THE ROLE OF WAVE (WASP-FAMILY-VERPROLIN HOMOLOGOUS PROTEINS) 1, 2 AND 3 ON CELLULAR MIGRATION AND INVASION OF COLORECTAL CANCER

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DOCTORATE OF MEDICINE

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Summary of Thesis

Colorectal carcinoma (CRC) is the second most common cause of cancer deaths in the UK. More than half of patients are diagnosed at a late stage with around a quarter of patients having metastases at diagnosis (stage IV). Approximately 50% of diagnosed patients will progress to metastatic disease. The metastatic spread of malignant cells to distant sites in the body accounts for the majority of cancer-related death and significantly decreases patient survival. Whilst cell migration is a physiologically important process, when uncontrolled, it can be a contributing factor to the metastatic phenotype. Actin polymerisation enables the dynamic restructuring of the cytoskeleton which is fundamental to cell migration and is stimulated by the Arp (actin-related protein) 2/3 protein complex which in turn is activated by members of the WAVE (WASP Verprolin homologous protein) family.

Clinico-pathological data was updated for a cohort of patients that had been involved in a previous colorectal tissue/carcinoma sampling study. The stored frozen tissue samples were analysed using histological and molecular biology techniques including conventional polymerase chain reaction, quantitative polymerase chain reaction, immunohistochemistry and *in vitro* gene knockdown studies. WAVE 1 and 3 expression was targeted separately in the RKO and CaCo2 cell lines utilising ribozyme transgene transfection to assess the effect knockdown on cell functions.

A high WAVE 2 expression level is associated with more aggressive and higher stage primary tumours and also a shorter overall survival time and disease free survival time. WAVE 3 expression is higher in colorectal tissues compared to normal tissues but otherwise showed no significant difference. WAVE 1 did not show an increase in expression levels compared to normal colorectal tissues. The *In vitro* functional assays revealed a significant reduction in cell invasion and motility following WAVE 3 knockdown in CaCo2 cells. Knockdown of WAVE 1 in RKO cells resulted in a significant reduction in invasion and a moderate reduction in motility that was not significant.

These results suggest WAVE1 and 3 proteins are involved in several metastatic traits and that WAVE 2 has significant correlation with higher stage disease. The data outlined here provides justification to further explore WAVE1, 2 and 3 as potential contributors of colorectal cancer progression.

Presentations/Publications

7th Chinese Clinical Oncology Annual Congress and 13th Cross-Strait Cancer Symposium 2012.
China National Convention Centre, Beijing, China
Presentation - Wiskott-Aldrich Verprolin-Homologous (WAVE) proteins in Colorectal Cancer 2012

7th Chinese Clinical Oncology Annual Congress and 13th Cross-Strait Cancer Symposium 2012
China National Convention Centre, Beijing, China
Poster - WAVE (WASP-family verprolin-homologous protein) expression in Colorectal Cancer 2012

Royal Society of Medicine, Coloproctology Section, Overseas Meeting, Geneva – Hospital Healthcare in the UK vs Switzerland
Hotel Royal, Geneva
Presentation - WAVE Expression in Colorectal Cancer 2012

The Society of Academic and Research Surgery (SARS) Annual Meeting 2012.
University of Nottingham Medical School
Presentation - WAVE 3 expression in human colorectal cancer 2012

Royal Society of Medicine, Surgery Section, Norman Tanner and MIA prize meeting 2011.
Royal Society of Medicine, London
Presentation - WAVE 1 expression in human colorectal cancer 2011

National Cancer Research Institute cancer conference, Liverpool, 2011
BT Convention Centre, Liverpool
Poster - WAVE 2 expression in Colorectal Cancer 2011

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Martin T, Toms A.M., et al,. ***The clinical and biological implications of N-WASP expression in human colorectal cancer****. Translational Gastrointestinal Cancer, 2012 April 1(1), pp 10-20*

Abbreviations

ABC - Avidin-Biotin staining Complex
Abi – ABL-interactor protein
ACF – Aberrant crypt foci
ADF – Actin-depolymerising factor
ADP – Adenosine diphosphate
AJ – Adherens junction
APC – Adenomatous polyposis coli
Arp 2/3 – Actin-related protein 2/3
ATP – Adenosine triphosphate
BRAF - v-Raf murine sarcoma viral oncogene homolog B
BSS – Balanced saline solution
cDNA – Complementary DNA
CIMP – CpG Island Methylator Phenotype
CIMP-H – High level CpG island methylator phenotype
CIMP-L – Low level CpG island methylator phenotype
CR16 – Corcticosteroids and regional expression-16
CRC – colorectal cancer/ carcinoma
CRIB – Cdc42 and Rac Interactive Binding site
CYFIP 2 – Cytoplasmic FMR1 interacting protein 2 (also known as Sra 1)
DAB - Diaminobenzidine
DEPC – Diethyl Pyrocarbonate
DMEM - Dulbecco's Modified Eagle's medium
DMSO – Dimethyl sulphoxide
ECM – Extracellular matrix
EDTA - Ethylenediaminetetraacetic acid
EGF – Epidermal growth factor
EGFR – Epidermal growth factor receptor
EMT – Epithelial-to-mesenchymal transition
F-actin – Filamentous actin
FAP – Familial adenomatous polyposis
G-actin – Globular actin
GAP – GTPase-activating protein
GBD – GTPase-binding domain
GDI – Guanine nucleotide dissociation inhibitor
GDP – Guanosine diphosphate
GEF – Guanine nucleotide exchange factor
GI – Gastrointestinal
GTP – Guanosine-5'-triphosphate
HCC – Hepatocellular carcinoma
HCT - Haematopoietic cell transplantation
HGF – Hepatocyte growth factor
HNPCC – Hereditary non-poyposis colorectal cancer
HSPC300 – Haematopoietic stem/progenitor cell protein 300 (also known as Brick 1)
IRSp53 – Insulin Receptor Substrate protein of 53kDa (Also known as BAIAP2)
ITP – Idiopathic thrombocytopaenic purpura
IXLT – Intermittent X-linked thrombocytopaenia
JMY – junction-mediating and regulatory protein
KRAS - V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

LCRMP-1 – Long isoform of collapsing response mediator protein-1
LIMK – LIM domain kinase
MET – Mesenchymal-to-epithelial transition
MGMT gene –O^6-methylguanine DNA methyltransferase gene
MGMT protein – O^6-alkylguanine DNA alkyltransferase
mRNA – messenger RNA
miR31 – Metastasis suppressor miRNA
miRNAs – MicroRNAs
MMP – Matrix-metalloproteinase
MMR – Mismatch repair
MSI – Microsatellite instability
MSI-L – Low level microsatellite instability
MSI-H – High level microsatellite instability
MSS – Microsatellite stable
MUD – Matched unrelated donor
Nap – NCK-associated protein
NCK – Non-catalytic kinase
NPF – Nucleation promoting factor
N-WASP – Neural – Wiskott-Aldrich Syndrome protein
PAK – P21-activated kinase
PBS – Phosphate buffered saline
PCR – polymerase chain reaction
PDGF – Platelet derived growth factor
PH – Pleckstrin homology
PI3K – Phosphoinositide 3-kinase
PIRI121 – 121F-specific p53 inducible RNA (Also known as CYFIP 2 and Sra 1)
PIP2 – Phosphatidylinositol [4,5] bisphosphate
PIP3 - Phosphatidylinositol [3,4,5] trisphosphate
PRD – Proline rich domain
Q-PCR – Quantitative polymerase chain reaction
Q-RT-PCR – Quantitative-RT-PCR
RbBMR - Ribozyme specific reverse primer
RBToP - Ribozyme specific forward primer
ROCK – Rho-associated protein kinase
RTK – Receptor Tyrosine Kinase
RT-PCR – Reverse transcription-Polymerase chain reaction
SCAR – Alternative name for WAVE
SH – Src Homology
SH3 – Src homology 3
SHD – SCAR homology domain
SSL – Sessile serrated lesion
TβRII – TGFβ II receptor
TGFβ – Transforming growth factor-beta
TIAM1 – T-lymphoma invasion and metastasis 1
TJ – Tight junction
TNFα – Tumour necrosis factor-alpha
TOCA 1 – Transducer of Cdc42-dependent actin assembly 1 (also known as FNBP1L)
TSA – Traditional serrated adenoma
TSG - Tumour suppressor gene

UK – United Kingdom
VCA – also known as WCA
VHD – Verprolin homology domain
WAS – Wiskott-Aldrich syndrome
WASH – WASP and SCAR Homologue
WASP – Wiskott-Aldrich Syndrome Protein
WASPs – WASP and N-WASP
WAVE – WASP-family verprolin-homologous protein
WCA – Also known as VCA. A conserved carboxy-terminal “catalytic core” consisting of a VHD (also known as the WH2 domain), a Central region; and an Acidic region
WH1 – WASP homology 1
WH2 – WASP homology 2
WHD – WAVE homology domain
WHAMM – WASP homologue associated with actin, membranes and microtubules
WICH/WIRE – WIP- and CR16-homologous protein/WIP-related protein
WIP – WASP-Interacting protein
Wnt - Wingless-related integration site
WRC – WAVE Regulatory Complex
XLN – X-linked neutropaenia
XLT – X-linked thrombocytopaenia

CHAPTER 1

INTRODUCTION TO COLORECTAL CARCINOMA

1.1 Introduction

Colorectal cancer (CRC) is a term used by the medical profession to refer to cancers, specifically carcinomas, of the colon and rectum. Metastasis is currently not completely understood but is thought to be a complex multi-stage process involving cell invasion, cell migration and changes in cell adhesive properties, ultimately leading to distant spread of malignant cells and the formation of secondary tumour deposits. Within this thesis, the author wishes to examine the relationship between the expression of WASP (Wiskott-Aldrich syndrome protein) family proteins, more specifically the WAVE (WASP-family verprolin-homologous) proteins, and the disease stage and subsequent prognosis of the patients concerned. The author also wishes to examine the relationship between expression of WAVE proteins and the role this expression may play in the aggressive behaviour of colorectal cancer cells that ultimately lead to the metastatic process. This thesis is a continuation of findings in the host centre that WAVE proteins can play a role in the aggressive behaviour of cancer cells.

1.2 Definition

In spite of the significant knowledge available regarding the anatomy of the colon and rectum there is still dispute over the accurate definitions of carcinomas of these regions. Although the colon consists of the large bowel proximal to the rectum, the definition of the rectum differs (Steele, 2010). Anatomical description of the rectum varies from the surgical description as does the definitions used by various countries (Enker and Paty, 1993).

Anatomically, the proximal end of the rectum is defined as the point where the sigmoid mesocolon ends or as the segment of large bowel level with the third sacral vertebrae (Williams et al, 1980). Surgically, the rectum is seen to start where the two antemesenteric taenia on the sigmoid colon fuse together (Phillips, 2010).

In the United Kingdom, rectal cancer is defined as a tumour within 15cm of the anal verge using a rigid sigmoidoscope (The Association of Coloproctology of Great Britain and Ireland, 2007; Phillips R. 2010). Whereas, in the USA, within 11 to 12cm is preferred (Enker and Paty, 1993).

The distinctions are important as the modes of treatment differ depending on the tumour location and CRC surgery outcome data is impossible to compare unless uniform definitions are utilised by all. This problem has yet to be tackled by international consensus.

1.3 Incidence and Mortality

1.3.1 Incidence

Colorectal cancer (CRC) is the third most common malignancy worldwide, with more than an estimated 1.36 million new cases of CRC being diagnosed in 2012, with incidence rates varying across the world. (Cancer Research UK)

CRC incidence rates are highest in Australia/New Zealand and lowest in Western Africa. This partly reflects varying data quality worldwide and may reflect different prevalence of risk factors, use of screening, and diagnostic methods. (Cancer Research UK)

CRC is the second most common malignancy in Europe, with around 447,000 new cases diagnosed in 2012. United Kingdom (UK) CRC incidence rates are estimated to be the 20th highest in males in Europe, and 17th highest in females. (Cancer Research UK)

CRC is one of the most common malignancies in the UK, after breast, lung and prostate cancer (2012), with around 40,000 new cases registered each year. (Cancer Research UK; NICE 2011)

CRC incidence is strongly related to age, with the highest incidence rates occurring in older patients (Figure 1.1). In the UK between 2010 and 2012, 95% of CRC cases were diagnosed in those aged 50 and over, with approximately 20% of cases occurring between the ages of 50 and 64, 32% of cases arising in people aged between 65 and 74 and 43% of cases being diagnosed in those aged 75 years and over. (Cancer Research UK; NICE 2011)

More than half of patients are diagnosed at a late stage (55% diagnosed at stage III or IV), compared to an early stage (45% diagnosed at stage I or II), with around a quarter (26%) of patients having metastases (distant spread) at diagnosis (stage IV). (Cancer research UK)

Approximately 50% of diagnosed patients will progress to metastatic disease (Arvelo et al., 2015)

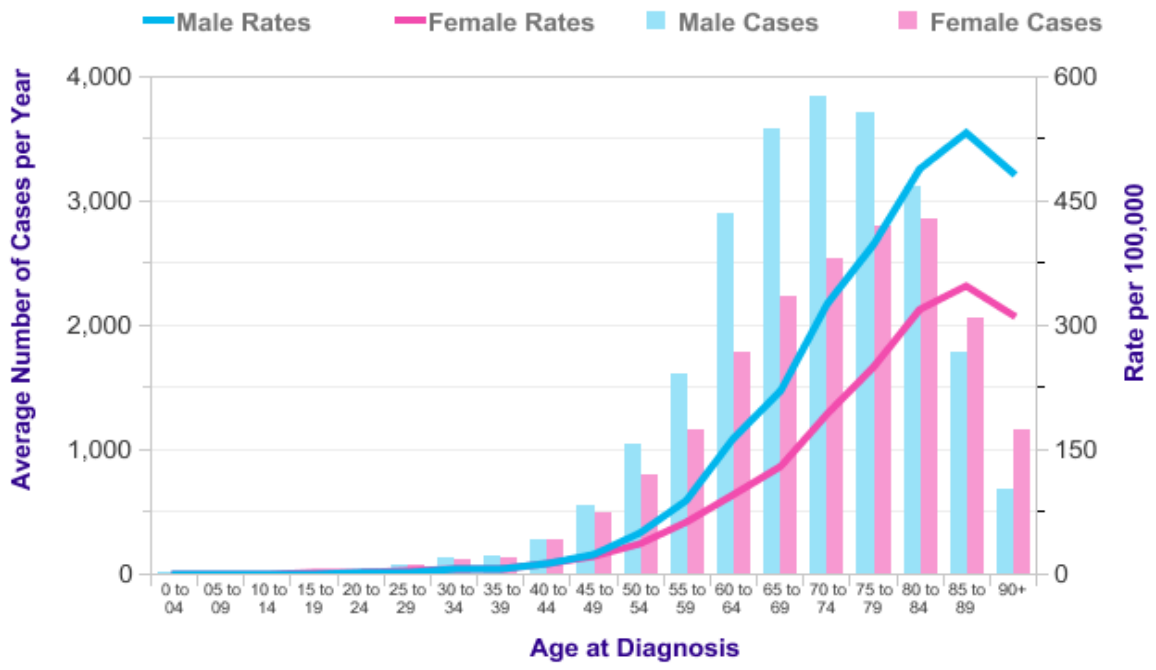


Figure 1.1 - Relationship between colorectal cancer incidence and age (Figure taken from Cancer Research UK, 2014)

1.3.2 Mortality

CRC was the second most common cause of cancer related deaths in the UK in 2012. In that year 16,187 people died from the disease in the UK, most with metastatic malignancy (Cancer Research UK; Office for National Statistics, 2014).

Mortality from CRC is higher than that for both prostate and breast having a five year survival of 59% in men and 58% in women (Cancer Research UK; Office for National Statistics, 2014).

1.4 Natural History of Colorectal Carcinoma

1.4.1 Location

Colorectal carcinomas arise throughout the large bowel and rectum with variations in frequency depending on the locations. In the United Kingdom there is also a slight variation between the sexes.

In men, carcinomas are most frequently found in the rectum (31.5%) followed by the sigmoid colon (23.1%) and then the caecum and ascending colon (12.2% and 7.3%). In women the rectum (23.1%) is followed by the sigmoid colon (20.4%) and then the caecum and ascending colon (17.2% and 9.8%). (Office for National Statistics, 2014; Welsh Cancer Intelligence and Surveillance Unit, 2014; ISD Scotland, 2014; Northern Ireland Cancer Registry, 2014)

As can be seen, in the caecum and ascending colon, the proportions are higher in females than males. There are no further marked variations between the sexes in other parts of the bowel.

In men, approximately 60% of colorectal carcinomas occur on the left side of the large bowel, as do just under 60% in women.

1.4.2 Aetiology

Colorectal cancer is a multifactorial disease process. No single risk factor accounts for the majority of cases of colorectal cancer.

Increasing age and male sex are the main sociodemographic risk factors.

A number of further risk factors, which are not mutually exclusive and can interact, have been identified and established in epidemiological studies.

The risk is found to be strongest in those with a family history of colorectal cancer (Taylor et al., 2010), particularly those with first-degree relatives affected, those with multiple affected relatives or relatives diagnosed at a young age. People with inflammatory bowel disease such as ulcerative colitis or crohn's disease are also at higher risk of CRC. In these patients the risk for developing colorectal cancer increases with the duration of disease and the greater amount of colon involved (Jess et al., 2012).

The other risk factors, which are seen frequently and are potentially amenable to change are smoking (Liang et al., 2009), excessive alcohol intake (Fedirko et al., 2011), high consumption of red and processed meat (Chan et al., 2011), obesity (Ma et al., 2013) and diabetes (Jiang et al., 2011). These factors account for a relatively large amount of the disease burden within the population despite the relative risk being less than is seen in those with a family history of CRC or with inflammatory bowel disease (Brenner, 2014).

It is also thought that there may be an increased risk of colorectal cancer associated with infection by Helicobacter pylori, fusobacterium species and other possible infectious agents (Sonnenberg et al., 2013; Kostic et al., 2012; Boleij et al., 2011).

Recognised preventive factors include physical activity (Boyle et al., 2012), use of hormone replacement therapy (Lin et al., 2012) and aspirin (Bosetti et al., 2012; Rothwell et al., 2011). Large bowel endoscopy with removal of precancerous lesions (adenomas) has been found to have the greatest risk reduction (Brenner, 2014; 2011).

There is some data to suggest a weak protective effect of diets rich in fruit, vegetables, cereal fibre and whole grains (Aune et al., 2011; 2012), dairy products (Aune, 2012) or fish (Wu et al., 2012) and possible statin therapy (Lochhead et al., 2013) but it is not as consistent as other data.

Colorectal cancer has a considerable genetic correlation, and up to 35% of colorectal cancer risk may be attributable to this (Lichtenstein, 2000; Arvelo, 2015).

The current estimate is that 15–30% of colorectal cancers may have a major hereditary component, given the occurrence of colorectal cancer in first- or second-degree relatives. However, only about a quarter of these familial cases (i.e. about 3-5% of all colorectal cancers) occur in a setting with a family history and/or clinical features that indicate a highly penetrant cancer syndrome that predisposes to colorectal cancer and that can be attributable to a known hereditary form. The large proportion of these highly penetrant cases are due to the hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome) syndromes, which pose about a 75% lifetime risk for developing colorectal cancer and are due to an inherited mutation in one of the mismatch repair genes. Another significant subset is associated with familial adenomatous polyposis (FAP) and closely related variant syndromes, where an hereditary mutation of the adenomatous polyposis coli (APC) gene is the cause and in which affected patients carry an almost 100% risk of developing colon cancer

by the age of 40 years (Walsh, 2015). A few other rare syndromes constitute the remainder of such highly penetrant cases.

Studying the genetic and molecular pathogenesis of the hereditary forms of colorectal cancer has proven highly valuable to understanding the genetic and molecular pathogenesis of sporadic colorectal carcinomas and has been shown to have an impact on the prognosis and treatment response of patients (Sadanandam et al., 2013; De Sousa et al., 2013).

1.4.3 Genetics of Colorectal Carcinoma

The current understanding of colorectal carcinoma and the genetics underpinning how it arises divides it into a variety of subtypes. Approximately 95% of colorectal carcinomas arise sporadically (i.e. they develop due to repeated environmental insults over the years that cause somatic gene mutations that predispose to the development of growths (adenomas) arising from the epithelial lining of the large bowel. Over time, with further somatic mutations, these adenomas will develop into colorectal carcinomas). The remaining 5% arise from the inheritable disorders of which FAP and Lynch syndrome are the main contributors (Brenner, 2014).

1.4.3.1 Adenoma-Carcinoma sequence

The colorectal polyp is considered to be the precursor lesion for most malignancies of the large bowel. These polyps take the form of conventional adenomas or a variety of lesions with serrated morphology, some of which take a sessile form.

