laniauskas, S., Coleman, K. A., . . . Crowe, J. P. (2007). The expression of the cytoskeletal focal adhesion protein paxillin in breast cancer correlates with HER2 overexpression and may help predict response to chemotherapy: a retrospective immunohistochemical study. *The breast journal, 13*(2), 130-139.

Sibony-Benyamini, H., & Gil-Henn, H. (2012). Invadopodia: the leading force. *European journal of cell biology, 91*(11), 896-901.

Sica, A., Schioppa, T., Mantovani, A., & Allavena, P. (2006). Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *European journal of cancer, 42*(6), 717-727.

Siegel, R., DeSantis, C., & Jemal, A. (2014). Colorectal cancer statistics, 2014. *CA: a cancer journal for clinicians, 64*(2), 104-117.

Siveen, K., & Kuttan, G. (2009). Role of macrophages in tumour progression. *Immunology letters, 123*(2), 97-102.

So, F. V., Guthrie, N., Chambers, A. F., Moussa, M., & Carroll, K. K. (1996). Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices.

Soengas, M. S., Capodieci, P., Polsky, D., Mora, J., Esteller, M., Opitz-Araya, X., . . . Lazebnik, Y. A. (2001). Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature, 409*(6817), 207-211.

Solinas, G., Germano, G., Mantovani, A., & Allavena, P. (2009). Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *Journal of leukocyte biology, 86*(5), 1065-1073.

Soucek, L., Lawlor, E. R., Soto, D., Shchors, K., Swigart, L. B., & Evan, G. I. (2007). Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nature medicine, 13*(10), 1211-1218.

Sounni, N. E., & Noel, A. (2013). Targeting the tumor microenvironment for cancer therapy. *Clinical chemistry, 59*(1), 85-93.

Staunton, M. J., & Gaffney, E. F. (1998). Apoptosis: basic concepts and potential significance in human cancer. *Archives of pathology & laboratory medicine, 122*(4), 310-319.

Stetler-Stevenson, W. G., Hewitt, R., & Corcoran, M. (1996). *Matrix metalloproteinases and tumor invasion: from correlation and causality to the clinic.* Paper presented at the Seminars in cancer biology.

Stoimenov, I., & Helleday, T. (2009). PCNA on the crossroad of cancer. *Biochem Soc Trans, 37*(Pt 3), 605-613.

Stone, R. C., Pastar, I., Ojeh, N., Chen, V., Liu, S., Garzon, K. I., & Tomic-Canic, M. (2016). Epithelial-mesenchymal transition in tissue repair and fibrosis. *Cell and Tissue Research, 365*(3), 495-506.

Straub, K. L., Stella, M. C., & Leptin, M. (1996). The gelsolin-related flightless I protein is required for actin distribution during cellularisation in Drosophila. *Journal of cell science, 109*(1), 263-270.

Strzalka, W., & Ziemienowicz, A. (2011). Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. *Annals of botany, 107*(7), 1127-1140.

Subauste, M. C., Pertz, O., Adamson, E. D., Turner, C. E., Junger, S., & Hahn, K. M. (2004). Vinculin modulation of paxillin–FAK interactions regulates ERK to control survival and motility. *The Journal of cell biology, 165*(3), 371-381.

Sun, X., Fa, P., Cui, Z., Xia, Y., Sun, L., Li, Z., . . . Cai, Z. (2013). The EDA-containing cellular fibronectin induces epithelial mesenchymal transition in lung cancer cells through integrin α9β1-mediated activation of PI3-K/AKT and Erk1/2. *Carcinogenesis*, bgt276.

Sung, B. H., Zhu, X., Kaverina, I., & Weaver, A. M. (2011). Cortactin controls cell motility and lamellipodial dynamics by regulating ECM secretion. *Current Biology, 21*(17), 1460-1469.

Sung, S.-Y., Hsieh, C.-L., Law, A., Zhau, H. E., Pathak, S., Multani, A. S., . . . Figg, W. D. (2008). Coevolution of prostate cancer and bone stroma in three-dimensional coculture: implications for cancer growth and metastasis. *Cancer research, 68*(23), 9996-10003.

Tachibana, K. e. K., Gonzalez, M. A., & Coleman, N. (2005). Cell-cycle-dependent regulation of DNA replication and its relevance to cancer pathology. *The Journal of pathology, 205*(2), 123-129.

Takanami, I., Takeuchi, K., & Kodaira, S. (1999). Tumor-associated macrophage infiltration in pulmonary adenocarcinoma: association with angiogenesis and poor prognosis. *Oncology, 57*(2), 138-142.