Colorectal cancer often develops over more than ten years, and dysplastic adenomas are the most common form of premalignant precursor lesions (Jass, 2007). Most colorectal carcinomas are presumed to arise in these premalignant adenomas the majority of which are amenable to endoscopic resection.

The APC gene plays a prominent role in the development of sporadic colorectal adenomas and subsequently carcinomas. Approximately 70-80% of sporadic colorectal adenomas and carcinomas have somatic mutations that inactivate both wildtype alleles of APC. It appears that these somatic APC mutations are an early and perhaps rate limiting event in the development of most adenomas. They are

found throughout the spectrum of lesions, from early microscopic adenomas up to and including large carcinomas (Brenner, 2014; Fearon, 2011).

The APC gene encodes for the APC protein which may be involved in the regulation of cell-cell adhesion, cell migration, chromosomal segregation and apoptosis in the colonic crypts (Polakis, 2006; Aoki, 2007; Brocardo, 2009). Although APC may have a number of cellular functions, the best established role for APC in the colorectal cancer process is as a major binding partner and regulator of the β-catenin protein in the β-catenin-dependent Wnt signalling pathway (Polakis, 2006; Aoki, 2007). In the absence of Wnt ligand signalling, wildtype APC would normally be involved in the process by which β-Catenin is eventually degraded. However, with the somatic mutations inactivating both of the APC alleles, this coordinated process of phosphorylation and destruction no longer occurs and there is a build-up of β-Catenin within the cell cytoplasm. β-catenin also functions as a transcriptional coactivator and so migrates to the nucleus, activating the expression of many different genes (Mossiman et al., 2009). The transcriptional program induced by β-catenin once in the nucleus resembles the transcriptional program in stem cells at the base of the colonic crypts thus establishing a crypt progenitor phenotype. It also is involved in the spatial organization and migratory pattern of the cells in the continuous renewal of crypts (Van de Wetering et al., 2002; Batlle et al., 2002). All of this goes some way to explain the development of adenomas.

The adenoma-carcinoma sequence is driven by the progressive accumulation of further somatic mutations within the adenoma and subsequent carcinoma, such as activation of the KRAS oncogene and inactivating mutations of the p53 tumour suppressor gene (TSG).

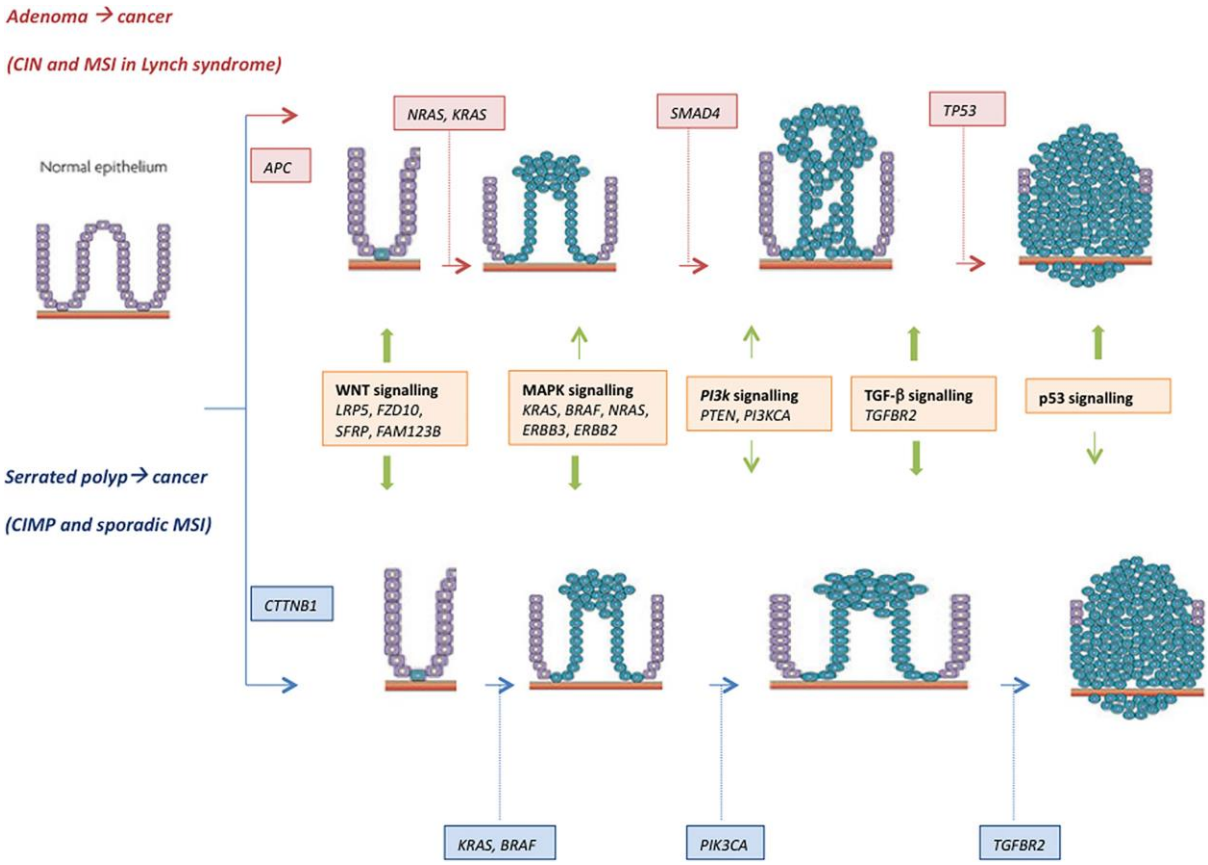


Figure 1.2 Schematic diagram of the polyp to colorectal cancer sequences. Currently, two discrete normal colon to colorectal cancer sequences have been identified. Both sequences involve the progression of normal colon epithelial cells to aberrant crypt foci (ACF), followed by early and advanced polyps with subsequent progression to early cancer and then advanced cancer. The classic or traditional pathway is the pathway originally identified and involves the development of tubular adenomas that can progress to adenocarcinomas. An alternate pathway that involves serrated polyps and their progression to serrated colorectal cancer has been described in the last 5–10 years. The genes mutated or epigenetically altered are indicated for each pathway. Some genes are shared between the two pathways and others are unique (ie, BRAF mutations and CpG Island Methylator Phenotype (CIMP) only in the serrated pathway). The signalling pathways deregulated during the progression sequence are also shown with the width of the arrow reflecting the significance of the signalling pathway in tumour formation. (Image taken from Dickinson et al., 2015.)

1.4.3.2 Microsatellite instabilities

The majority of colorectal carcinomas, 85%, show microsatellite stable (MSS) or low level microsatellite instability (MSI-L) phenotypes, but are often accompanied by chromosomal instability (i.e. changes in the number of chromosomes and structural changes of the chromosomes). The large majority of these cancers develop sporadically through the classic adenoma-carcinoma pathway. Approximately 1% develop because of the inherited familial adenomatous polyposis (FAP) syndrome. (Brenner, 2014; Tomlinson, 2015; Frayling et al., 2015; Walsh, 2015)

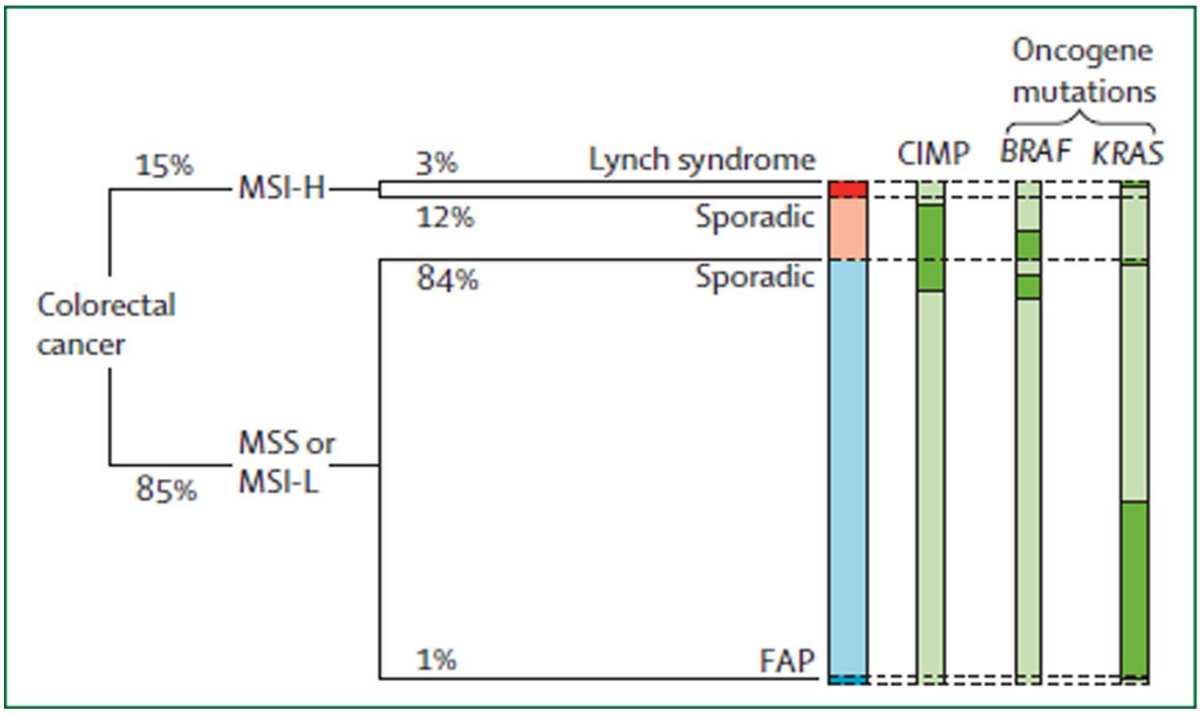


Figure 1.3*:* Molecular subtypes of colorectal cancer

Most colorectal cancers (85%, light blue and dark blue) show MSS or MSI-L phenotype, but are characterised by chromosomal changes. Most of these cancers develop through the classic adenoma–carcinoma pathway, but about 1% develop with inherited syndrome FAP (dark blue). About 15% of colorectal cancers (red and pink) have the MSI-H phenotype as a result of DNA mismatch repair defi ciency. About 3% of colorectal cancers have MSI-H in context of the inherited Lynch syndrome (red), whereas 12% develop as sporadic tumours (pink), with sessile serrated adenomas as a typical precursor lesion. The distribution of typical molecular changes including the CIMP and mutations of the *BRAF* or *KRAS* oncogenes are sketched in green. Dark green is the proportion of

positive or mutant changes and light green is the proportion of negative or wildtype changes. MSI-H=high-level microsatellite instability in relation to the phenotypes in the first bar. CIMP=CpG island methylator phenotype. MSS=microsatellite-stable. MSI-L=low-level microsatellite instability. FAP=familial adenomatous polyposis. (Image taken from Brenner et al., 2014)

Focus is also on a small number of oncogenes and tumour suppressor genes (TSGs), most notably APC, KRAS, BRAF and p53. These are mutated in a large number of colorectal cancers. The mutations are of two types: changes that lead to new or increased function of oncogenes and changes that lead to loss of function of TSGs. The transformation of genes into their oncogenic variants can be as a result of specific point mutations or rearrangements that alter the gene structure and function or from chromosome rearrangements or amplifications that disrupt gene expression. So far, only somatic oncogene mutations have been found in colorectal carcinomas. TSG inactivation can result from localized mutations, complete loss of the gene, or epigenetic alterations that interfere with gene expression. The vast majority of TSG defects in CRC are somatic (Fearon, 2011)

The other subtype of colorectal carcinoma are those with the presence of microsatellite instabilities within the genome. Microsatellites are regions where a short DNA sequence (up to five nucleotides) is repeated. There are large numbers of such sequences in the human genome, the majority in non-coding DNA. Instability of these regions arise when the cellular DNA repair mechanisms, that usually catch problems during DNA replication, are deficient (Tomlinson, 2015; Frayling et al., 2015; Walsh., 2015).

Base-pair mismatches occurring during DNA replication are normally repaired by the mismatch repair (MMR) proteins which are expressed by the MMR genes (Peltomaki

et al., 1993; Peltomaki, 2001). These are tumour suppressor genes and their role is to correct errors in base-pair matching during replication of DNA or to initiate apoptosis when DNA damage is beyond repair. In tumours with a deficiency of the MMR proteins this mechanism fails and microsatellites become mutated, resulting in a change in the number of sequence repeats and hence the length of the microsatellite, resulting in microsatellite instability (MSI). Therefore, a distinguishing feature of tumours with defective MMR genes is the presence of microsatellite instability (Brenner, 2014; Tomlinson, 2015; Frayling et al., 2015; Walsh, 2015).

Approximately 15% of colorectal cancers have a high-level microsatellite instability (MSI-H) phenotype as a result of DNA MMR deficiency. About 3% of colorectal cancers have MSI-H due to the inherited Lynch syndrome where a defective MMR gene copy is inherited, whereas the remaining 12% develop as sporadic tumours with sessile serrated adenomas as a typical precursor lesion. Within this MSI-H subgroup of colorectal carcinomas, a distinguishing test can be to look for a BRAF oncogene mutation, as the presence of this is almost exclusively restricted to sporadic MSI-H colorectal carcinomas (Brenner, 2014; Parsons et al., 2012). These MSI-H sporadic tumours occur in older patients as a consequence of inactivation of MMR genes by promoter methylation, which is not related to any inherited factor (Brenner, 2014; Tomlinson, 2015; Frayling et al., 2015; Walsh, 2015). Clinically, MSI-H malignancies tend to occur in the proximal colon, are observable in patients younger than 50 years of age (hereditary form) or in older patients (sporadic form), can have synchronous malignancies and large local tumours are only rarely accompanied by metastases (Jung et al., 2012).

The MSI-H phenotype is associated with a more vigorous anti-tumoural immune response identified by a high density of tumour-infiltrating lymphocytes (Shia et al.,

2003; Dolcetti, 1999). It is thought to be related to microsatellite instabilities leading to frameshift mutations and the production of altered proteins which are foreign to the immune system and seen as tumour antigens (Schwitalle et al, 2008). This vigourous immune response could be contributory to the improved prognosis of those patients with MSI-H colorectal carcinoma.

Together with DNA-methylation and chromosomal instability, these mutations can influence a number of phenotypic traits.

1.4.3.3 Lynch Syndrome (Hereditary non-polyposis colorectal carcinoma)

Lynch syndrome is an autosomal dominant inherited syndrome associated with a high risk of gastrointestinal (GI) tract malignancies and also malignancies in other organ systems (Lynch and Krush, 1967). Lynch syndrome accounts for approximately 3% (2.8% - 3.3%) of colorectal cancers. Patients with Lynch syndrome inherit a defective mismatch repair (MMR) gene copy from one parent. Tumorigenesis is triggered when the solitary normal gene in a cell suffers a somatic hit and becomes mutated or lost. Once this happens the defective MMR mechanism no longer repairs the DNA mismatches within that cell, resulting in an accumulation of mutations in a number of other genes particularly TSGs and oncogenes. There are at least seven MMR genes identified which function together as a complex to eliminate DNA base pair errors. The commonest genes affected by mutations are MLH1, MSH2, MSH6 and PMS2. Of these, mutations in MLH1 and MSH2 account for the vast majority of mutations in Lynch syndrome (Walsh, 2015; Tiwari et al., 2016).

1.4.3.4 Familial adenomatous polyposis (FAP)

Familial adenomatous polyposis is an autosomal dominant inherited syndrome characterised by the presence of adenomatous polyps in the colon and rectum with the inevitable development of colorectal carcinoma if not treated. Patients with FAP inherit a defective APC gene copy from one parent (Bodmer et al., 1987). The main function of the expressed APC protein is to provide a scaffold for the phosphorylation of the Wnt pathway effector, β-catenin which is subsequently degraded. APC mutations result in abnormal function and disrupt this scaffold (Fearnhead et al., 2001). APC is a TSG and FAP polyps usually start to develop after the solitary wildtype allele of APC suffers a somatic hit that inactivates it. Classical FAP is a disease of 100's-1000's of adenomatous polyps throughout the colorectum, although sufferers of the attenuated variant of FAP develop fewer polyps (10's-100's). This change in polyp burden is associated with the location of the APC gene mutation (Nieuwenhuis et al., 2007; Tomlinson, 2015). As well as colorectal adenomas and carcinomas, FAP sufferers are also at risk of developing upper gastrointestinal polyps and carcinomas, desmoids and extra-gastrointestinal disease and malignancies.

1.4.3.5 Serrated pathway (SP): Serrated lesions – Serrated adenocarcinoma

Serrated lesions of the colorectum are the precursors of approximately 30% of colorectal cancers (Rex et al., 2012; O'Brien et al., 2015; Bettington et al., 2013).

Precursor lesions consist of sessile serrated lesions (SSLs), SSLs with dysplasia and traditional serrated adenomas (TSAs).

SSLs are usually found in the proximal colon, but can occur throughout the length of the large bowel. They are usually sessile in nature and may be over 10 mm in diameter, although, around one-third of SSLs are 5 mm or less across (Bateman, 2014). The sessile morphology of these proximal lesions makes them easier to overlook and they tend to have ill-defined borders, which can lead to incomplete resection at endoscopy.

TSAs are uncommon lesions, occurring most frequently in the left colon. High-grade dysplasia can arise within them and progression to carcinoma may take place, showing a serrated appearance.

Precursor serrated lesions can progress to colorectal carcinoma and the term 'serrated adenocarcinoma' has been used to describe these tumours. Cancers arising in serrated lesions show a diverse range of genetic profiles and biological behaviours. The genetic alterations occurring during progression to colorectal carcinoma along the 'serrated pathway' are distinct to those occurring within the classical 'adenoma-carcinoma sequence', and there is evidence that this progression occurs more quickly within the 'serrated pathway' (Bettington et al., 2013)

A small number of serrated adenocarcinomas arise within the caecum and ascending colon and are thought to develop from SSLs. Sessile serrated lesions

may show dysplasia or not. Those lesions containing a focus of dysplasia tend to show early BRAF mutation and may develop loss of the MMR protein hMLH-1 secondary to inactivation of the hMLH-1 gene through DNA methylation (the “CpG island methylator phenotype” - CIMP) which would subsequently cause microsatellite instability and show the MSI-H phenotype. (Bateman, 2014). SSLs in which hMLH-1 expression is not lost may alternatively show p16 and MGMT loss. The resulting CRC again contain BRAF mutations and exhibit CIMP-H but are MSS.

The CIMP is a state in which extensive methylation (a physiological process important in the regulation of gene activity) of the promoter sequences of genes occurs. CpG islands are pairs of cytosine and guanine nucleotides that are present mainly within the promoter regions of genes such as the DNA MMR enzyme-encoding gene hMLH-1. When methylation of these CpG islands occurs, this results in inactivation of the corresponding gene. Methylation may be present at either a low (CIMP-L) or high (CIMP-H) level (Bateman, 2014).

The BRAF gene encodes a protein called B-Raf, which is a member of the Raf kinase family of phosphorylating enzymes that are involved in the control of cell division and differentiation. Acquired BRAF mutations have been identified in many human cancers, including malignant melanoma and carcinomas of the lung and colorectum. These mutations result in BRAF acting as an oncogene. The V600E mutation is the most common (90% of BRAF mutations) (Pakneshan et al,. 2013).

The p16 gene is a tumour suppressor gene encoding a protein (cyclin-dependent kinase inhibitor 2A) that is involved in cell cycle control and that may be mutated in several different cancers. The O6-methylguanine DNA methyltransferase (MGMT) gene encodes the O6-alkylguanine DNA alkyltransferase (MGMT) protein that is

involved in DNA repair and that can be inactivated via hypermethylation of its promoter sequence (Bateman, 2014).

The majority of serrated adenocarcinomas arise in the distal colon or rectum. These tumours are believed to develop from TSAs. TSAs are particularly associated with early KRAS mutations and Wnt abnormalities. The resulting serrated adenocarcinoma contain KRAS mutations, exhibit CIMP-L and are MSS or MSI-L (Bateman, 2014).

The KRAS gene encodes the KRAS protein, which is a member of the Ras family of proteins that are important for signalling in normal cells. Mutations within the KRAS gene are commonly seen in carcinomas of the pancreas, lung and colorectum and result in KRAS acting as an oncogene. In colorectal carcinoma, the presence of a KRAS mutation is a predictor of a poor response to EGFR inhibitors such as cetuximab (Bateman, 2014).

1.4.4 Invasion and Metastases

The ability of a tumour to invade neighbouring tissue is a pre-requisite for the development of metastases. Colorectal cancer tends to metastasise to the liver and lungs.

Direct invasion of a tumour by the progression of its margin or by the movement of individual cells through a tissue is important in the development of distant and regional metastases. In order for a tumour to metastasise to a distant organ or lymph node it must first reach a vascular or lymphatic channel. To achieve this, malignant cells must penetrate the basement membrane and travel through the extracellular matrix. A part of this process is aided by the family of endopeptidases known as matrix-metalloproteinases (MMPs). MMPs are a group of structurally related proteins which are commonly involved in normal tissue remodelling during growth and wound healing or repair (Boedefeld et al., 2003). MMPs exert a proteolytic activity and have the ability to rupture intercellular junctions and break down the molecules of the extracellular and adhesion matrices.

Over-expression of MMP's and induction of expression in other cell types of MMP's are important processes in the development of invasiveness in neoplastic disorders. A number of associations have been found between colorectal cancer and the different MMPs. MMP-1 is associated with a worse prognosis as it favours haematogenous metastasis. MMP-2 is related to tumour invasion and it has a greater expression on the invasive front of a tumour (Arvelo et al., 2015). MMP-7 is related to invasion and metastasis in colorectal cancer also.

Cell motility and migration is another crucial factor in the development of direct invasion of a tumour.

Haematogenous and lymphatogenous metastases occurs when tumour cells come across blood or lymphatic vessels, either new or original, and invade into them. Embolisation of individual cells or small groups of cells is then possible. All that is then required is an appropriate organ and micro-environment in which these tumour emboli can begin to form a micro-metastasis before developing into a more substantial deposit. Colorectal carcinomas have the ability to stimulate angiogenesis in and around the edge of the tumour which would aide this process immeasurably.

Despite significant improvements in diagnostic procedures, more than 50% of patients with colorectal cancer have liver metastases either at presentation or will go on to develop it (Bramhall et al., 2003). In many parts of the world, most cancer-related deaths are still due to metastases that are resistant to conventional therapy (Kassahun, 2015).

Surgery for metastases confined to the liver or lung can be curative when carried out by specialists with experience of this type of work. Although such resection is only appropriate for a minority of patients, it can increase five-year survival rates from close to zero to over 30%. Pre-operative chemotherapy can produce a similar increase in survival rates in selected patients whose liver metastases are initially too extensive for surgery, by shrinking the tumour so that curative resection becomes possible (NICE guidelines 2004)

Gaining a further understanding of the mechanisms by which malignant colorectal cancer cells are able to metastasise to locations within the body which are distant

from the primary tumour site could help point to the development of therapeutic agents which may help prevent further metastases from developing in those with Stage IV disease, and prevent distant metastases from developing in those with Stage III and below colorectal cancer.

1.5 Staging

Staging systems are utilised by clinicians to determine the extent of spread of disease. This includes local invasion to adjacent structures, lymph node metastases and distant metastases. It aides clinicians together with patients to make the best informed decisions on the appropriate course of clinical management. Furthermore, staging also allows comparisons between different therapeutic modalities in order to assess novel and improved techniques for management of cancers and to compare outcomes in a like for like manner.

There are two main classification systems in use for the staging of colorectal carcinoma. The oldest system is the Dukes classification (Figure 1.4) (Dukes, 1932; Dukes, 1950). The most widespread in use and internationally recognised is the TNM staging system (Figures 1.5 & 1.6) (UICC [International Union Against Cancer], 2002; Wittekind et al., 2004).

The Dukes staging system requires pathological assessment of the surgical specimen and therefore cannot be used for pre-operative disease staging or for the staging of advanced disease not suitable for operative management. In these situations, the TNM system is preferred.

Stage	Features	5-year survival
A	Tumor confined to the mucosa	90–95%
B1	Tumor growth into muscularis propria	75–80%
B2	Tumor growth through muscularis propria and serosa (full thickness)	60%
C1	Tumor spread to 1–4 regional lymph nodes	25–30%
C2	Tumor spread to more than 4 regional lymph nodes	
D	Distant metastases (liver, lung, bones)	<1%

Figure 1.4 Dukes Classification

Definitions

Primary Tumor (T)

- **TX** Primary tumor cannot be assessed
- **T0** No evidence of primary tumor
- **Tis** Carcinoma in situ: intraepithelial or invasion of lamina propria[1]
- **T1** Tumor invades submucosa
- **T2** Tumor invades muscularis propria
- **T3** Tumor invades through the muscularis propria into pericolorectal tissues
- **T4a** Tumor penetrates to the surface of the visceral peritoneum[2]
- **T4b** Tumor directly invades or is adherent to other organs or structures[2,3]

Regional Lymph Nodes (N)[4]

- **NX** Regional lymph nodes cannot be assessed
- **N0** No regional lymph node metastasis
- **N1** Metastasis in 1–3 regional lymph nodes
- **N1a** Metastasis in one regional lymph node
- **N1b** Metastasis in 2–3 regional lymph nodes
- **N1c** Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
- **N2** Metastasis in 4 or more regional lymph nodes
- **N2a** Metastasis in 4–6 regional lymph nodes
- **N2b** Metastasis in 7 or more regional lymph nodes

Distant Metastasis (M)

- **M0** No distant metastasis
- **M1** Distant metastasis
- **M1a** Metastasis confined to one organ or site (for example, liver, lung, ovary, nonregional node)
- **M1b** Metastases in more than one organ/site or the peritoneum

Figure 1.5 TNM staging system with definition (American joint committee on colon cancer, 7th Ed)

Clinical Stage	T	N	M
0	Tis	N0	M0
I	T1-2	N0	M0
IIA	T3	N0	M0
IIB	T4	N0	M0
IIIA	T1-T2	N1	M0
IIIB	T3-T4	N1	M0
IIIC	Any T	N2	M0
IV	Any T	Any N	M1

Figure 1.6 TNM overall stage

CHAPTER 2

INTRODUCTION TO THE WISKOTT ALDRICH SYRNDROME PROTEIN (WASP) FAMILY

2.1 Introduction

Cell migration is required for many biological processes, such as embryonic morphogenesis, immune surveillance and tissue repair and regeneration. Aberrant regulation of cell migration drives progression of many diseases, including cancer invasion and metastasis. Cancer cells possess a broad spectrum of migration and invasion mechanisms that include both individual and collective cell-migration strategies. Understanding the mechanisms of cell migration is critical for our understanding of the pathology of cancer during metastasis. This greater understanding can help direct further investigation and potential therapeutic approaches that could inhibit the metastatic process.

2.2 Cell Migration

2.2.1 The role of Actin Filaments in cell migration

Among eukaryotic cells, actin is one of the most conserved proteins with close to 90% identity between species as diverse as yeast and mammals (Campellone et al., 2010; Skarp et al., 2010; Erickson 2007; Lowe et al., 2009). The vast majority of eukaryotes have genes for actin and most have genes for myosin motor proteins that generate forces on actin filaments. They were initially discovered in the 1940's in skeletal muscle and later research identified how myosin produced force from the hydrolysis of adenosine triphosphate (ATP) (Pollard et al., 2009). Actin is the most abundant protein in eukaryotic cells. A 42 kDa monomeric ATP-binding protein, globular actin (G-actin), can undergo spontaneous polymerisation into long, stable filamentous actin (F-actin) (Figure 2.1). The double helical arrangement of globular subunits all arranged head to tail give the filament a molecular polarity (Campellone et al., 2010). Regulation of actin polymerisation is essential as cell cytoplasm contains high concentrations of G-actin that could potentially self assemble (Pollard 2007).

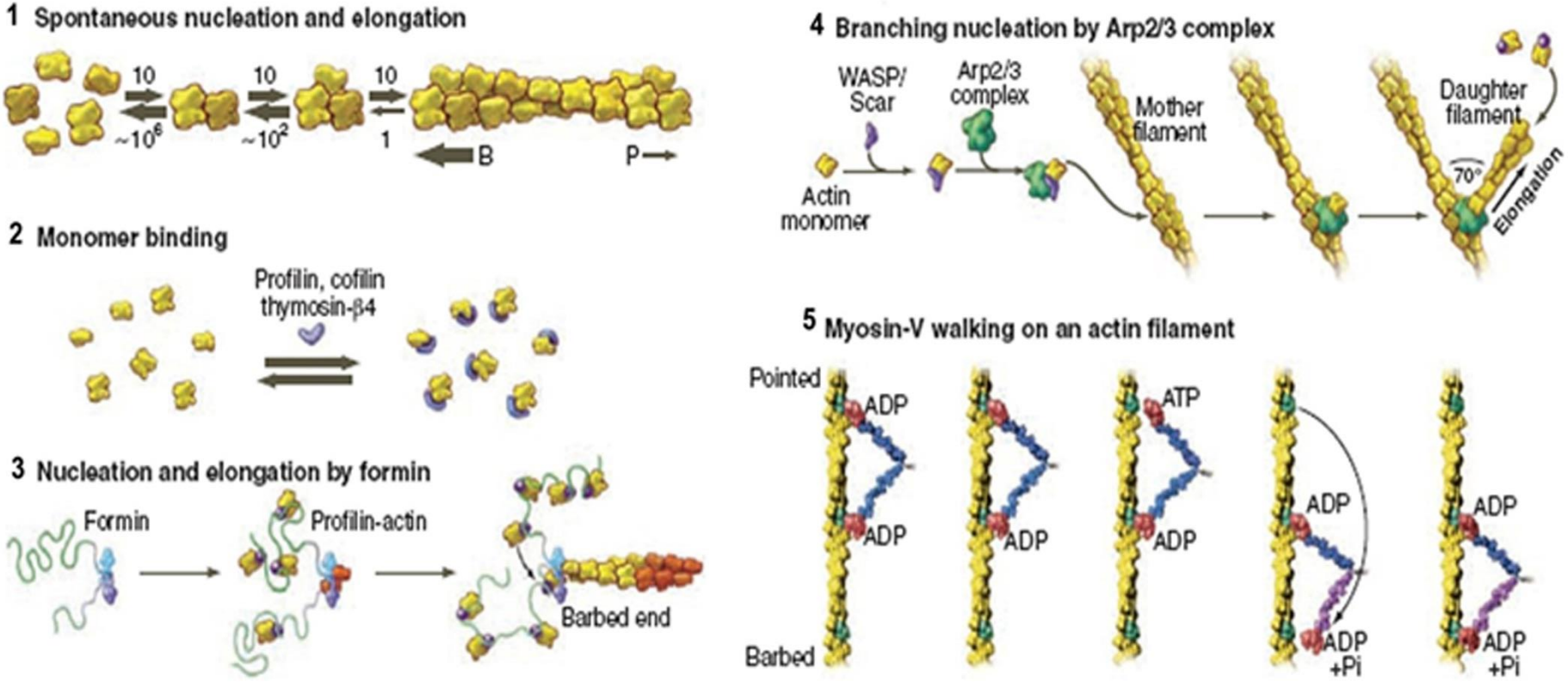


Figure 2.1. Structures of Actin and diagrams of reactions. (1) Spontaneous nucleation and elongation. Dimers and trimers are unstable. Longer polymers grow rapidly at the barbed end. (2) Actin monomer binding proteins. Thymosin-beta4 blocks all assembly reactions; profiling promotes nucleotide exchange and inhibits pointed-end elongation and nucleation but not barbed-end elongation; cofilin inhibits nucleotide exchange and promotes nucleation. (3) Nucleation and elongation by formins. Formins initiate polymerisation from free actin monomers and remain associated with the growing barbed end. Profilin-actin binds to formin and transfers actin onto the barbed end of the filament. (4) Nucleation by Arp2/3 complex. Nucleation promoting factors such as WASP and WAVE bind an actin monomer and the Arp2/3 complex. Binding to the side of a filament completes activation, and the barbed end of the daughter filament grows from Arp2/3 complex. (5) Myosin motors, such as myosin V, use cycles of ATP hydrolysis to walk along actin filaments, generally toward the barbed end. (Adapted from images in Pollard et al., 2009)

Actin filaments provide cells with structural support and contribute to biological processes such as sensing environmental forces, internalizing membrane vesicles and dividing cells in two. A major role played by actin filaments is in that of cellular migration. Directional motility is a fundamental cellular process essential for embryonic development, wound healing, immune responses and development of tissues. Cancer cells also utilise this function to invade and metastasise through the body (Pollard et al., 2009). Actin filaments have a fast growing barbed end and a slower growing pointed end, differentiated by their biochemical characteristics. The barbed end is favoured for rapid growth and actin filaments in cells are strongly oriented with the barbed ends directed towards the cell membrane (Pollard et al., 2003). With both the aforementioned rapid growth and orientation, these filaments produce protrusions of the cell membrane, the first step in cell locomotion (Millard et al., 2004; Pollard et al., 2009; Pollard et al., 2003). Cellular motility involves a cycle of four steps: protrusion of the leading edge, adhesion to the substratum, retraction of the rear and de-adhesion (Figure 2.2) (Wong et al., 2010).

The terms “pointed” and “barbed” come from the appearance of the filament structure as seen in electron micrographs. These show the arrow-like appearance of the actin filaments adorned with the motor domain of myosin (Millard et al., 2004; Campellone et al., 2010). Myosin motor proteins interact with actin filaments. This produces two types of movement. First, myosin generates force between actin filaments, producing contraction that can pinch dividing cells in two, it enables a change in cellular shape to form tissues and during migration it retracts the rear of cells, one of the essential steps in the cell motility cycle (Figure 2.1 and 2.2). Second, myosins move intracellular cargos along actin filaments over short distances (Pollard et al., 2007, 2009).

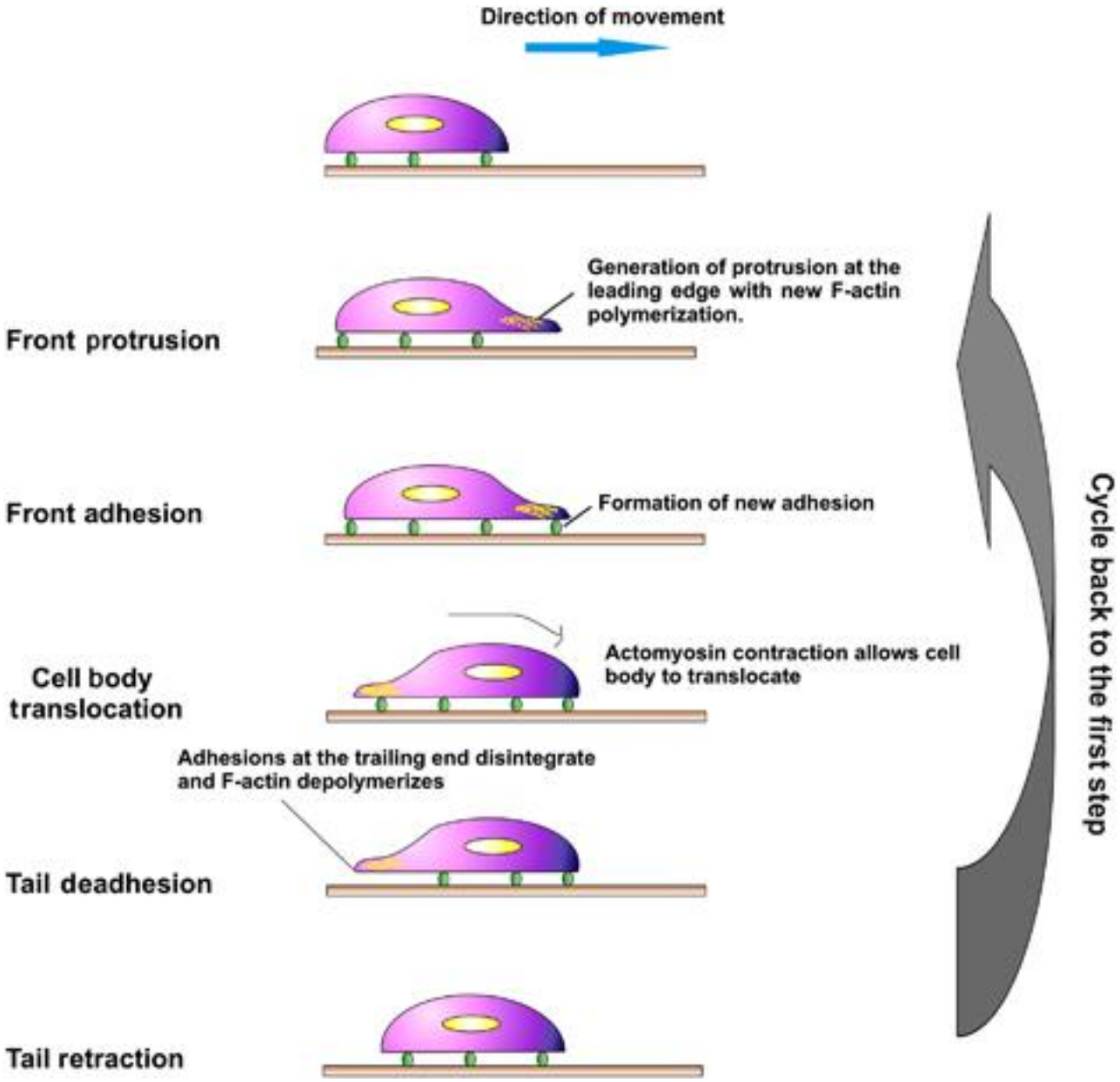


Figure 2.2. Fundamental steps in cell motility. Front protrusion: at the new protrusion, actin polymerization is required and new focal adhesion molecules are recruited to adhere the cell front to the substratum. Cell body translocation: the cell body translocates by actomyosin contraction. Tail de-adhesion and retraction: Actin filaments depolymerize, focal adhesion molecules at the trailing/posterior edge (tail) disassemble and the trailing edge retracts. (Image from Wong et al., 2010)

G-actin monomers bind ATP molecules. After assembly into filaments, following recruitment at the barbed end, G-actin hydrolyses the terminal phosphate from the bound ATP and slowly dissociate the phosphate. Subtle structural changes in the actin subunits related to this hydrolysis prepare the now adenosine diphosphate (ADP) bound actin filaments for disassembly by regulatory proteins at the pointed end (Campellone et al., 2010). Cells use a large number of accessory/regulatory proteins to maintain a pool of actin monomers, initiate polymerisation, restrict the length of actin filaments, regulate the assembly and turnover of actin filaments and cross-link filaments into networks or bundles (Figure 2.1).

New filaments can be formed with a variety of mechanisms:

1) Creating a branch from the side of an existing filament (actin nucleation): The actin-related protein 2/3 (Arp2/3) complex constructs a dense network of short, branched actin filaments that grow in successive generations like the twigs of a bush. Most filaments are capped before growing longer than 0.5µm presumably to prevent buckling under the force (Pollard et al., 2009). Each filament can produce picoNewton forces, allowing the front end of cells to move at rates up to about 1µm per second (Pollard et al., 2003, 2007, 2009; Millard et al., 2004). A short distance behind the cell's leading edge the network of branched filaments is turned over within a few seconds, replaced by one composed of longer unbranched filaments (Figure 2.1).

2) Severing a filament to create two free barbed ends: ADF (actin-depolymerising factor)/cofilin has a severing activity which creates free ends (Millard et al., 2004)

3) Starting a filament from monomers: Spontaneous actin assembly is inefficient due to the inherent instability of actin dimers and trimers. However, once larger than a trimer, actin filaments grow rapidly (Campellone et al., 2010).

4) Nucleation and elongation by membrane bound formin proteins which initiate the formation of F-actin from free actin monomers and remain associated with the growing free end. This method produces long, unbranched actin filaments (Figure 2.1).

2.2.2 Arp2/3 Complex: Nucleator of Actin Reorganisation

The ubiquitous and evolutionarily conserved 220 kDa Arp2/3 complex consists of seven subunits (seven stably associated polypeptides). These include two Arp's, Arp2 and Arp3 each of which has a structural homogeneity of approximately 45% with actin, and five additional subunits named ARPC1 to ARPC5 which form a scaffold that supports both of the Arps (Campellone et al., 2010; Pollard 2007; Xu et al., 2012). Crystallographic studies have shown that the two Arps lie distant from each other in the inactive complex (Pollard 2007; Derivery et al., 2010).

Among the known actin nucleators, the Arp2/3 complex plays a unique role in its ability to both nucleate actin filaments and generate branched F-actin networks at the leading edge of motile cells (Campellone et al., 2010). These organised and branched actin networks are particularly seen in lamellipodia and other similar leading edge protrusions.

In the presence of nucleation promoting factors , pre-existing actin filaments, actin monomers and ATP, the Arp2/3 complex will initiate new branching filaments that grow at an angle of 78° from the pre-existing mother filaments (Xu et al., 2012). However, a complete understanding of the structural pathway taken to accomplish this has not yet been achieved.

Actin nucleation and branched network formation are the ultimate responses to a cellular stimulation from the external environment. Signalling pathways are initiated

via plasma membrane receptors, causing activation of membrane based small GTPase molecules (e.g. Cdc42, RhoA, Rac1). This results in a signalling cascade which activates nucleation promoting factors (NPFs) and finally the Arp2/3 complex (Figure 2.2).