Tehrani, S., Faccio, R., Chandrasekar, I., Ross, F. P., & Cooper, J. A. (2006). Cortactin has an essential and specific role in osteoclast actin assembly. *Molecular biology of the cell, 17*(7), 2882-2895.

Thiery, J. P. (2003). Cell adhesion in cancer. *Comptes Rendus Physique, 4*(2), 289-304.

Tiwari, N., Gheldof, A., Tatari, M., & Christofori, G. (2012). *EMT as the ultimate survival mechanism of cancer cells.* Paper presented at the Seminars in cancer biology.

Tlsty, T. D., & Coussens, L. M. (2006). Tumor stroma and regulation of cancer development. *Annu. Rev. Pathol. Mech. Dis., 1*, 119-150.

Tojkander, S., Gateva, G., & Lappalainen, P. (2012). Actin stress fibers–assembly, dynamics and biological roles. *J Cell Sci, 125*(8), 1855-1864.

Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C., & Brown, R. A. (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nature reviews Molecular cell biology, 3*(5), 349-363.

Torisu, H., Ono, M., Kiryu, H., Furue, M., Ohmoto, Y., Nakayama, J., . . . Kuwano, M. (2000). Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: Possible involvement of TNFα and IL-1α. *International journal of cancer, 85*(2), 182-188.

Toschi, L., & Bravo, R. (1988). Changes in cyclin/proliferating cell nuclear antigen distribution during DNA repair synthesis. *The Journal of cell biology, 107*(5), 1623-1628.

Tsuchiya, S., Fujiwara, T., Sato, F., Shimada, Y., Tanaka, E., Sakai, Y., . . . Tsujimoto, G. (2011). MicroRNA-210 regulates cancer cell proliferation through targeting fibroblast growth factor receptor-like 1 (FGFRL1). *Journal of Biological Chemistry, 286*(1), 420-428.

Tsuji, T., Mimura, Y., Wen, S., Li, X., Kanekawa, A., Sasaki, K., & Shinozaki, F. (1995). The significance of PCNA and p53 protein in some oral tumors. *International journal of oral and maxillofacial surgery, 24*(3), 221-225.

Tsujino, T., Seshimo, I., Yamamoto, H., Ngan, C. Y., Ezumi, K., Takemasa, I., . . . Monden, M. (2007). Stromal myofibroblasts predict disease recurrence for colorectal cancer. *Clinical Cancer Research, 13*(7), 2082-2090.

Turner, S., & Sherratt, J. A. (2002). Intercellular adhesion and cancer invasion: a discrete simulation using the extended Potts model. *Journal of Theoretical Biology, 216*(1), 85-100.

Ueno, T., Toi, M., Saji, H., Muta, M., Bando, H., Kuroi, K., . . . Matsushima, K. (2000). Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clinical Cancer Research, 6*(8), 3282-3289.

Vadlamudi, R., Adam, L., Tseng, B., Costa, L., & Kumar, R. (1999). Transcriptional up-regulation of paxillin expression by heregulin in human breast cancer cells. *Cancer research, 59*(12), 2843-2846.

Van Cutsem, E., Cervantes, A., Nordlinger, B., & Arnold, D. (2014). Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, mdu260.

Van den Abbeele, A., De Corte, V., Van Impe, K., Bruyneel, E., Boucherie, C., Bracke, M., . . . Gettemans, J. (2007). Downregulation of gelsolin family proteins counteracts cancer cell invasion in vitro. *Cancer letters, 255*(1), 57-70.

Van den Abbeele, A., De Corte, V., Van Impe, K., Bruyneel, E., Boucherie, C., Bracke, M., . . . Gettemans, J. (2007). Downregulation of gelsolin family proteins counteracts cancer cell invasion in vitro. *Cancer Lett, 255*(1), 57-70. doi: 10.1016/j.canlet.2007.03.023

Van den Hooff, A. (1988). Stromal involvement in malignant growth. *Adv Cancer Res, 50*(159), 96.

van Rossum, A. G., Moolenaar, W. H., & Schuuring, E. (2006). Cortactin affects cell migration by regulating intercellular adhesion and cell spreading. *Experimental cell research, 312*(9), 1658-1670.

Van Zijl, F., Mair, M., Csiszar, A., Schneller, D., Zulehner, G., Huber, H., . . . Mikulits, W. (2009). Hepatic tumor–stroma crosstalk guides epithelial to mesenchymal transition at the tumor edge. *Oncogene, 28*(45), 4022-4033.

Velnar, T., Bailey, T., & Smrkolj, V. (2009). The wound healing process: an overview of the cellular and molecular mechanisms. *Journal of International Medical Research, 37*(5), 1528-1542.

Verjans, E., Noetzel, E., Bektas, N., Schütz, A. K., Lue, H., Lennartz, B., . . . Bernhagen, J. (2009). Dual role of macrophage migration inhibitory factor (MIF) in human breast cancer. *BMC cancer, 9*(1), 230.

Vermes, I., Haanen, C., & Reutelingsperger, C. (2000). Flow cytometry of apoptotic cell death. *Journal of immunological methods, 243*(1), 167-190.

Vicente-Manzanares, M., Choi, C. K., & Horwitz, A. R. (2009). Integrins in cell migration–the actin connection. *Journal of cell science, 122*(2), 199-206.

Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., . . . Bos, J. L. (1988). Genetic alterations during colorectal-tumor development. *New England Journal of Medicine, 319*(9), 525-532.

Wang, L.-F., Chai, C.-Y., Kuo, W.-R., Tai, C.-F., Lee, K.-W., & Ho, K.-Y. (2006). Correlation between proliferating cell nuclear antigen and p53 protein expression and 5-year survival rate in nasopharyngeal carcinoma. *American journal of otolaryngology, 27*(2), 101-105.

Wang, L., Yi, T., Kortylewski, M., Pardoll, D. M., Zeng, D., & Yu, H. (2009). IL-17 can promote tumor growth through an IL-6–Stat3 signaling pathway. *The Journal of experimental medicine, 206*(7), 1457-1464.

Wang, S.-C. (2014). PCNA: a silent housekeeper or a potential therapeutic target? *Trends in pharmacological sciences, 35*(4), 178-186.

Wang, T., Chuang, T.-H., Ronni, T., Gu, S., Du, Y.-C., Cai, H., . . . Chen, X. (2006). Flightless I homolog negatively modulates the TLR pathway. *The Journal of Immunology, 176*(3), 1355-1362.

Wang, W., Goswami, S., Sahai, E., Wyckoff, J. B., Segall, J. E., & Condeelis, J. S. (2005). Tumor cells caught in the act of invading: their strategy for enhanced cell motility. *Trends in cell biology, 15*(3), 138-145.

Weaver, A. M. (2006). Invadopodia: specialized cell structures for cancer invasion. *Clinical & experimental metastasis, 23*(2), 97-105.

Weaver, A. M. (2008). Cortactin in tumor invasiveness. *Cancer letters, 265*(2), 157-166.

Weber, C., Telerman, S. B., Reimer, A. S., Sequeira, I., Liakath-Ali, K., Arwert, E. N., & Watt, F. M. (2016). Macrophage infiltration and alternative activation during wound healing promote MEK1-induced skin carcinogenesis. *Cancer research, 76*(4), 805-817.

Wei, S. C., Fattet, L., Tsai, J. H., Guo, Y., Pai, V. H., Majeski, H. E., . . . Engler, A. J. (2015). Matrix stiffness drives epithelial-mesenchymal transition and tumour metastasis through a TWIST1-G3BP2 mechanotransduction pathway. *Nature cell biology, 17*(5), 678-688.

Welch, M. D., & Mullins, R. D. (2002). Cellular control of actin nucleation. *Annual review of cell and developmental biology, 18*(1), 247-288.

Wells, A. (1999). Tumor invasion: role of growth factor-induced cell motility. *Advances in cancer research, 78*, 31-101.

Wells, A. (2006). *Cell motility in cancer invasion and metastasis* (Vol. 8): Springer.

Wells, A., Chao, Y. L., Grahovac, J., Wu, Q., & Lauffenburger, D. A. (2011). Epithelial and mesenchymal phenotypic switchings modulate cell motility in metastasis. *Frontiers in bioscience (Landmark edition), 16*, 815-837.

Wells, A., Grahovac, J., Wheeler, S., Ma, B., & Lauffenburger, D. (2013). Targeting tumor cell motility as a strategy against invasion and metastasis. *Trends in pharmacological sciences, 34*(5), 283-289.

Wilson, J. M., Coletta, P. L., Cuthbert, R. J., Scott, N., MacLennan, K., Hawcroft, G., . . . Lolis, E. (2005). Macrophage migration inhibitory factor promotes intestinal tumorigenesis. *Gastroenterology, 129*(5), 1485-1503.

Wong, G., & Rustgi, A. (2013). Matricellular proteins: priming the tumour microenvironment for cancer development and metastasis. *British journal of cancer*.

Wong, M. E., Hollinger, J. O., & Pinero, G. J. (1996). Integrated processes responsible for soft tissue healing. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology, 82*(5), 475-492.

Woods, D. B., & Vousden, K. H. (2001). Regulation of p53 function. *Experimental cell research, 264*(1), 56-66.