Nucleation promoting factors (NPFs) are proteins that bind to and activate the Arp2/3 complex via a WCA-domain. Mammalian NPFs (Class I) consist of Wiskott-Aldrich syndrome protein (WASP) and neural-WASP (N-WASP), WASP-family verprolin-homologous proteins (WAVEs) 1,2 & 3 and the recently identified factors WASH WASP and SCAR homologue (WASH) and (WASP homologue associated with actin, membranes and microtubules (WHAMM). All of these proteins together are known as the WASP superfamily. These important proteins are detailed further on along with the clinical syndrome from which they take their name (the Wiskott-Aldrich Syndrome).

The WCA-domain consists of one or more WASP homology 2 (WH2) domains that bind actin monomers, plus an amphipathic connector region and an acidic peptide that together are thought to bind to Arp2/3 (Campellone et al., 2010). It is generally considered that the act of the WCA-domain binding to the Arp2/3 complex will bring about substantial conformational changes within the complex which prime it for nucleation (Campellone et al., 2010).

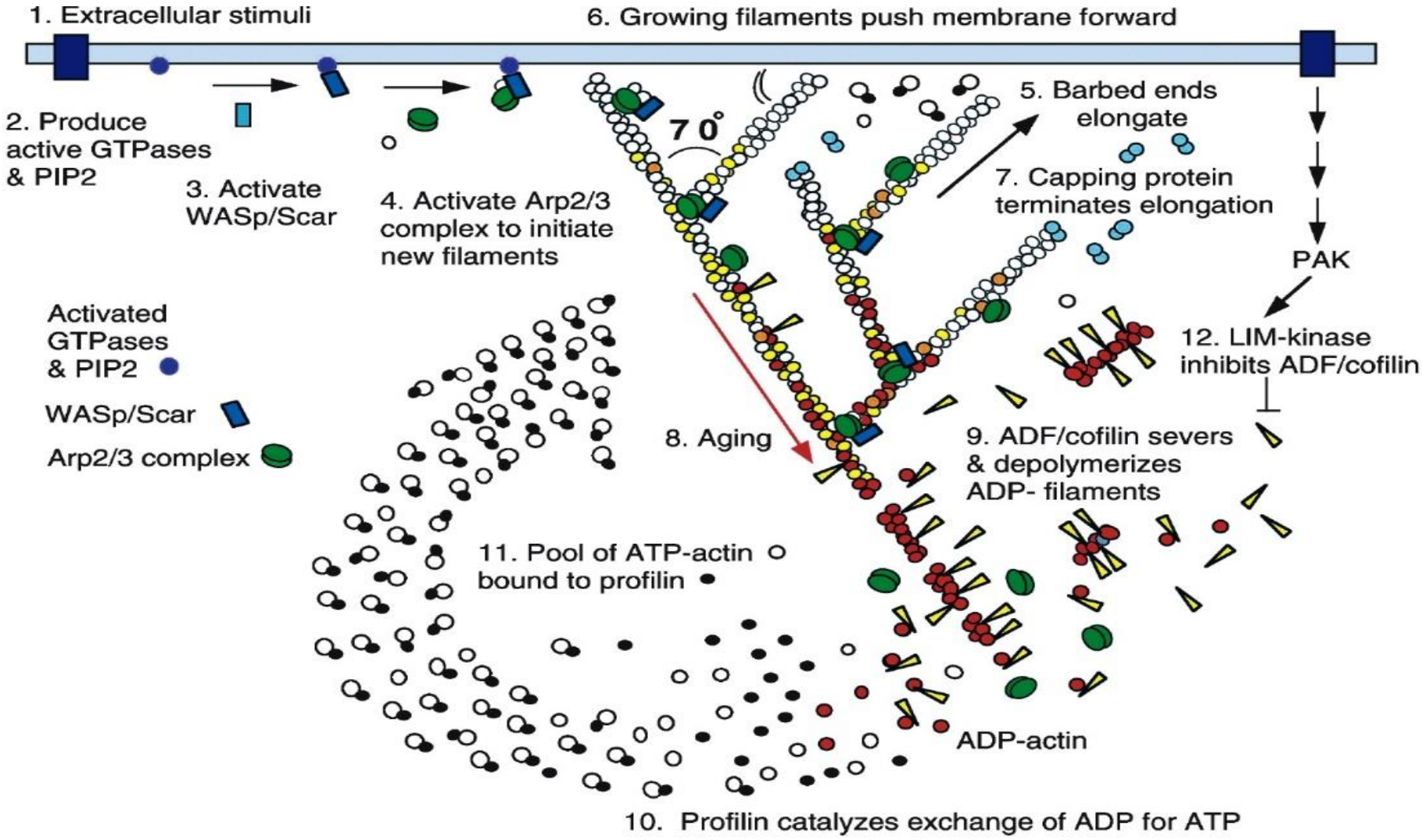


Figure 2.3. Overview of the functions of Arp2/3 complex. Nucleation-promoting factors such as WASP and WAVE bring together the Arp 2/3 complex with an actin monomer on the side of a filament to nucleate a branch. The free barbed end of the branch grows until it is capped. (Adapted from Pollard, 2007)

On activation the Arp2/3 complex is bound to the side of a pre-existing mother filament. Arp2 and Arp3, through considerable conformational changes, are thought to mimic two of the three actin monomers required for the nucleation of actin, through apposition of Arp2 and Arp3. Polymerisation and the formation of the new (daughter) filament is the end result (Rouiller et al., 2008; Takenawa et al., 2007; Derivery et al., 2010) (Figure 2.3). In this active conformation, all of the subunits of the Arp2/3 complex, including the pointed ends of both Arps, contact the mother filament. The conformational changes that the Arp2/3 complex undergoes to achieve this and the exact sites of NPF interactions, have yet to be defined (Xu et al., 2012).

2.2.3 The Rho-family small GTPases: Key Regulators of Actin

2.2.3.1 Reorganisation

Small GTPases of the Ras superfamily are divided into smaller subfamilies (Ras, Rho, Arf, Rab, Ran) (Hall 1998; Ellenbroek et al., 2007). The Rho subfamily of p21 small GTPases are approximately 21 kDa in size and the majority of information relating to them has been collected from studies on the best characterised members, Cdc42, Rac1 and RhoA. These have been found to regulate a broad range of cellular functions including mitosis, proliferation, apoptosis, regulation of the cytoskeleton and gene transcription (Ellenbroek et al., 2007).

The Rho family proteins differ from the other subfamilies because their sequence contains a Rho insert domain in the GTPase domain. This is suggested to be involved in the activation of downstream effectors (Saskia et al., 2007; Spiering et al., 2011). There are currently 23 identified members of the Rho family, divided into 6 groups:

The Rho proteins (RhoA, RhoB, RhoC), Rac proteins (Rac1, Rac2, Rac3, RhoG), The Cdc42-like proteins (Cdc42, TC10, TCL, Wrch1, Chp), the Rnd proteins (Rnd1, Rnd2, Rnd3/RhoE), The RhoBTB proteins (RhoBTB1, RhoBTB2, RhoBTB3) and the Miro proteins (Miro1, Miro2). Some Rho proteins don’t belong to any subgroup – RhoD, Rif and RhoH/TTF (Ellenbroek et al., 2007).

Rho GTPases function as molecular/binary switches in a large variety of signalling pathways. These signalling cascades are initiated by the stimulation of cell surface

receptors. Rho GTPases can be in an active conformation, bound to GTP, or in an inactive conformation, bound to guanosine diphosphate (GDP) (Ridley et al., 2003). They have an inherent phosphatase activity which catalyzes GTP to GDP. Only in the GTP bound state are these proteins able to interact with downstream effector proteins and propagate signals from a large variety of membrane receptors.

2.2.3.2 Regulators of Rho GTPases

The activity of Rho GTPases is strictly controlled in order to stimulate specific downstream signalling pathways in cells. The activity regulators consist of three classes of proteins: GEFs (guanine nucleotide exchange factors), GAPs (GTPase-activating proteins) and GDIs (guanine nucleotide dissociation inhibitors) (Hall 1998).

GAPs enhance the very low intrinsic ability of Rho GTPases to hydrolyse bound GTP to GDP. Thus GAPs promote inactivation and reverse effector binding, thereby shutting down signalling pathways. GAPs are usually multidomain proteins and are able to interact with many other proteins (Ellenbroek et al., 2007; Ridley et al., 2003; Spiering et al., 2011).

GEFs facilitate the exchange of GDP for GTP (Figure 2.4). This alters the conformation of the switch regions of the GTPases, thereby increasing the binding affinity of effector proteins leading to downstream signalling. GEFs, Like GAPs, also contain various additional domains which enable them to influence and determine the signalling route downstream of Rho GTPases. This is done either by direct binding to effector molecules, or by acting as scaffold proteins which interact with components of downstream effector signalling pathways (Ellenbroek et al., 2007; Ridley et al., 2003; Spiering et al., 2011).

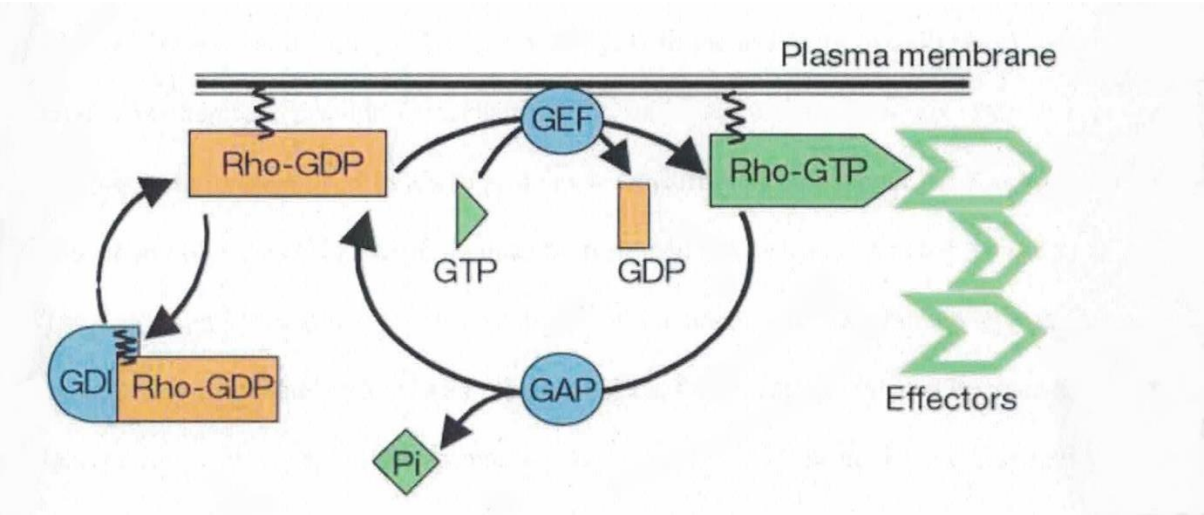


Figure 2.4 The cyclic transition between an active GTP-bound state and inactive GDP-bound state of RHO GTPases facilitated by guanine nucleotide exchange factors (GEF); GTPase activating proteins (GAP) and guanine nucleotide dissociation inhibitors (GDI). Pi denotes a phosphate group which is released upon GTPase hydrolysis (Figure taken from Etienne-Manneville and Hall, 2002)

GDIs are cytosolic proteins which form complexes with inactive GDP-bound Rho GTPases. They prevent the dissociation of GDP for GTP and stop cycling of the GTPases between the cytosol and the plasma membrane. This prevents the Rho GTPases from localising to the membrane, which is their place of action, and thereby prevents their activation by GEFs (Ellenbroek et al., 2007; Ridley et al., 2003; Spiering et al., 2011). GDIs are also able to form complexes with active, GTP-bound Rho GTPases. In this situation they prevent hydrolysis of GTP to GDP and inhibit interaction with downstream effectors.

Association with GDIs keeps Rho GTPases in the cytoplasm, inactive or unable to signal to downstream effectors. Phosphorylation of Rho GDIs breaks down the complex, releasing the Rho GTPases and allowing translocation from the cytoplasm to the plasma membranes were GEFs can activate them and enable binding of effectors (Raftopoulou et al., 2004; Ellenbroek et al., 2007; Spiering et al., 2011).

In normal conditions the activity of Rho GTPases is tightly regulated by a large number of GEFs and GAPs, which themselves are also strictly controlled. Activation of GTPases depends on the manner of cell and on the specific receptor signalling pathways. There are many more GEFs, GAPs and effector proteins than there are Rho GTPase family members and so activation is dependent on the balance of the activities of the regulators. The local amount of GTP-bound protein and the time during which the protein remains active determines the downstream signalling at specific sites in cells (Ellenbroek et al, 2007; Spiering et al., 2011).

2.2.3.3 Rho GTPases and the Actin Cytoskeleton

The Rho GTPases have best been characterised by their particular function in the regulation of the actin cytoskeleton in response to receptor signalling. The vast majority of Rho proteins play a role in organising the actin filament system (Ellenbroek et al., 2007; Ridley et al., 2003; Spiering et al., 2011).

RhoA regulates formation of contractile actomyosin bundles (stress fibres) and focal adhesions. Rac1 regulates formation of actin rich protrusions (lamellipodia and membrane ruffling) and Cdc42 regulates formation of filopodia (Ellenbroek et al., 2007; Ridley et al., 2003; Spiering et al., 2011). These actin dynamics are regulated by coordinated activation of different signalling pathways downstream of the small GTPases. RhoA, B and C interact with Rho-associated protein kinase (ROCK) which subsequently activates myosin light chain kinase, resulting in activation of myosin (by phosphorylation), increased contractility and formation of stress fibres. ROCK is also upstream of LIM domain kinase (LIMK) whose activation has been linked to the phosphoregulation of ADF/cofilin. This is one of the key regulators of actin severing, nucleation and capping (Raftopoulou et al., 2003). RhoA, B and C also interact with the effector proteins mDia1 and mDia2, which catalyze F-actin and help produce filopodia and lamellae. This stimulates actin polymerisation, producing straight, unbranched actin filaments.

Cdc42 activates the Arp2/3 complex via its effector N-WASP, which results in actin polymerisation and the formation of filopodia.

Rac1 regulates actin organisation via the WAVEs or P21-activated kinase (PAK), resulting in altered actin nucleation activity of the Arp2/3 complex and formation of lamellipodia. WAVE proteins don't have a GTPase binding domain, therefore, their activation requires binding of Rac1 to the adaptor molecule IRSp53, followed by binding of this combined complex to the WAVE regulatory complex (WRC) to activate the WAVE protein. Binding and activation of the Arp2/3 complex then occurs and produces dendritic actin network formation (Ellenbroek et al., 2007; Ridley et al., 2003; Spiering et al., 2011).

The GEF Tiam1, which activates Rac, can bind IRSp53 and p21Arc (one of the components of the Arp2/3 complex) thereby providing a mechanism to directly regulate Tiam1/Rac-mediated actin polymerisation processes by the Arp2/3 complex.

Rho GTPases have been shown to regulate various cytoskeleton-dependent processes. They have been shown to regulate the formation and maintenance of adherens junctions (AJs) and tight junctions (TJs). Rho GTPase signalling can stabilise or disassemble AJs leading to epithelial-mesenchymal transition (EMT). The reciprocal balance between Rac and Rho activity determines the epithelial or mesenchymal phenotype of epithelial cells.

Many effector proteins of Rho GTPases are serine/threonine kinases which phosphorylate downstream targets thereby initiating various signalling cascades that

regulate different cellular processes. P21-activated kinases (PAKs) are such serine/threonine kinase effectors (Ellenbroek et al., 2007). There are 6 members, PAK1-6. Some of these are able to bind active Rac1 (PAK1-3), and all of which can bind active Cdc42 (PAK1-6). PAKs are important organisers of the cytoskeleton. Rho associated coiled-coil-containing protein kinases (ROCK-I and ROCK-II), also serine/threonine kinases, bind RhoA. Activation of ROCK may lead to different cellular events that regulate cytoskeletal changes affecting cell-cell or cell-substrate adhesions and cell migration. These effects are mediated via actomyosin-mediated cell contraction. N-WASP is a specific Cdc42 effector protein that upon activation by Cdc42 binds other proteins such as the Arp2/3 complex to regulate actin polymerisation.

The balance between Rac1 and RhoA activity (between two small GTPases of the Rho family) is crucial for several processes such as cell-cell and cell-matrix adhesions, cell migration and EMT. Classically, it was suggested that RhoA was thought to be activated mainly at the retracting tail of a motile cell, to promote tail contraction, while Rac1 was thought to be activated at the front of the cell to promote lamellipodial protrusion. However, studies of fibroblasts and cancer cells show a more complex and dynamic pattern of Rac1/RhoA GTPase coordination that is regulated not only spatially but temporally over very small time scales in those areas that undergo cytoskeletal rearrangements. Cross talk between Ras and Rho proteins (between different GTPase subfamilies) has also been observed in several biological processes, including cell transformation, cell migration and EMT (Sahai et al., 2002).

2.2.4 Cell migration and invasion in cancer

As commented upon by Chambers et al. (2002) “Metastases rather than primary tumours are responsible for most cancer deaths”. Cell motility has been implicated in the spreading of cancer cells and is therefore an essential step in cancer metastasis. Understanding cellular motility and the migrational processes used by cancer cells can potentially form the basis for new ideas relating to diagnostic, prognostic and therapeutic approaches in the management of metastases (Wang et al., 2005). Cancer cells in vivo migrate through three dimensional (3D) networks of extracellular matrix (ECM) fibres and sometimes move collectively as a tumour mass. Fundamental aspects of the molecular mechanisms of cancer cell motility have been largely revealed in simple two dimensional (2D) environments, but recent 3D studies greatly expand upon the area.

To migrate, the cell body must change its shape and stiffness as it interacts with its surrounding environment. This is a vital role undertaken by the actin cytoskeleton that exists throughout the cell. Cancer cells typically develop alterations in their shape and in their attachments to other cells and the ECM. The loss of cell-to-cell adhesion molecules (such as E-cadherin) is an initial first step. The ECM provides the substrate along which the cell will migrate, but it also acts as a barrier (Hanahan et al., 2011)

Cell migration can be visualised as a cyclical process (Figure 2.5). Initially protrusions are extended by the cell in the direction of migration which recognise and

interact with the external environment. These are driven by actin polymerisation, part of the cell cytoskeleton. These protrusions are then stabilised by transmembrane receptors which are linked to the actin cytoskeleton intracellularly and adhere to the ECM or adjacent cells. These adhesions then act as traction sites for migration. As the cell migrates forward over the adhesion sites they are disassembled at the cell rear, allowing the cell to detach at that point and retract the protrusion (Ridley et al., 2003). This results in a motility cycle and overall produces a forward motion in the direction of the initial protrusion. It has been noted however, that cancer cells can modify their morphology and nature of migration in order to respond to environmental changes as necessary (Ridley et al., 2003; Friedl et al., 2003).

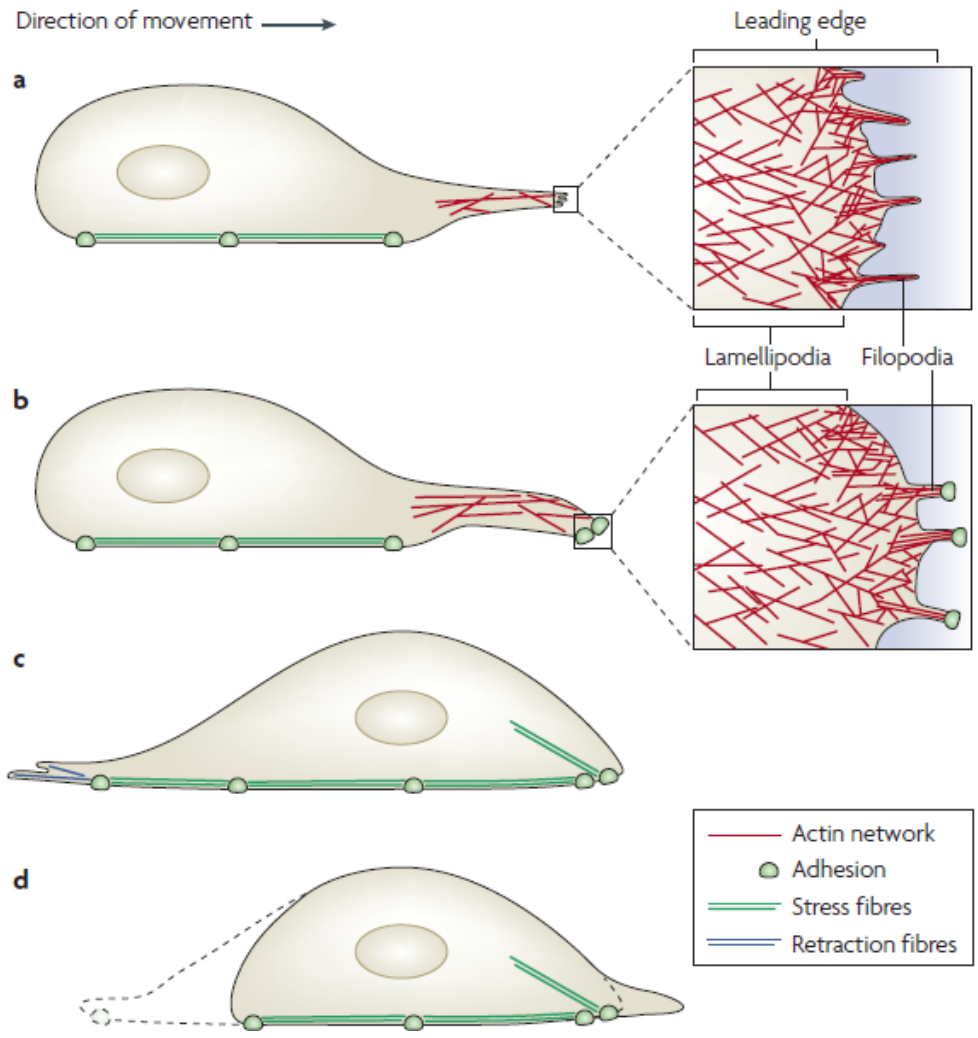


Figure 2.5 Cell migration is driven by cyclic steps dependent on cytoskeletal reorganisation. (a) Protrusive structure such as filopodia and lamellipodia at the leading edge extend out in the direction of movement. (b) The leading edge adheres to the substrate. (c) (d) The tail end of the cell is detached and retracted (Image taken from Mattila and Lappalainen, 2008)

The initial protrusions formed by cells can be quite diverse in their morphology and dynamics. In 2D environments the main protrusions seen are lamellipodia and filopodia. Lamellipodia are flat, broad sheets of membrane constructed from polymerized actin filaments in branched networks that protrude forward from the leading edge of cells during cell migration. Filopodia are finger-like protrusions of up

to 50μm in length or more, constructed from a core of actin filaments that are bundled in parallel (Friedl et al., 2003).

Cancer cell migration and invasion of the ECM in 3D environments (which more closely mimics the *in vivo* cancer microenvironment) show a wide range of processes which help to further understand this mechanism. Initially cell-cell adhesions become weakened and disrupted, which allow solitary cells to depart from the primary tumour mass. This is followed by changes in cell morphology which imply different “modes” of motility/invasion: elongated/mesenchymal invasion and round-shape/amoeboid invasion (Figure 2.6) (Wolf et al., 2003; Sahai, 2007; Sanz-Moreno et al., 2008; Kurisu et al., 2010; Hanahan et al., 2011). Each mode conveys different properties to the cells which enable them to invade surrounding tissues of varying matrix density (Wang et al., 2005). A further third invasion mode has been described, that of collective invasion. This refers to cells moving in a group and has been speculated to mimic the invasive front of tumours (Sanz-Moreno et al., 2008; Kurisu et al., 2010; Hanahan et al., 2011). The fact that a group of cells is involved suggests that the integrity of cell-cell adhesions has not been completely lost in this motility mode. Finally, invasion of the ECM requires the ability to enzymatically degrade and sever ECM fibres to remodel the surrounding matrix. Two main structures have been identified in cells which carry out this process. These are pseudopodia and podosomes (or invadopodia). These actin based cellular protrusions are active zones for ECM proteolysis. Pseudopodia are formed by activity of WAVE 2 and the Arp2/3 complex in cells from the mesenchymal/elongated mode of motility and are structures where proteolytic enzymes are concentrated (Sahai et al., 2003; Sanz-Moreno et al., 2007; Ridley et al., 2003). Invadopodia are

unique protrusive structures which are different from pseudopodia (Kurisu et al., 2010). They have an actin-rich core structure which concentrates proteases (Kurisu et al., 2010; Chen, 1989; Bowden et al., 1999) and are likely supporters of elongated/mesenchymal invasion rather than amoeboid (Li et al., 2010).

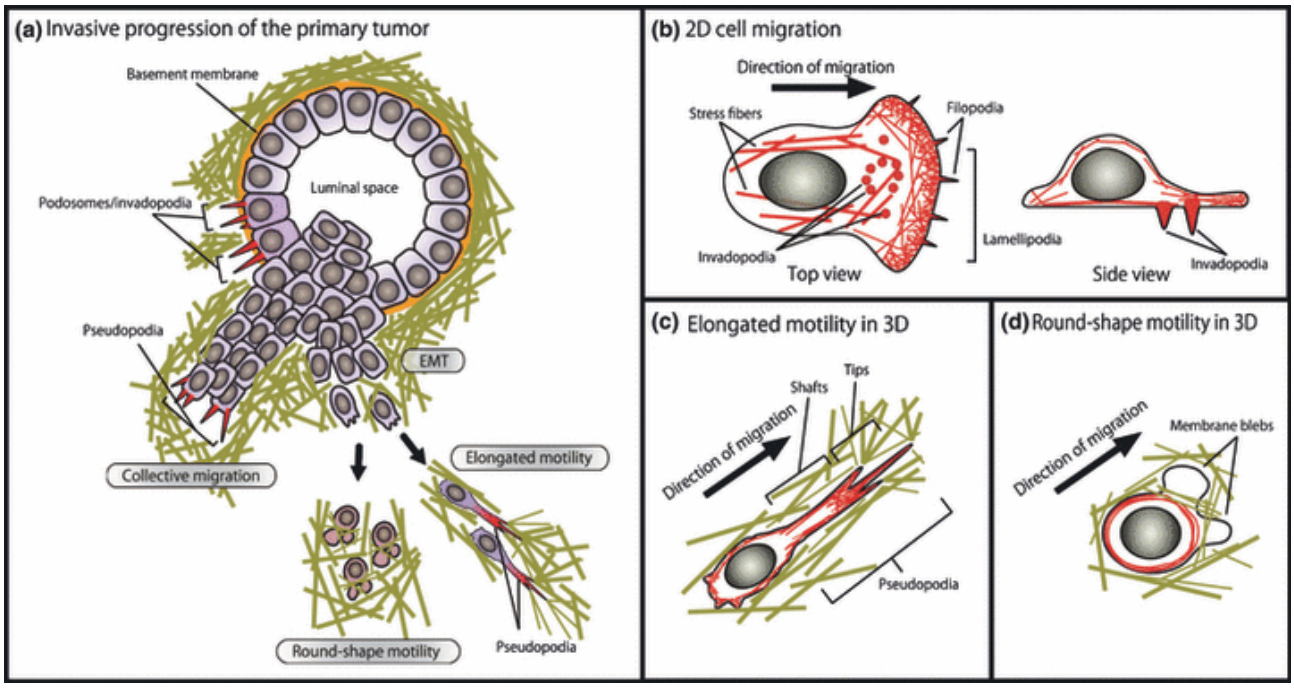


Figure 2.6 Multiple modes of cancer cell motility in 3D. (a) Cancer progression is illustrated from the stand point of cell motility mode. A primary tumour initially invades in a mode called collective migration. If cancer cells are released from a primary tumour mass and move as single cells, the cells can adopt two modes of invasion, round-shape motility or elongated motility. EMT, epithelial-to-mesenchymal transition. (b) The actin cytoskeleton of a two dimensionally migrating cell. In 2D circumstances, characteristic actin structures are commonly seen in many cell types. They generally form lamellipodia, filopodia and stress fibres. In malignant cancer cells, invadopodia are sometimes evident as large actin dots that show proteolytic activity around them. (c) The actin cytoskeleton in cells migrating in the elongated motility mode. The tip contains dense actin networks and its biogenesis is dependent on Wiskott-Aldrich syndrome protein family verprolin homologous protein and actin-related protein 2/3 activities. (d) The actin cytoskeleton in cells migrating in the round-shape motility mode. Protrusions used for migration are, in this case, membrane blebs. Both the formation and retraction of membrane blebs rely on actomyosin contractility (Image taken from Kurisu and Takenawa, 2010)

The two main modes of migration in cancer cell are mesenchymal and amoeboid. These modalities of migration appear to be interchangeable, depending on the microenvironment of the cell. Mesenchymal invasion is characterised by an elongated cellular morphology and is dependent on extracellular proteolysis. Broad lamellipodia are no longer formed at the leading edge, instead long finger-like protrusions rich in F-actin are extended called pseudopodia. The tip of pseudopodia seemingly contain miniature versions of the lamellipodium found in 2D studies and filopodium-like spikes insert into the pericellular ECM. The GTPase Rac has been implicated in this type of motility which signals through WAVE2 to commence actin polymerisation (Sanz-Moreno et al., 2008; Kurisu et al., 2010; Hanahan et al., 2011). Amoeboid invasion is where individual cancer cells show morphological plasticity, allowing them to slither through existing interstices in the ECM rather than clearing a path for themselves, as occurs in mesenchymal invasion (Friedl et al., 2003; Yamazaki et al., 2009; Sahai et al., 2003). Cells tend to have a rounded morphology with no obvious polarity and are far less dependent on proteases. These cells rapidly inflate their membrane in the direction of movement in a balloon-like shape called a membrane bleb. Actin networks gradually fill the cortex of these blebs and the actin mesh generates a contractile force by way of myosin II activity. They require high ROCK signalling to drive the elevated levels of actomyosin contractility needed (Sanz-Moreno et al., 2008; Kurisu et al., 2010; Hanahan et al., 2011).

Due to the plasticity of these modes of invasion and the different signalling pathways involved, the effectiveness of single therapeutic agents aimed at reducing invasion may be limited.

The members of the Rho family of small GTPases are key regulators of cell movement through their actions on actin assembly, actomyosin contractility and microtubules. The three main members of this family Rho, Rac and Cdc42 have all been linked to cell movement. Rac1 drives motility by promoting lamellipodia formation via WAVE2 and the Arp2/3 complex, whereas RhoA signals to the Rho-kinases (ROCKI and II) promoting the formation of actin stress fibers and generation of the actomyosin contractile force required for cell movement (Sanz-Moreno et al., 2008; Hanahan et al., 2011).

As described, cell protrusions are a requirement for initiation and maintenance of cell migration in all cells. These can be formed spontaneously or induced by chemokines or growth factors (Friedl et al., 2003; Ridley et al., 2003; Wang et al., 2005). Therefore, cancer cell migration and invasion can be enhanced by chemotaxis. Cell surface receptors are engaged by extracellular molecules (such as growth factors, chemokines or ECM molecules) which are detected by intracellular signalling pathways. These coordinate cell migration along the gradient path of the molecule (Condeelis et al., 2003; Maghazachi, 2000). Growth factor signalling at the cell membrane is detected by receptor tyrosine kinases (RTKs). These then recruit PI3K (phosphoinositide 3-kinase) to the plasma membrane-anchored recpetors where it is activated. The active PI3K phosphorylates PIP2 (Phosphatidylinositol [4,5]

bisphosphate) to generate PIP3 (Phosphatidylinositol [3,4,5] trisphosphate). Through its pleckstrin homology (PH) domain, the nodal kinase AKT (also known as PKB) binds to PIP3, where it is activated by 2 phosphorylation events and triggers a complex cascade of signals that regulate growth, proliferation, survival and motility (Yuan et al., 2008). In the case of motility one of the pathways through which actin polymerisation is triggered involves the increased PIP3 levels at the plasma membrane. This leads to localised activation of Rac (Rossman et al., 2005; Cote et al., 2007). The activated Rac recruits WAVEs to the plasma membrane where they are activated, leading to formation of membrane protrusions such as lamellipodia and pseudopodia.

Macrophages and cancer cells have been reported to migrate together towards chemoattractants such as growth factors or colony-stimulating factors. They become locked in a paracrine loop whereby each signals to the other and encourages joint migration. This interaction can provide a mechanism for attracting cancer cells to blood vessels where those macrophages in the vicinity will help to signal and direct cancer cells to them (Wang et al., 2005).

In vivo invasion studies have been performed where solitary invasive cells were isolated from a live primary tumour using microneedles containing chemoattractants such as EGF to mimic signals from blood vessels and surrounding tissues. It isolated a subpopulation of highly motile cells that at that stage of invasion were neither proliferating nor apoptotic (Wang et al., 2005; 2004; Goswami et al., 2004). The lack of proliferation would suggest that treatments aimed at growth pathways might be

ineffective at killing invasive tumour cells. Gene expression patterns in these migrating, invasive cancer cells were compared to those in the general primary tumour cancer cell population. A unique pattern of gene expression in the invasive cells was identified (Wang et al., 2004). This pattern showed that genes involved in the motility of cells were markedly upregulated. These include the Arp2/3 pathway, the cofilin pathway and the capping protein pathway. All of which help regulate the actin polymerization process located in cell protrusions and at leading edges. This study gave credence to the idea of highly motile and chemotactic invasive cancer cells.

2.2.4.1 Epithelial-Mesenchymal Transition (EMT)

The epithelial to mesenchymal transition (EMT), while being a critical process during embryonic development and wound healing, also plays a fundamental role in cancer metastasis. It has been prominently implicated as the process by which epithelial cells acquire their invasive phenotype (the abilities to invade, resist apoptosis and disseminate), undergoing a change from the differentiated to a more undifferentiated state. During the process of invasion and metastasis cancer cells can undergo the EMT in varying degrees. It can be transient or it can be more permanent and stable (Klymkowsky et al., 2009; Polyak et al., 2009; Thiery at al., 2009; Yilmaz et al, 2009; Barallo-Gimeno et al., 2005).

EMT is regulated by a variety of signalling pathways originating from the stroma surrounding cancer cells. These include Transforming Growth Factor-beta (TGFβ), Tumour Necrosis Factor-alpha (TNFα), Hepatocyte Growth Factor (HGF), Platelet Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF) and Integrin engagement, all of which converge at the level of key transcription factors such as SNAIL, SLUG, TWIST and ZEB (Kalluri et al., 2009; Bullock et al., 2012). TGFβ appears to play a dominant role. It binds the TGFβ II receptor (TβRII) ultimately resulting in the phosphorylation of SMAD2 and 3 which form a complex with SMAD4 that is capable of translocation into the nucleus and exerting transcriptional control over numerous genes, thereby directly activating transcription factors (Zavadil et al., 2005; Bullock et al., 2010; Kalluri et al., 2009; Bullock et al., 2012). These are normally active during embryogenesis and their activation coordinates the EMT and related migratory processes through initial repression of epithelium-specific gene

expression including cytoskeletal and cell surface proteins (Thiery et al., 2009). Transcription factors have been found expressed in various combinations in a number of malignant tumours and are important in invasion and metastasis (Micalizzi et al., 2010; Taube et al., 2010; Schmalhofer et al; 2009; Yang et al., 2008). The traits induced by these transcription factors include loss of adherens junctions (E-cadherin and cytokeratin are downregulated in mesenchymal cells, and replaced during EMT by the mesenchymal specific markers vimentin and fibronectin [Kalluri et al., 2009; Iwatsuki et al., 2010]), conversion from an epithelial/polygonal to a fibroblastic/spindly morphology, increased motility, expression of matrix-degrading enzymes and increased resistance to apoptosis.

An area of significant progress has been identification of the critical role played by microRNAs (miRNAs) in the EMT process. MiRNAs are a class of small, highly conserved non-coding RNAs that provide widespread control of gene expression through translational repression or degradation of mRNA. MiRNAs have fundamental roles in the regulation of cellular processes and are recognised as playing a critical role in malignant transformation with a growing number of oncogenes and tumour suppressor genes found to be under miRNA control (Bullock et al., 2012). Over 1400 miRNAs have been identified and de-regulation of them has profound consequences, as each individual miRNA targets multiple genes and is capable of inducing broad downstream and feedback effects simultaneously. Several miRNAs have now been described as crucial regulators in EMT. These miRNAs dynamically influence the balance between EMT and the reverse process of mesenchymal-epithelial transition (MET) (Gibbons et al., 2009).

Cancer cells at the invasion margins of certain carcinomas appear to have undergone an EMT. This suggests these cells are subjected to microenvironmental stimuli that promote the transition (such as TGFβ), compared to cells at the core of the primary tumour who do not show evidence of an EMT and therefore have not been subjected to the stimuli that promotes this transition (Hlubeck et al., 2007).

The transcription factors which induce EMT appear able to coordinate most aspects of the invasion-metastasis cascade and with the discovery of miRNAs further regulatory processes contributing to cancer cells acquiring their migratory and invasive abilities are being discovered.

2.3 Wiskott-Aldrich syndrome

As already described, nucleation promoting factors (NPFs) are proteins that bind to and activate the Arp2/3 complex, part of the signalling cascade necessary for cell motility and therefore invasion and metastasis in cancers. These important NPFs are known as the WASP (Wiskott-Aldrich syndrome protein) superfamily. Details of these proteins are discussed later, but the clinical syndrome from which they take their name will be discussed here.

The Wiskott-Aldrich syndrome was first described in 1937 by Dr. Alfred Wiskott, a German paediatrician, who described a case of three brothers that presented with eczema, thrombocytopenia, bloody diarrhoea and recurrent ear infections. They all died at a young age of sepsis or gastrointestinal haemorrhage. Wiskott associated the haemorrhagic symptoms with a platelet dysfunction and on observing that all three brothers were afflicted, but none of the four sisters showed any symptoms, this led him to propose that the syndrome was due to a hereditary thrombopathia (Wiskott, 1937). Later, in 1954, Dr. Robert Anderson Aldrich reported a family case study. Over six generations of a Dutch-American family, 16 out of 40 males, but no females, died of a disease very similar to that which Wiskott described. Aldrich also attributed the disease to a recessive X-linked mode of inheritance (Aldrich et al., 1954). Currently, the Wiskott-Aldrich syndrome (WAS) is described as a hereditary X-linked disease where affected males exhibit microthrombocytopaenia, varying degrees of eczema, combined immunodeficiency and an increased risk of autoimmune disorders and lymphoid malignancies (Albert et al., 2011; Ochs et al., 2009, Sullivan et al., 1994). The incidence of WAS has been estimated to be

between 1 and 10 in 1 million individuals (Stray-Pedersen et al., 2000; Ryser et al., 1988). However, this could potentially be much higher.

2.3.1 Molecular basis of Wiskott-Aldrich Syndrome

The disease is caused by a mutation in the WAS gene which codes for the Wiskott-Aldrich syndrome protein (WASP). This was first isolated in 1994 (Derry et al., 1994) and since then more than 150 unique mutations in the WAS gene have been identified, correlating with the variable clinical phenotypes associated with WAS (Albert et al., 2011; Ochs et al., 2006). Missense mutations in exons 1-3 seem to be the most common, followed by nonsense and splice mutations (mainly in exons 6-11) and short deletions and insertions (Ochs et al., 2006).

WASP is a key regulator of actin polymerization in haematopietic cells. The involvement of the actin cytoskeleton in cell migration and cell trafficking of myeloid cells, macrophages, dendritic cells and Langerhans cells make these cell lines particularly vulnerable to WASP mutations. The WASP gene consists of 12 exons, encodes a 502 amino acid protein, and contains several unique domains with characteristic functional properties.

2.3.2 Clinical Spectrum of the Wiskott-Aldrich syndrome

Once the molecular defect was identified in 1994 the clinical spectrum of Wiskott-Aldrich syndrome broadened. Depending on the type of mutation and its effect on WASP expression a number of overlapping but distinct phenotypes could be seen. These include classic WAS, chronic or intermittent X-linked thrombocytopaenia, a relatively mild form of WAS, and X-linked neutropenia caused by an arrest of myelopoiesis (Ochs et al., 2006). It is also possible for the disease to progress at a later age, therefore patients diagnosed with a mild form earlier in life could develop autoimmunity or cancer as they get older (Albert et al., 2011).

2.3.2.1 Classic Wiskott-Aldrich syndrome

Classic WAS is based on the original description by Wiskott in 1937. Young males present in their early childhood with bleeding diathesis due to thrombocytopaenia, recurrent often severe infections and difficult to treat eczema. There is a gradual reduction of lymphocyte (T and B) numbers over the years and a drop in the proliferative response of T lymphocytes to specific antigens (polysaccharide antigens and bacteriophage) and anti-CD3.

IgG and IgM levels usually remain at normal levels but their response to antigens may be impaired. IgA and IgE serum levels are usually increased, reflecting the immune dysfunction (Sullivan et al., 1994).

Unless haematopoietic cell transplantation or gene therapy is carried out, patients with classic WAS have a poor prognosis and the median life expectancy is only 15 years (Moratto et al., 2011). Without such therapy they tend to develop autoimmune disorders and lymphoma or other malignancies which greatly reduce life expectancy and leads to an early death (Sullivan et al., 1994).

2.3.2.2 X-linked thrombocytopaenia

X-linked thrombocytopaenia (XLT) is seen in male patients with congenital thrombocytopaenia, sometimes associated with eczema. Very rarely the thrombocytopaenia may be intermittent (IXLT). These patients are often misdiagnosed as being affected with idiopathic thrombocytopaenic purpura (ITP), resulting in a late diagnosis.