Wu, L., Chen, H., Zhu, Y., Meng, J., Li, Y., Li, M., . . . Tong, X. (2013). Flightless I homolog negatively regulates ChREBP activity in cancer cells. *Int J Biochem Cell Biol, 45*(11), 2688-2697. doi: 10.1016/j.biocel.2013.09.004

Wu, L., Chen, H., Zhu, Y., Meng, J., Li, Y., Li, M., . . . Tong, X. (2013). Flightless I homolog negatively regulates ChREBP activity in cancer cells. *The international journal of biochemistry & cell biology, 45*(11), 2688-2697.

Wynn, T. A., & Ramalingam, T. R. (2012). Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nature medicine, 18*(7), 1028-1040.

Xiang, L., Xie, G., Ou, J., Wei, X., Pan, F., & Liang, H. (2012). The extra domain A of fibronectin increases VEGF-C expression in colorectal carcinoma involving the PI3K/AKT signaling pathway. *PloS one, 7*(4), e35378.

Xiong, G., Deng, L., Zhu, J., Rychahou, P. G., & Xu, R. (2014). Prolyl-4-hydroxylase α subunit 2 promotes breast cancer progression and metastasis by regulating collagen deposition. *BMC cancer, 14*(1), 1.

Yamaguchi, H., & Condeelis, J. (2007). Regulation of the actin cytoskeleton in cancer cell migration and invasion. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1773*(5), 642-652.

Yamaguchi, H., Wyckoff, J., & Condeelis, J. (2005). Cell migration in tumors. *Current opinion in cell biology, 17*(5), 559-564.

Yang, G., Rosen, D. G., Zhang, Z., Bast, R. C., Mills, G. B., Colacino, J. A., . . . Liu, J. (2006). The chemokine growth-regulated oncogene 1 (Gro-1) links RAS signaling to the senescence of stromal fibroblasts and ovarian tumorigenesis. *Proceedings of the National Academy of Sciences, 103*(44), 16472-16477.

Yang, J., & Weinberg, R. A. (2008). Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Developmental cell, 14*(6), 818-829.

Yano, H., Uchida, H., Iwasaki, T., Mukai, M., Akedo, H., Nakamura, K., . . . Sabe, H. (2000). Paxillin α and Crk-associated substrate exert opposing effects on cell migration and contact inhibition of growth through tyrosine phosphorylation. *Proceedings of the National Academy of Sciences, 97*(16), 9076-9081.

Yilmaz, M., & Christofori, G. (2009). EMT, the cytoskeleton, and cancer cell invasion. *Cancer and Metastasis Reviews, 28*(1-2), 15-33.

Young, J. P., Win, A. K., Rosty, C., Flight, I., Roder, D., Young, G. P., . . . Ruszkiewicz, A. (2014). Rising Incidence of Early-Onset Colorectal Cancer in Australia over Two Decades: Report and Review. *Journal of gastroenterology and hepatology*.

Zaidel-Bar, R., Ballestrem, C., Kam, Z., & Geiger, B. (2003). Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *Journal of cell science, 116*(22), 4605-4613.

Zaidel-Bar, R., Milo, R., Kam, Z., & Geiger, B. (2007). A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. *Journal of cell science, 120*(1), 137-148.

Zamir, E., & Geiger, B. (2001). Molecular complexity and dynamics of cell-matrix adhesions. *Journal of cell science, 114*(20), 3583-3590.

Zhang, F., Yang, Q., Meng, F., Shi, H., Li, H., Liang, Y., & Han, A. (2013). Astrocyte elevated gene-1 interacts with β-catenin and increases migration and invasion of colorectal carcinoma. *Molecular carcinogenesis, 52*(8), 603-610.

Zhang, L.-H., Tian, B., Diao, L.-R., Xiong, Y.-Y., Tian, S.-F., Zhang, B.-H., . . . Ji, J.-F. (2006). Dominant expression of 85-kDa form of cortactin in colorectal cancer. *Journal of cancer research and clinical oncology, 132*(2), 113-120.

Zhang, Z., Izaguirre, G., Lin, S.-Y., Lee, H. Y., Schaefer, E., & Haimovich, B. (2004). The phosphorylation of vinculin on tyrosine residues 100 and 1065, mediated by SRC kinases, affects cell spreading. *Molecular biology of the cell, 15*(9), 4234-4247.

Ziegler, W. H., Liddington, R. C., & Critchley, D. R. (2006). The structure and regulation of vinculin. *Trends in cell biology, 16*(9), 453-460.

Zigrino, P., Kuhn, I., Bäuerle, T., Zamek, J., Fox, J. W., Neumann, S., . . . Mauch, C. (2009). Stromal expression of MMP-13 is required for melanoma invasion and metastasis. *Journal of Investigative Dermatology, 129*(11), 2686-2693.