XLT patients could potentially have a very mild and benign disease course with excellent long term survival, however, they still carry an increased risk for severe disease-related events. These include life-threatening sepsis (especially following splenectomy), severe haemorrhage or autoimmunity and cancer (Albert et al., 2010, 2011). Those patients misdiagnosed with ITP often do not have the therapeutic decisions made that reflect this increased risk of cancer or severe disease-related events, as such it is important for those patients noted to have a microthrombocytopaenia to be assessed for WAS gene mutations and WASP expression.

2.3.2.3. X-linked neutropaenia

Missense mutations in the GTPase (Cdc42)-binding domain of WASP impairs the autoinhibitory conformation of the protein and leads to a “gain of function”, resulting in increased actin polymerization. These activating mutations in the WAS gene result in a variant of congenital X-linked neutropaenia (XLN). (Albert et al., 2011)

XLN patients suffer from profound neutropaenia. It can be associated with lymphopaenia, reduced in-vitro proliferation to anti-CD3 and an increased risk for myelodysplastic changes in the bone marrow (Beel et al., 2009).

2.3.3 Pathophysiology

As described previously, WASP is a cytoplasmic protein involved in cell motility, cell-cell interactions, immune regulation, cell signalling and cytotoxicity. These can affect adaptive and innate immunity, immune surveillance and platelet function.

2.3.3.1 Immunodeficiency of Wiskott-Aldrich syndrome

Patients suffering from WAS display a variety of characteristics resulting in immunodeficiency. They have a reduced number of T lymphocytes that function poorly and disturbed B cell homeostasis. Over time a lymphopaenia can develop, in those patients with classic WAS, of variable severity. IgG and IgM are usually at normal serum levels, but IgA and IgE are raised. Antibody responses to some antigens (such as vaccinations) may be inadequate. Absent or reduced WASP also affects innate immunity, causing problems for natural killer (NK)-cells and aberrant regulatory T-cell function due to the impact on the immunological synapse which WASP plays an important role in producing and maintaining (Orange et al., 2002; Albert et al 2011; Dupre et al., 2002) . A lack of WASP causes an inability to produce podosomes resulting in defects of movement of phagocytic cells (Albert et al., 2011; Ochs et al., 2009).

2.3.3.2 Thrombocytopaenia

Microthrombocytopaenia is seen in all patients with WAS mutations (except XLN patients) and the platelets that are present also show defective procoagulatory activity. Therefore, bleeding and the risk of haemorrhage are common clinical problems. This can range from minor problems such as petechiae, haematomas or bleeding from the gums to more life threatening manifestations such as severe gastrointestinal haemorrhage or an intracranial bleed. These can occur spontaneously or as a consequence of trauma.

The exact mechanisms resulting in the thrombocytopaenia of WAS/XLT patients is still not clearly understood. It is generally accepted that platelets in these patients are more rapidly destroyed and *in vitro* studies of mice completely devoid of WASP have attributed this accelerated platelet turnover to both intrinsic platelet abnormalities and to an immune-mediated mechanism (Marathe et al., 2009).

2.3.3.3 Autoimmunity

The mechanisms underlying autoimmunity in WAS have recently begun to be elucidated. WASP is required to regulate and maintain the function of regulatory T cells (Marangoni et al., 2007; Maillard et al., 2007; Humblet-Baron et al., 2007). *In vitro* studies of WASP deficient regulatory T cells have found them to express lower levels of CD25 and following activation they fail to suppress effector T lymphocyte proliferation and interferon-γ production (Marangoni et al., 2007; Maillard et al., 2007; Humblet-Baron et al., 2007). Furthermore, it has been postulated that the lack of expression of a variety of “homing” molecules explains why the regulatory T cells

lacking in WASP are unable to migrate to inflamed tissues *in vivo* (Marangoni et al., 2007; Maillard et al., 2007; Humblet-Baron et al., 2007).

A further possible contributing cause to the autoimmunity in WAS has been suggested by *in vivo* studies. These have found that WASP negative murine T cells have a defective Fas ligand expression, thereby reducing Fas-mediated apoptosis. This is an important mechanism as self-reactive lymphocytes are eliminated in the periphery via this process (Nikolov et al., 2010). Defective phagocytosis of apoptotic cells in WASP deficient patients may result in chronic inflammation and autoimmunity (Albert et al., 2011).

2.3.4 Treatment of Wiskott-Aldrich syndrome

2.3.4.1 Haematopoietic cell transplantation

Haematopoietic cell transplantation (HCT) has become the treatment of choice for those patients suffering with classic WAS phenotype. The first allogeneic HCT in a WAS patient occurred over 40 years ago and since then there have been gradually improving outcomes (Parkman et al., 1978; Moratto et al., 2011; Albert et al., 2011).

Outcomes for recipients of a matched sibling donor are now similar to those recipients of a matched unrelated donor (MUD) when recipients are under the age of 5 years at time of HCT (99% 5 year survival vs. 94%) . However, The 5 year survival for recipients over the age of 5 does decrease in those who receive a MUD-HCT (73.3%) but is better than seen in earlier studies (Filipovich et al., 2001). HLA-mismatched family donors have been less satisfactory in the past but since 2000 survival has increased to over 90% (Moratto et al., 2011). Partially matched unrelated cord blood as a source of stem cells has also been increasingly used as a treatment (Knutsen et al., 2003).

A recent international study has shown that patients with WAS who were treated with all types of HCT, since 1980, had an overall survival of 84%. This was even higher (89.1% 5 year survival) for those who received HCT from 2000, reflecting better HLA-typing techniques, better donor-recipient matching and advances in supportive care (Moratto et al., 2011; Albert et al., 2011).

2.3.4.2 Gene therapy

The current concept of gene therapy for WAS involves the introduction of a normal WAS gene copy into haematopoietic CD34+ stem cells isolated from a WAS patient. The patient is subjected to submyeloablative doses of chemotherapy before the manipulated cells are infused back into the same patient (Botzug et al., 2006).

2.3.4.3 Splenectomy

Undertaking splenectomies in WAS/XLT patients results in an increase in platelet numbers the majority of the time. However, it has not been shown in any prospective studies that it reduces the risk for severe bleeding although retrospective case evaluations strongly suggest that it might (Mullen et al., 1993). As already explained, WAS/XLT patients have a higher risk for systemic infections, particularly so after a splenectomy. They would therefore require lifelong antibiotic prophylaxis and this should be taken into account along with family situation and possible future compliance issues.

2.3.5 Development of new therapeutic strategies

Current focus lies on improving gene therapy and delivery systems. Clinical trials using improved lentiviral vectors are in the final stages to start enrolling classic WAS patients (Albert et al., 2011). This makes gene therapy a potentially viable option for those patients lacking a suitable stem cell donor. However, to succeed, gene therapy for WAS requires a balance between the risks of insertional mutagenesis and

reduced clinical efficacy associated with insufficient promoter activity (Astrakhan et al., 2012).

2.4 The Wiskott-Aldrich Syndrome Protein (WASP) Family

As previously mentioned, NPFs are proteins which bind to and activate the Arp2/3 complex via a carboxy (C)-terminal WCA-domain to initiate actin nucleation. They have diverse amino-terminal sequences that enable different modes of regulation and functions in cells. The WASP super-family are important regulators and promoters of actin nucleation through their role as NPFs. They function downstream of Rho family GTPases to activate the Arp2/3 complex.

The WASP super-family consists of WASP, which is expressed exclusively in haematopoietic lineages and its ubiquitous homologue Neural-(N-)WASP; brain enriched WASP-family verprolin-homologous protein (WAVE)1 and WAVE 3, and ubiquitous WAVE 2; and newly characterised members WASP and SCAR homologue (WASH), WASP homologue associated with actin, membranes and microtubules (WHAMM), and junction-mediating and regulatory protein (JMY) (Kurisu et al., 2010).

The WASP super-family genes are located on different chromosomes. WASP is carried on the X chromosome, N-WASP on chromosome 7, WAVE 1 on chromosome 6, WAVE 2 on chromosome 1 and WAVE 3 on chromosome 13. The expressed proteins are between 498 and 559 amino acids long and are encoded by 9 to 12 exons. The length of the genes is relatively similar, ranging from 67.1kb for N-WASP to 131.2kb for WAVE 3, with the exception of WASP which is a compact 7.6kb (figure 2.7).

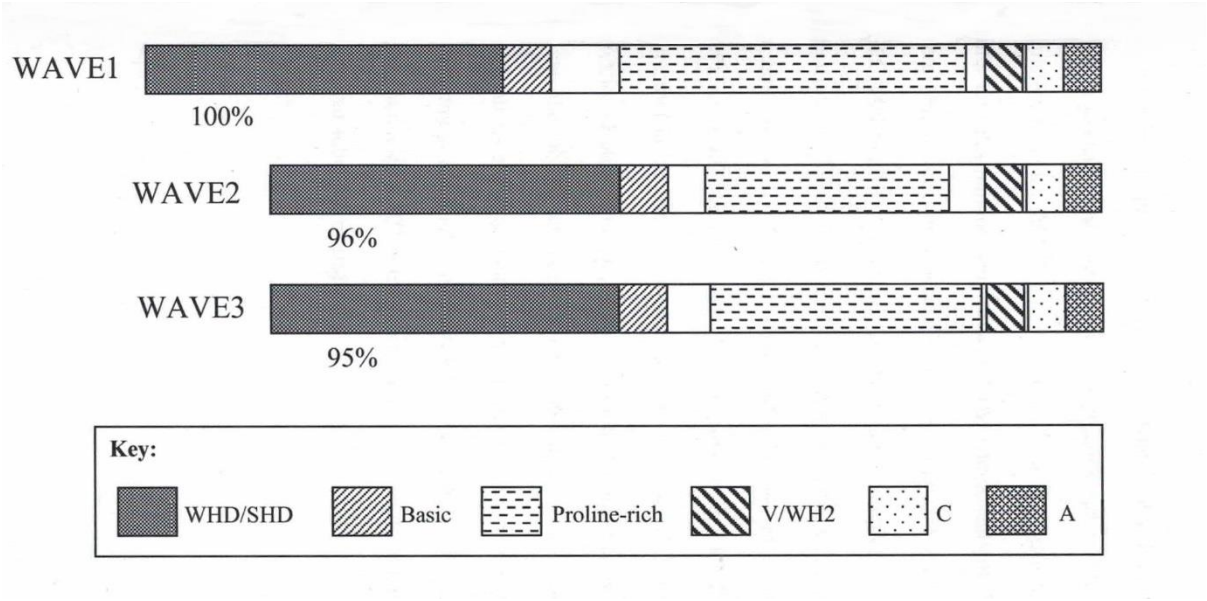


Figure 2.7 Human WAVE protein domain structure. Key denotes conserved domains. The percentage shown below the WHD/SHD domain indicates the amino acid similarity of that domain (Figure modified from Kurisu and Takenawa., 2009)

WASP super-family proteins share two main regions of homology: a central proline-rich segment followed by a conserved C-terminal sequence called the WCA (or VCA in some papers) domain. This consists of one or more WASP homology 2 (WH2) domains (also called verprolin homology domain, hence the alternative name) that bind G-actin monomers, plus an amphipathic connector or central region and an acidic peptide region that together bind to the Arp2/3 complex (Campellone et al., 2010; Kurisu et al., 2009, 2010). This binding action brings these two important components for actin nucleation into close proximity, activates the Arp2/3 complex and initiates actin polymerisation. As all WASP super-family members contain this WCA domain, they are all capable of initiating Arp2/3 complex–mediated actin polymerisation to produce intricate branched actin networks.

WASPs and WAVEs are known as effectors of Rho family small GTPases, specifically Cdc42 and Rac1. When activated intracellularly in studies Cdc42 and Rac1 induce the formation of lamellipodia and filopodia, respectively.

2.4.1 WASP and N-WASP

WASP and N-WASP are found in animals, fungi and protists with N-WASP sharing 50% homology with WASP. Mammalian WASP is expressed specifically in haematopoietic cells as previously discussed. By contrast, N-WASP is expressed in most cell types and its deletion results in neurological and cardiac abnormalities and embryonic lethality in mice (Campellone et al., 2010).

Endogenous WASP and N-WASP (WASPs) form a heterodimer complex with WASP-interacting protein (WIP) family proteins. These consist of WIP (A G-actin and F-actin binding molecule), CR16 (corticosteroids and regional expression-16), and WICH/WIRE (WIP- and CR16-homologous protein/WIP-related protein). Structurally, WASPs have a modular domain organisation consisting of an N-terminal WASP homology 1 (WH1) domain, which directly interact with the WIPs to bind them together to form the WASP or N-WASP complex in a 1:1 molar ratio (Ho et al., 2001; Kurisu et al., 2010). Following on from this domain is a basic region, a Cdc42 and Rac interactive binding (CRIB) site and autoinhibitory motifs that are collectively called the GTPase-binding domain (GBD). The proline rich domain (PRD) is located next, adjacent to the WCA domain found at the C-terminal (Campellone et al., 2010).

The WASPs are autoinhibited in a resting state via an intramolecular interaction and therefore have very little NPF activity. This is thought to prevent unregulated actin polymerisation, anathema to normal cellular function, by masking part of the WCA domain.

Activation of the N-WASP-WIP complex is via signal transduction pathways initiated at the plasma membrane which converge on cellular effectors that interact with the GBD or the PRD. N-WASP directly binds via the GBD/CRIB domain with activated Cdc42, which is localised at the cell membrane. This interaction results in partial activation and conformational changes of the GBD in preparation to free the WCA domain from its autoinhibition, this interaction is enhanced by the binding of PIP2 to the basic region (Campellone et al., 2010). A similar mechanism has been reported for the haematopoietic WASP-WIP complex (Higgs et al., 2000). In addition, diverse PRD-binding proteins with Src homology 3 (SH3) domains are capable of activating N-WASP. These include the adaptor proteins non-catalytic kinase (NCK) 1 and NCK 2 (Tomasevic et al., 2007), membrane-deforming factors such as transducer of Cdc42-dependent actin assembly 1 (TOCA1; also known as FNBP1L) (Ho et al., 2004; Takano et al., 2008), and the kinase-interacting protein ABL-interactor 1 (Abi1) (Innocenti et al., 2005). All of these factors work synergistically in different combinations to activate N-WASP, With multiple inputs being integrated to promote higher activity (Ho et al., 2004).

TOCA1 is an effector of Cdc42 and it binds to N-WASP through an SH3 domain. TOCA1 (and its paralogous proteins FBP17 and CIP4) belongs to a family of proteins containing an F-BAR domain, also known as an EFC domain, which is a domain of dimerisation that has affinity for negatively charged lipids, namely phopsphatidylserine and PIP2. The rigid F-BAR dimer forms a concave surface which allows proteins containing them to sense a particular membrane curvature or to deform membranes until they reach the curvature imposed by this concavity. Through the F-BAR domain, TOCA1 induces long invaginations from the plasma

membrane that are covered by a helicoidal scaffold formed by these proteins (Shimada et al., 2007; Itoh et al., 2005; Tsujita et al., 2006; Frost et al., 2008).

Activation of N-WASP ultimately results in the WCA domain binding to the Arp2/3 complex, where actin polymerization results in a number of cellular functions (Takenawa et al., 2007; Kurisu et al., 2010). These include the formation of filopodia, dorsal membrane ruffling and membrane invagination. Another important N-WASP function is that of vesicle trafficking. N-WASP is now known to mediate actin polymerisation on endocytic vesicles and thereby mobilise them to be pinched off from the plasma membrane (Takenawa et al., 2007; Kurisu et al., 2010).

WASPs are required for actin assembly in invadopodium formation and possibly for bleb formation in round-shape movement in 3D ECM (Gadea et al.,2008; Oikawa et al., 2008; Mizutani et al., 2002; Kaverina et al., 2003; Yamaguchi et al., 2005). Invadopodia act not just as protrusive cellular structures but also as storage of proteases and their secreting machinery for cancer cell invasion into the ECM (Kurisu et al., 2010). This suggests that WASPs are multimodal promoters in cancer cell invasion.

2.4.2 Wiskott-Aldrich verprolin-homologous proteins (WAVEs)

There are three mammalian isoforms of the WAVE-family NPFs expressed in numerous cell types. WAVE 1, WAVE 2 and WAVE 3. WAVEs 1 and 2 are distributed most broadly, whilst WAVE 3 is more restricted, although all are enriched in brain tissue. Because of this, the majority of investigations and studies have used either WAVE 1 or 2 as their subjects. Some, however, have utilised all three WAVE isoforms with the outcome that they behave very similarly and are likely to participate in the same kinds of protein complexes. Any differences are likely to be at the level of tissue distribution or subtle differences in the affinity for specific binding partners (Stovold et al., 2005).

Structurally WAVEs have an N-terminal WAVE-homology domain (WHD) /SCAR-homology domain (SHD) followed by a basic region. They lack a GBD but have a proline-rich segment which is quite distinct from the regulatory WCA portion that lies on the C-terminus of the proteins.

Endogenous WAVEs exist within a heterologous multiprotein complex, called the WAVE Regulatory Complex (WRC). This was initially purified from bovine brain extracts and WAVE 1 was shown to form a stable heteropentameric protein complex with 121F-specific p53 inducible RNA (PIRI121; also known as cytoplasmic FMR1 interacting protein 2 [CYFIP2] and Sra1), NCK-associated protein 1 (Nap1), Abl-interactor 2 (Abi2), and hematopoietic stem/progenitor cell protein 300(HSPC300; also known as Brick 1) in a 1:1:1:1:1 molar ratio (Eden et al., 2002). This pioneering

work led to the understanding that essentially all endogenous, mammalian WAVE isoforms tightly form a WAVE complex with four other subunits or their paralogues. The WAVE isoforms characteristic N-terminal WAVE-homology domain (WHD) serves as a docking site for Abi and HSPC300 and is therefore highly homologous between all three WAVEs. In both WAVEs and WASPs, forming a multiprotein complex seems to protect each subunit from protease-dependent degradation (Figure 2.8) (Kurisu et al., 2010; Derivery et al., 2010).

The WAVE complex is intrinsically inactive, reconstitution revealed that the WCA domain is masked by the Sra/Nap subcomplex through a weak interaction (Figure 2.8). Similar to the WASP/N-WASP complex's autoinhibited state, this would prevent unregulated actin polymerisation within the cell (Derivery et al., 2009, 2010; Ismail et al., 2009). In contrast to N-WASP, most signalling to the WAVE proteins occurs through interactions with the WRC, rather than direct binding.

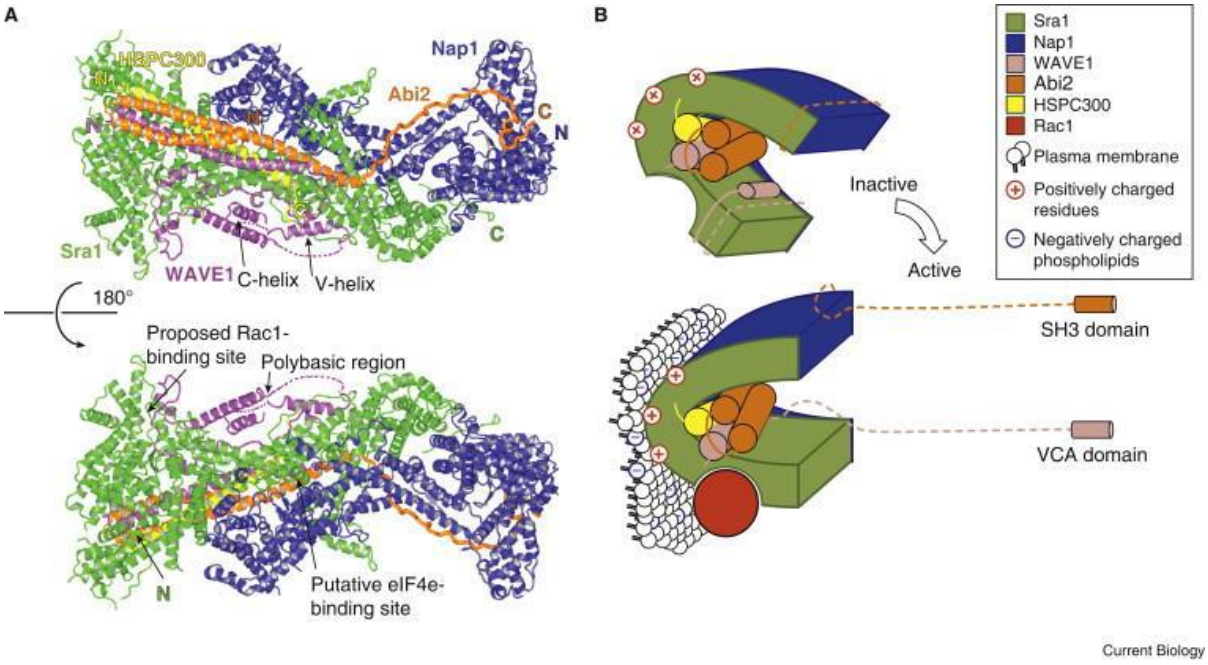


Figure 2.8 WAVE regulatory complex (WRC) structure and regulation. (A) Structure of WRC. Sra1 (green), Nap1 (blue), HSPC300 (yellow), WAVE 1 (magenta) and Abi2 (orange). The WAVE proline rich domain has been replaced with a short linker (dashed line) whilst the Abi2 SH3 domain has been removed. This image is taken from Chen et al, 2010. (B) Simplified schematic demonstrating protein interactions between components of the WRC in addition to mode of activation. The WAVE VCA region is sequestered by WRC components in its inactive state. Upon Rac1 association with Sra1, WRC is recruited to the plasma membrane and the VCA is released whereby interactions with the negatively charged phospholipids at the plasma membrane induce the VCA domain into the correct orientation for actin polymerisation at the cell leading edge (This image is taken from Davidson and Insall., 2011).

The mechanism of activation of the WAVE complex is beginning to be understood, particularly now that it is confirmed that the WRC is inactive, but it is still not completely transparent. The WRC is thought to be regulated by the small GTPase Rac. As previously mentioned, Rac controls the formation of plasma membrane projections such as lamellipodia and ruffles, and the WRC is a critical effector in the formation of these Rac-dependent membrane structures (Innocenti et al., 2004; Miki et al., 1998; Steffen et al., 2004; Derivery et al., 2010). The GTP bound form of Rac binds to the WRC via the Sra subunit. This binding most likely displaces the WCA

domain of WAVE either directly or through a conformational change, but *In vitro* assays required very high concentrations of active Rac to achieve this so it is probable that other factors are involved *in vivo (Derivery et al., 2010)*. A recent study by Koronakis et al (2011) suggested that Rac1 is necessary to induce WAVE-dependent actin nucleation, but alone it is insufficient. A key requirement for Arf GTPases has been suggested by their work, as a much stronger activation was achieved with the addition of both Rac1 and Arf GTPases.

The main function of WAVE NPFs is to activate Arp2/3 during plasma membrane protrusion and cell motility. In these processes WAVE 1 and 2 have partially overlapping functions, as WAVE 2 primarily activates peripheral membrane ruffling and lamellipodia formation (Innocenti et al., 2005; Yamazaki et al., 2003; Yan et al., 2003; Suetsugu et al., 2003; Steffan et al., 2006) and WAVE 1 is related to dorsal ruffling and migration through the extracellular matrix (Suetsugu et al., 2003). Generally, WAVEs localise to the leading edges of lamellipodia and are essential for cell motility.

Clustering mechanisms to recruit WAVEs to the cell membrane, particularly in the case of WAVE 2, have been revealed. WAVE 2 can bind to SH3 domain-containing proteins such as insulin receptor substrate protein of 53 kDa (IRSp53; also known as BAIAP2) via its PRD. IRSp53m also contains a modified BAR domain which dimerises, binds to PIP2, and promotes the formation of outward protrusions of the membrane, presumably through polymerisation onto the membrane as described for F-BAR proteins which interact with N-WASP (Campellone et al., 2010; Kurisu et al.,

2009). IRSp53 has also been implicated in the recruitment and activation of the WAVE 2 complex at the membrane and as a consequence in the formation of lamellipodia. PIP3, the product of Phosphatidylinositol 3-kinase, is another obvious candidate for the clustering effect. PIP3, the WRC and Rac are entangled in a positive feedback loop *in vivo*. PIP3 binds directly to the WAVE 2 protein at the Basic region and recruits the WRC at the plasma membrane. PIP3 and IRSp53 have been shown to synergize with Rac to activate the WAVE 2 complex at the plasma membrane. WAVE 2 has a much stronger affinity for IRSp53 than have WAVE 1 and 3, therefore the interaction with it is likely to contribute specifically to the localisation of of WAVE 2 at lamellipodial tips (Kurisu et al., 2009). Finally, a complex pattern of phosphorylations also appear to modulate the activity of the WRC.

2.5 WAVE Family proteins in cancer cell migration

The WASP family proteins have an important role in cell motility, but their dysregulation results in aberrant cell-motility phenotypes. Cancer invasiveness and metastasis are promoted by enhanced cell motility caused by aberrant upregulation of WAVEs (Kurisu et al., 2005, 2009).

WAVE 2 is required for formation of cell-cell adhesion in epithelial cells (Kurisu et al., 2010), presumably assisiting in blocking the transition from epithelial to mesenchymal phenotype. Thus it appears to act as a tumour suppressor in epithelial-like benign tumours. However WAVEs, especially WAVE 2, induce actin meshwork at the leading edge in single elongated cells and enhance the invasive capacity of cancer cells (Kurisu et al., 2005, 2010; Yamazaki et al., 2009). Therefore, WAVE 2 also works as an invasion promoter once cancer cells start to invade as a single elongated cell in a later stage of cancer progression.

A quantitative regulation of WAVE expression levels has been hypothesised by Kurisu et al. (2010) to help explain the seemingly opposing effects of WAVE 2 in cancer invasion. This regulation is based on the understanding that WAVE isoforms all share the same components for the WRC. Therefore, the expression level of total WAVEs is capped by the level of the least expressed complex component. Excess WAVEs that fail to form a complex are degraded in proteasomes. Kurisu et al. (2010) called this the “isoform sequestration model”. Sra1 has been reported as being depleted in some carcinomas (Silva et al., 2009) and so would result in a reduction

of the expression level of total WAVE isoforms to that of the level of the Sra1 homologue PIR121/CYFIP2.According to this model, overexpression of WAVE 3, as seen in the advanced stages of breast cancer, would lead to a reduction in the overall pool of WRC components available and therefore lead to a reduction in the amount of WAVE 2 stably complexed and available for activation, resulting in a change in the ratio of WAVE isoforms available within the cell.

The WAVE isoforms are activated downstream of Rac although activated Rac cannot bind directly to the WAVE proteins. Therefore IRSp53 has been identified as a linker molecule. Its N-terminal domain specifically binds to active forms of Rac1 and a C-terminal based SH3 domain binds to the proline rich sequence of WAVEs, thereby enhancing the activity of WAVEs. Rac recruits WAVEs and their complexes to the membrane. WAVE 2 has a much stronger affinity for IRSp53 than 1 or 3, therefore interactions are most likely to contribute primarily to activity regulation of WAVE 2. The specifically Rac1-associated protein (Sra 1) present in the WRC, more specifically the PIR121 paralogue, can interact and bind with Rac1.

Lebensohn and Kirschner (2009) provided evidence that the WAVE 2 WRC cannot be solely activated by Rac1 and a negatively charged phospholipid (such as PIP3) was needed also. PIP3 binds to the basic amino acid cluster found in WAVE 2 which is important for recruitment of WAVE 2 to the membrane. In a number of cancer types, signals that increase cellular levels of PIP3 are aberrantly augmented by mutations in genes that constitute the phosphoinositide 3-kinase (PI3K) pathway. In addition, PIP3 activates Rac through PIP3-responsive GEFs. Therefore WAVEs are

presumably hyperactivated in cancers with enhanced PI3K signalling and may thus contribute to cancer pathogenesis.

Phosphorylation has been reported as a WAVE regulation system. Cyclin dependent kinase 5 phosphorylates and inhibits WAVE 1 activity (Kim et al., 2006). Conversely, c-Abl tyrosine kinase activates WAVE 2 through phosphorylation (Leng et al., 2005). C-Abl is a proto-oncogene product best studied in chronic myeloid leukaemia (CML) implicating the association between WAVE 2 and CML (Li et al., 2007). WAVE 2 regulates lamellipodium-driven 2D cell motility and pseudopodia formation in 3D motility. A study by Pan et al. (2011) has shown that WAVE 1 appears to be regulated by LCRMP-1 (long isoform of collapsing response mediator protein – 1) which promotes the formation of filopodia via the WAVE/actin nucleation pathway. LCRMP-1 binds to the SH domain subunit of the WRC and the basic domain of WAVE 1.

WAVE 3 is regulated by the metastasis suppressor miRNA (miR-31) during invasion-metastasis cascade. miR-31 has been found to negatively regulate the expression of TIAM1 (T-lymphoma invasion and metastasis 1), a GEF for the Rac GTPase that has been implicated in the regulation of cancer cell invasion (Bullock et al., 2012).The miR-200 mediated down-regulation of WAVE 3 leads to a significant reduction in the invasive potential of breast cancer cells. Loss of WAVE 3 expression downstream of miR-200 also resulted in a dramatic change in cell morphology resembling that seen in the reverse process of EMT – the MET (Soussey et al., 2009; Bullock et al., 2012). Soussey et al. (2010) have hypothesised that during EMT, WAVE 3 will be transiently expressed to antagonise WAVE 2 at cell-cell

junctions, counteracting actin maintenance at these sites due to their findings that ectopic expression of WAVE 3 in breast cancer cells induces changes associated with disintegrating E-cadherin based adhesions.

WAVE 3 has been noted to promote cell motility through regulation of MMP-1, 3 and 9 expression (Sossey-Alaoui et al., 2005).

2.6 Clinical associations of WAVE proteins with human cancers

The WAVE isoforms have been linked with a number of different cancers with a developing understanding of their roles in cancer invasion and metastatsis.

WAVE 1 over-expression is present in epithelial ovarian cancer and is associated with an unfavourable prognosis. Those patients having a high WAVE 1 expression had a significantly worse survival compared to those with low WAVE 1 expression (Zhang et al., 2012). WAVE 1 up-regulation is evident in prostate cancer tissue samples and knockdown of endogenous WAVE 1 production in metastatic prostate cancer cell lines (DU-145 and PC-3) resulted in significantly reduced invasion (Fernando et al., 2008).

WAVE 2 has been identified as being over-expressed in breast cancer. Node positive cases as well as moderately and poorly differentiated tumours showed high, over-expressed levels compared to normal tissues. Those patients with high WAVE 2 levels had an overall poorer prognosis than those with a low expression (Fernando et al., 2007). Expression of WAVE 2 was investigated by Yang et al. (2006) in hepatocellular carcinoma (HCC). WAVE 2 is up-regulated in HCC tissues, which correlated with a poor prognosis as those patients with a high WAVE 2 level had a shorter survival period. The investigators felt WAVE 2 could be a candidate prognostic marker for HCC. Iwaya et al. (2007) investigated tissue samples from patients with colorectal carcinoma and liver metastases. These were assessed for their WAVE 2 and Arp 2 levels and co-localisation was found to be significantly

predictive of liver metastasis. Co-expression of WAVE 2 and Arp 2 was also found to be significantly higher in sections of lung adenocarcinoma taken from patients with lymph node metastases, compared to those sections taken from patients with lymph node negative disease. Those patients whose tissues showed co-expression of WAVE 2 and Arp2 had a shorter disease-free and overall survival time (Semba et al., 2006).

In a study on murine melanoma cells, Kurisu et al. (2005) showed that WAVE 1 and 2 were overexpressed and the malignant cell type used showed higher Rac activity than the non invasive, non metastatic parent cells. WAVE 2 knockdown resulted in suppression of membrane ruffling, cell motility and invasion into the ECM. *In vivo* studies showed that knockdown of WAVE 2 expression in the injected melanoma cells resulted in suppression of pulmonary metastases.

Elevated expressions of WAVE 3 were found in advanced breast cancer (Soussey et al 2007). Also elevated levels of WAVE 3 were found in grade 3 breast cancers and in tumours of patients who died from metastasis (Fernando et al., 2007).

CHAPTER 3

AIMS AND HYPOTHESIS

3.1 Aims

Cancer metastasis is incompletely understood but is of vital importance in ultimately determining patient outcome in most cancers including colorectal carcinoma. Metastasis is a complex multi-stage process involving cell invasion, cell migration and changes in cell adhesive properties. This study is a continuation of findings in the host centre that WASP family proteins play an important role in the aggressive behavior of cancer cells, more specifically the WAVE proteins.

3.1.1 Human Colorectal Cancer Tissue Analysis

As can be seen from the previous chapters, the ability of cancer cells to invade and migrate is considered among one of the major factors in the metastasis of any cancer, including colorectal. As such, the expression of WAVE proteins in colorectal carcinoma may be an important factor in the progression of colorectal carcinoma from a low stage disease to a more advanced and disseminated stage.

The aim of the tissue analysis experiments in this thesis is to use quantitative and descriptive techniques to analyse the relationship between the expression of WAVE proteins and the disease progression in patients from whom the tissue samples were taken.

3.1.2 Human Colorectal Cancer Cell Line Investigation

The cell line experiments in this thesis aim to analyse the expression of WAVE 1, 2 and 3 in human colorectal carcinoma cell lines and also to analyse the effects of gene knockout of WAVE 1 and 3 in human colorectal carcinoma cell lines on the cells ability to grow, invade, adhere and migrate.

3.2 Hypothesis

3.2.1 Human Colorectal Cancer Tissue Analysis Hypothesis

1) Increased WAVE protein expression is related to advancing stage or grade of tumour
2) Increased WAVE protein expression is related to poorer patient outcomes.

Whilst it is hypothesised that the expression levels of WAVEs 1, 2 and 3 will be significantly higher in advanced stage/grade tumour tissues and that significantly higher expression levels results in a poorer patient outcomes, for statistical purposes the null hypothesis is that there will be no difference in the expression levels in these tissues and there is no correlation between expression and patient outcomes.

3.2.2 Human Colorectal Cancer Cell Line Investigation Hypothesis

1) Knockout of WAVE 1, 2 or 3 expression from human colorectal carcinoma cell lines results in a reduction in the cells ability to grow, invade, adhere and migrate.

Whilst it is hypothesised that altering the expression levels of WAVEs 1, 2 and 3 will have a biological effect, for statistical purposes the null hypothesis is that expressional alteration of these proteins will have no effect on cellular traits such as growth, invasion, migration or adhesion.

Chapter 4
Materials and Methods

4.1 Standard solutions and reagents

All standard chemicals and reagents, unless otherwise stated, were obtained from Sigma-Aldrich (Dorset, UK).

4.1.1 Solutions for cell culture work

0.05M EDTA (Ethylenediaminetetraacetic acid)

One gram KCl (Fisons Scientific Equipment, Loughborough, UK), 5.72g Na_2HPO_4, 1g KH_2PO_4, 40g NaCl and 1.4g EDTA (Duchefa Biochemie, Haarlem, The Netherlands) were dissolved in distilled water to make a final volume of 5L. The solution was adjusted to pH 7.4 before autoclaving and storing for use.

Trypsin (25mg/ml)

Five hundred milligrams trypsin were dissolved in 20ml 0.05M EDTA. The solution was mixed and filtered through a 0.2µm Minisart Syringe filter (Sartorius, Epsom, UK), distributed into 10ml aliquots and stored at -20˚C. When required for cell detachment, one 5ml aliquot was diluted in 100ml of 0.05M EDTA.

Antibiotic and antifungal mix for tissue culture

An antibiotic and antifugal mixture for tissue culture were made consisting of 5g streptomycin, 3.3g penicillin and 12.5mg amphotericin B (2ml of 6.25mg/ml amphotericin B in dimethyl sulphoxide (DMSO)). These components were fully dissolved topped up to a total volume of 500ml with balanced saline solution (BSS), filtered through a 0.2µm Minisart Syringe filter (Sartorius, Epsom, UK) and pipetted into 5ml aliquots. When a 5ml aliquot of this 100x concentrated mix was added to

500ml medium the concentrations of the antibiotics and antifugal agents were as follows: 100U/ml penicillin, 0.1mg/ml streptomycin and 0.25µg/ml amphotericin B.

Balanced Saline Solution (BSS)

79.5g NaCl, 2.2g KCl, 2.1g KH_2PO_4, and 1.1g Na_2HPO_4 were dissolved in distilled water to make a final volume of 10L. The pH was adjusted to 7.2 before use.

4.1.2 Solutions for cloning work

LB agar

Ten grams of tryptone, 5g yeast extract, 10g NaCl and 15g agar were dissolved in distilled water to a final volume of 1L, the pH adjusted to 7.0 and the solution autoclaved. When required, the solution was heated to yield a liquid state and cooled slightly before adding selective antibiotic (if required). The solution was then poured into 10cm^2 petri dish plates (Bibby Sterilin Ltd., Staffs, UK), allowed to cool and solidify then inverted for storage at 4˚C until required.

LB broth

Ten grams of tryptone (Duchefa Biochemie, Haarlem, The Netherlands), 5g yeast extract (Duchefa Biochemie, Haarlem, The Netherlands) and 10g NaCl were dissolved in distilled water to a final volume of 1L and the pH adjusted to 7.0. This was autoclaved and allowed to cool before adding selective antibiotic (if required) and stored at room temperature.

4.1.3 Solutions for use in RNA and DNA molecular biology

Diethyl Pyrocarbonate (DEPC) water

Two hundred and fifty microlitres diethyl pyrocarbonate (DEPC) were added to 5ml distilled water. This solution was then autoclaved before use.

5x Tris, Boric acid, EDTA (TBE)

Five hundred and forty grams of tris-Cl (Melford Laboratories Ltd., Suffolk, UK), 275g Boric acid (Duchefa Biochemie, Haarlem, The Netherlands) and 46.5g of disodium EDTA were dissolved in distilled water, made up to a final volume of 10L and stored at room temperature. When required, the solution was diluted 1:5 in distilled water prior to use in agarose gel electrophoresis.

SYBR®Safe DNA Gel Stain

A 1:10,000 dilution of SYBR®Safe DNA Gel Stain (Invitrogen, Life Technologies Ltd, Paisley, UK) was used to stain DNA in the agarose gel following electrophoresis as specified by manufacture’s intructions.

4.1.4 Solutions for cell and tissue staining

Diaminobenzidine (DAB) chromagen

The DAB (Diaminobenzidine) chromagen was prepared by mixing the following reagents in order, 2 drops of wash buffer, 4 drops DAB (Vector Laboratories Inc., Burlingame, USA) and 2 drops of H_2O_2 to 5ml of distilled water. The mixture was shaken well after the addition of each reagent.

Avidin-Biotin staining Complex (ABC)

The ABC was prepared using a kit obtained from Vector Laboratories Inc., Burlingame, USA. 4 drops of reagent A were added to 20ml of wash buffer, followed by the addition of 4 drops of reagent B and thorough mixing. The ABC complex was then left to stand for approximately 30 minutes before use.

4.2 Cell line work

Cell lines used throughout this thesis were cultured under conditions listed in section 4.2.4. The cell lines are briefly outlined below:

4.2.1 Cell lines

The CaCo2 cell is isolated from a well differentiated primary colonic adenocarcinoma in a 72-year-old Caucasian male using the explant culture technique. CaCo2 cells are epithelial and adherent in morphology.

The RKO cell line is a poorly differentiated colon adenocarcinoma cell line. RKO cells are epithelial and adherent in morphology. They express wild type p53 and are tumorigenic.

HRT-18 is derived from human rectum adenocarcinoma. HRT-18 cells are epithelial-like and adherent in morphology.

The RKO, CaCo2, and HRT-18 cell lines were obtained from the American type culture collection (ATCC, Rockville, Maryland, USA).

4.2.2 Preparation of cell medium

Cells were routinely cultured in Dulbecco's Modified Eagle's medium (DMEM / Ham's F12 with L-Glutamine), pH 7.3 containing 2mM L-glutamine and 4.5mM $NaHCO_3$ supplemented with streptomycin, penicillin, amphotericin B and 10% heat inactivated foetal calf serum. Cell lines transfected with the pEF6 plasmid were cultured in blasticidin S (Melford Laboratories Ltd, Suffolk, UK) selection medium at a concentration of 5µg/ml for at least 7 days and subsequently in a blasticidin S maintenance medium at a concentration of 0.5µg/ml (according to manufacturer's recommendation and routine protocol in the research laboratory).

4.2.3 Revival of cells from liquid nitrogen

When cells were required, cryotubes (Greiner Bio-One Ltd, Gloucestershire, UK) containing the desired cells were removed from storage in liquid nitrogen and revived for culture using the following steps. Cells were thawed rapidly following their removal from liquid nitrogen before the transfer of contents into a universal container containing 10ml of pre-warmed medium to immediately dilute the DMSO present in the storage medium. This was then centrifuged at approximately 382g (1,800 RPM) for 10 minutes to form a cell pellet. The medium was aspirated to remove any traces of DMSO, the cell pellet re-suspended in 5ml of pre-warmed medium, placed into a fresh 25cm^2 tissue culture flask (Greiner Bio-One Ltd, Gloucestershire, UK) and incubated for 4 - 5 hours. Following examination under a microscope to determine adherence of cells to the flask, the medium was changed to remove dead cells and residual DMSO then returned to the incubator.

4.2.4 Maintenance of cells

Cells were maintained in supplemented DMEM medium prepared as described in Section 4.2.2, and routinely sub-cultured upon reaching 70-90% confluency as described later in Section 4.3.5. Confluence was assessed by visualising the approximate coverage of cells over the surface of the tissue culture flask using a light microscope. Cells were maintained and grown in either 25cm^2 or 75cm^2 tissue culture flasks (Greiner Bio-One Ltd, Gloucestershire, UK), in an incubator at 37°C, 5% CO_2 and 95% humidity. All tissue culture techniques were carried out following aseptic techniques using autoclaved and sterile equipment inside a Class II laminar flow cabinet which had been cleaned prior to and following use with 70% ethanol.

4.2.5 Detachment of adherent cells and cell counting

Upon reaching approximately 70-90% confluency, medium was aspirated and adherent cells were detached from the tissue culture flask by incubating with 1-2ml of trypsin/EDTA for several minutes. Once detached the cell suspension was placed in a 30ml universal container (Greiner Bio-One Ltd, Gloucestershire, UK) and centrifuged at approximately 382g (1,800 RPM) for 10 minutes to form a cell pellet. The cell pellet was typically resuspended in 1ml fresh medium to allow a determination of cell density. Cells were counted in a haemocytometer counting chamber (Hawksley, Sussex, UK) using an inverted microscope (Ceti Microscopes; Medline,Oxon, UK) under 10 x 10 magnification. Each 16 square area of the

haemocytometer counting chamber measuring 1mm x 1mm x 0.2mm allowed calculation of the number of cells per millilitre using the following equation:

$$\text{Cell no. / ml} = (\text{number of cell in 16 square area} \div 2) \times 10^4$$

Two 16 square areas of the haemocytometer counting chamber were counted and the mean was used to calculate cell number per millilitre which was then used to calculate volume of re-suspended cells for use in the appropriate *in vitro* cell function assays.

4.2.6 Storage of cell stocks in liquid nitrogen

Stocks of low passage cells were stored in liquid nitrogen. Cells were first detached from their flasks using EDTA/Trypsin as described in Section 4.2.5 and pelleted in a centrifuge at approximately 382g (1,800 RPM) for 10 minutes. These cells were resuspended in the required volume (dependent on the number of samples to be frozen) of a protective medium consisting of 10% DMSO in normal growth medium. Following resuspension, cells were aliquoted into pre-labelled 1.8ml cryotubes (Greiner Bio-One Ltd, Gloucestershire, UK), in 1 ml volumes, wrapped loosely in tissue paper and stored overnight at -80°C in a deep freezer. Cells were later transferred to liquid nitrogen tanks for long term storage.

4.3 Generation of mutant RKO, CaCo2, and HRT-18 cell lines

4.3.1 Production of ribozyme transgenes

Ribozyme transgenes were designed to specifically target and cleave either WAVE1 or WAVE3 messenger RNA transcripts to down regulate their expression. These ribozyme constructs were developed previous to this study by Fernando *et al* (2008; 2010) however the steps are outlined here. The secondary structure of the WAVE 1 and WAVE 3 transcript was initially predicted using Zuker's RNA mFold software (Zuker, 2003) (Predicted structures shown in Figures 4.1a and 4.1b). Doing so allowed identification of loop structures which are unpaired regions and are less stable than paired stemmed regions and therefore make them good ribozyme targets. Suitable GUC or AUC ribozyme target sites were selected from the predicted secondary structure loop structures and a ribozyme was designed for that region, allowing it to specifically bind to the sequence surrounding the target GUC or AUC codon regions. Doing so, allowed the hammerhead catalytic region of the ribozyme transgene to interact with and accurately cleave the mRNA transcript of interest at the specific GUC codon sequence. The secondary structure of the hammerhead ribozyme is shown in Figure 4.1c whilst its mode of action is depicted in Figure 4.2.

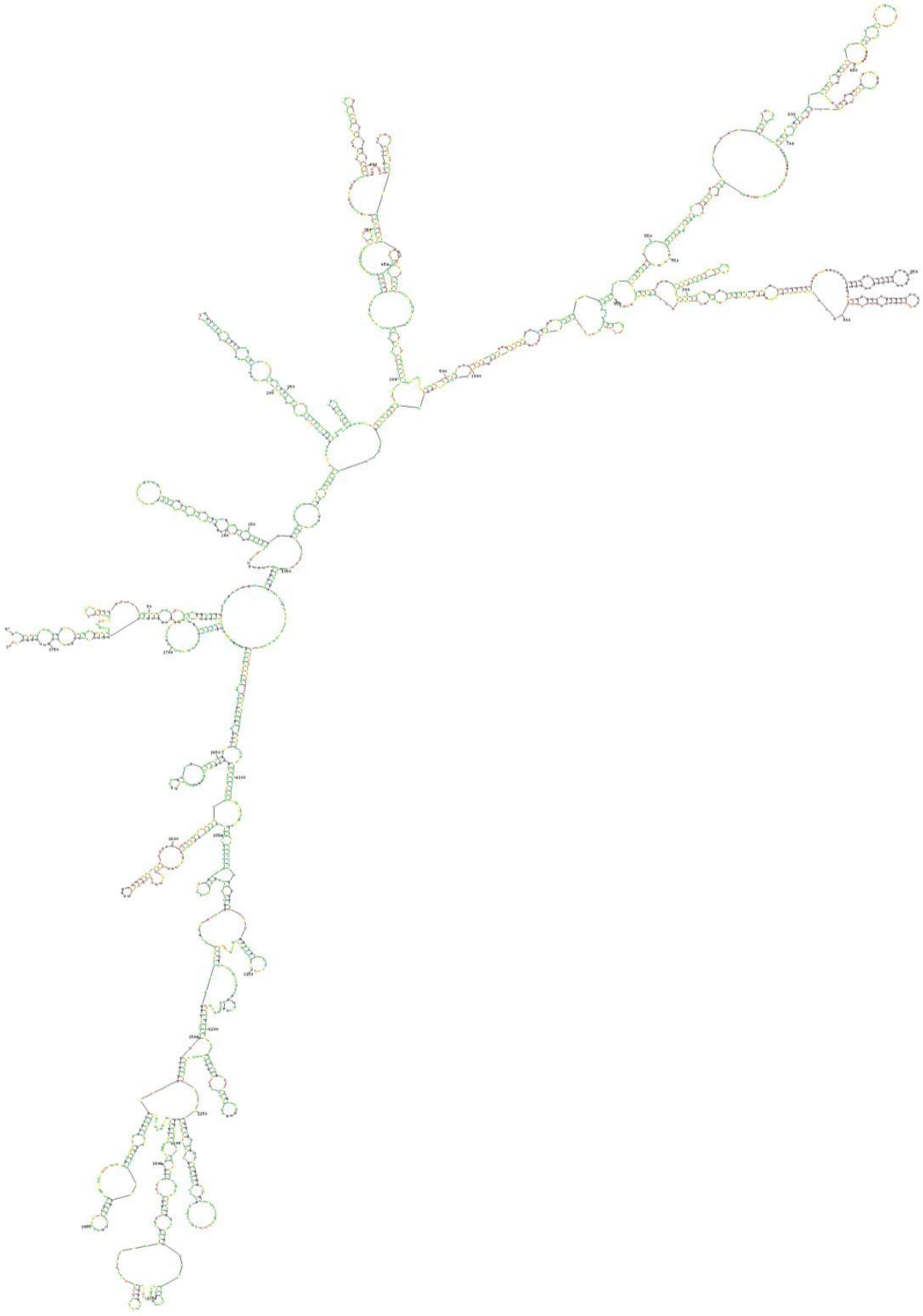


Figure 4.1a. Secondary structure of human WAVE 1 mRNA based on the Zuker programme

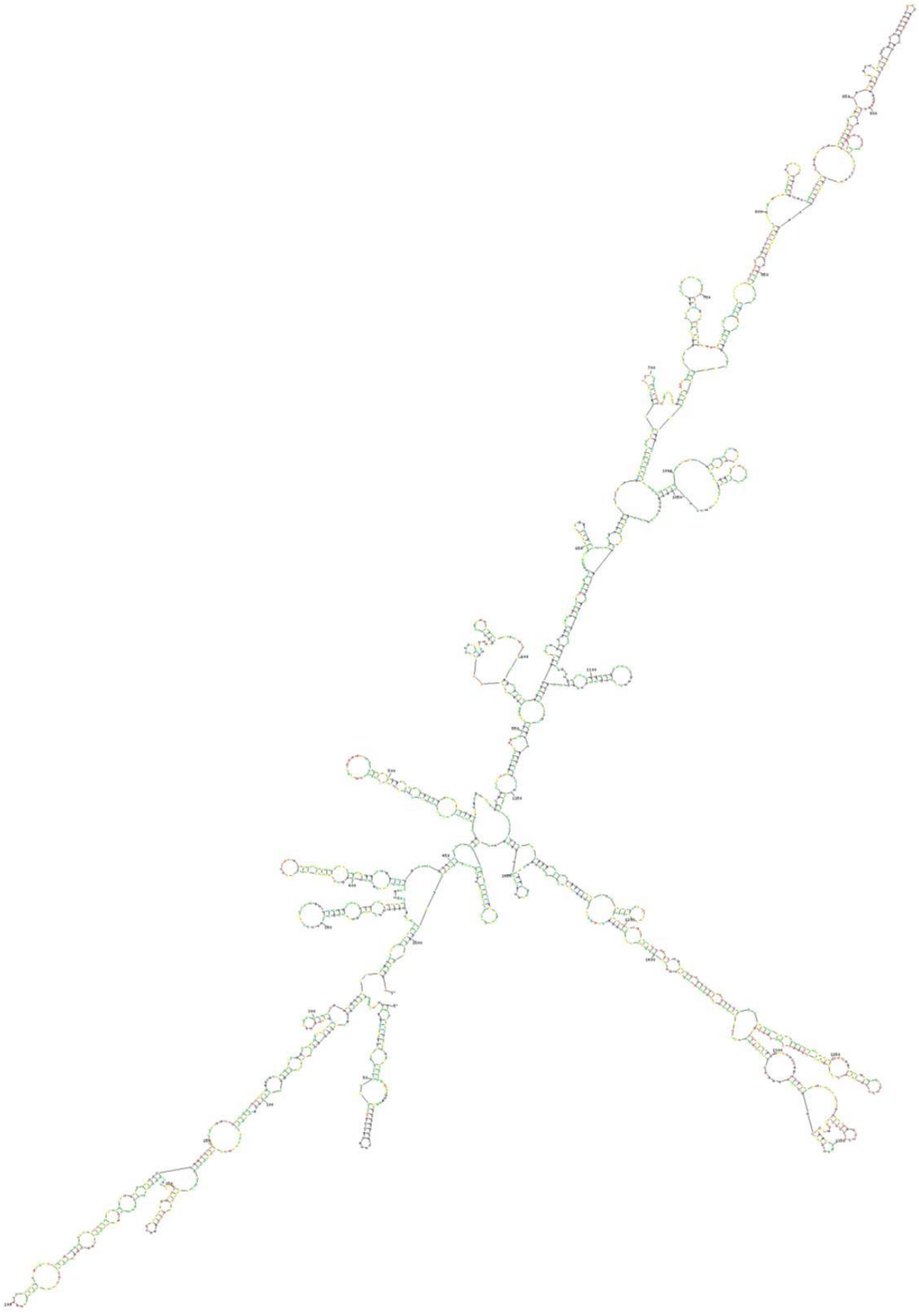


Figure 4.1b. Secondary structure of human WAVE3 mRNA based on the Zuker programme

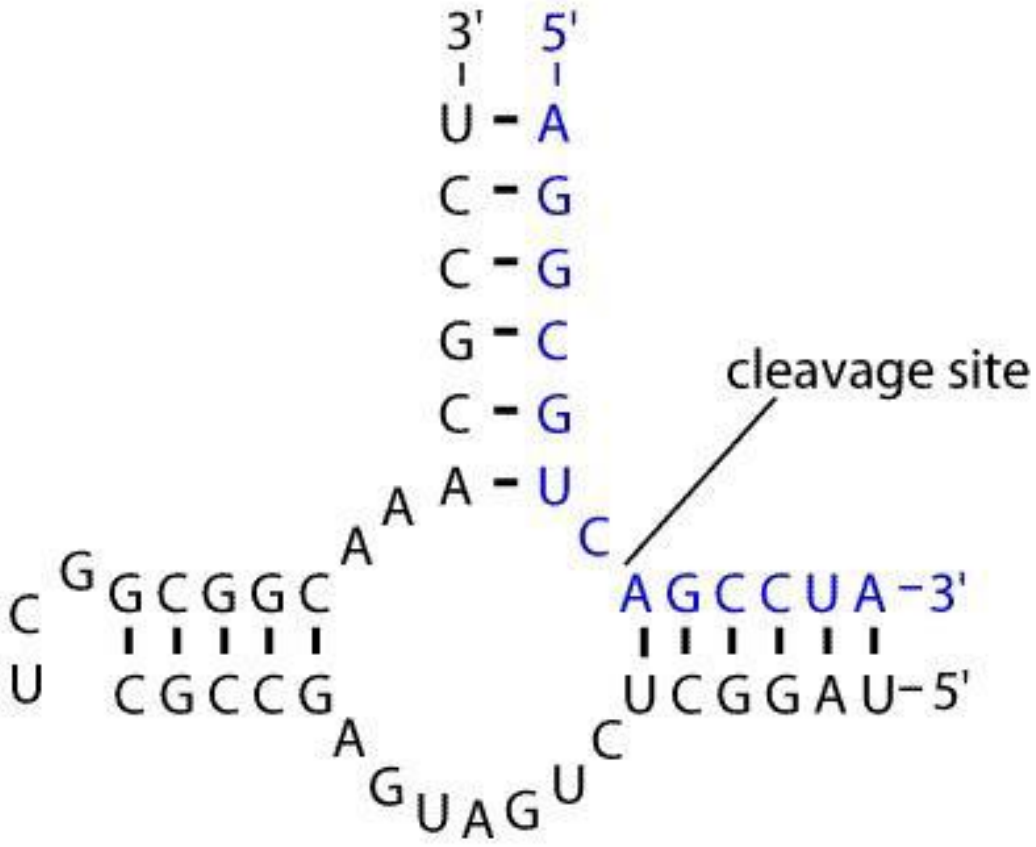


Figure 4.1c. Representative diagram of the secondary structure of a hammerhead ribozyme and its associated substrate (Figure taken from Shaw *et al*, 2001)

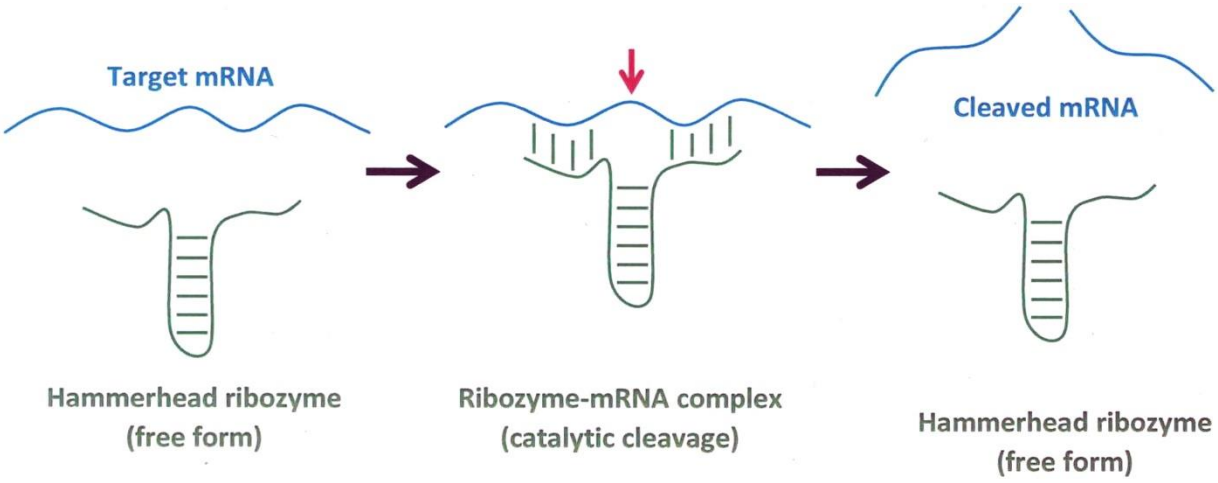


Figure 4.2 Schematic representation of the mode of action elicited on target mRNA by their specifically designed hammerhead ribozymes. The ribozyme hybridises to the substrate and enzymatically cleaves at the target site. Dissociation from the substrate sees the release of the cleaved product (Figure adapted from Mulbacher et al, 2010).

Once designed, the oligo sequences for the ribozyme transgene were synthesised by Invitrogen as sense/antisense strands (ribozyme transgene sequences are shown in Table 4.1). Incorporation of these strands into the transgene was achieved using touchdown PCR. The touchdown PCR parameters were as follows:

• Step 1: Initial denaturing period – 94°C for 5 minutes

• Step 2: Denaturing step – 94°C for 10 seconds

• Step 3: Various annealing steps – 70°C for 15 seconds, 65°C for 15 seconds, 60°C for 15 seconds, 57°C for 15 seconds, 54°C for 15 seconds and 50°C for 15 seconds.

• Step 4: Extension step – 72°C for 20 seconds

• Step 5: Final extension period – 72°C for 7 minutes

Step 2 – 4 was repeated over 48 cycles, each different annealing temperature comprising 8 cycles.

Once combined, the transgenes were electrophoresed on a 2% agarose gel to confirm presence and correct size before being inserted into the pEF6 plasmid in the TOPO cloning reaction, as described in a later section.

Target Gene	Ribozyme	Ribozyme sequence 5`-3`
WAVE 1	WAVE 1 Rib1F	CTGCAGCATCATCTTCAGCCAGCTCTGCTGATGAGTCCGTGAGGA
	WAVE 1 Rib1R	ACTAGTTGGCAGAAGCTGGCCCAAGTTTCGTCCTCACGGACT
	WAVE 1 Rib2F	CTGCAGTTCATGAGGAAGATCTACTGATGAGTCCGTGAGGA
	WAVE 1 Rib2R	CTAGTCATGACAGGCAGAAAAATTTCGTCCTCACGGACT
WAVE 3	WAVE 3 Rib1F	CTGCAGTTGTAAATATCAGCAACAGCTGATGAGTCCGTGAGGA
	WAVE3 Rib1R	ACTAGTTTCAAAGAACAGCATTCCTAATTTCGTCCTCACGGACT
	WAVE3 Rib2F	CTGCAGCCCCCTCTGGGGCCTGAGGGGCTGATGAGTCCGTGAGG
	WAVE3 Rib2R	ACTAGTCAGCCGCCCCCCCGGCGTTTCGTCCTCACGGACT

Table 4.1. Ribozyme transgene sequences used for the TOPO cloning step

4.3.2 TOPO cloning reaction

Cloning of all ribozyme transgene sequences was achieved using the pEF6/V5-His TOPO TA Expression Kit (Invitrogen, Life Technologies Ltd , UK) following the manufacturer's protocol as described here. This kit allows fast effective cloning of *Taq* polymerase amplified products for expression in mammalian cells. The following TOPO cloning reaction was set up in a pre-labelled eppendorf tube for each ribozyme transgene sequence used:

- PCR product (ribozyme transgene) – 4µl
- Salt solution – 1µl
- TOPO vector – 1µl

This reaction was gently mixed and incubated at room temperature for 30 minutes and stored in ice before proceeding to One Shot Chemical Transformation.

4.3.3 Transformation of chemically competent *Escherichia coli*

A 5µl volume from the TOPO cloning reaction outlined in Section 4.3.2 was added to a vial of One Shot TOP10 Chemically Competent *E. coli* and gently mixed by stirring the mixture in the eppendorf tube using the pipette tip as opposed to pipetting up and down to avoid damage to the bacteria. The vial was placed in ice for 30 minutes, exposed to heat-shock treatment at 42˚C for 30 seconds and immediately placed back into ice. To each tube, 250µl of SOC medium (2% Tryptone, 0.5% yeast extract, 10nM NaCl, 2.5mM KCl, 10mM MgCl2, 10mM MgSO4 and 20mM glucose) at room temperature were added followed by shaking at 200 RPM on a horizontal orbital shaker (Bibby Stuart Scientific, UK), at 37°C for 1 hour. Following this incubation period, the contents of the tube were spread at a high and low seeding density onto two separate selective agar plates containing 100µg/ml ampicillin (Melford Laboratories Ltd., Suffolk, UK) and allowed to grow overnight at 37˚C in an incubator. As the pEF6 plasmid contains two antibiotic resistance genes that allow cells containing the plasmid to grow in the presence of ampicillin and blasticidin S selection, any colonies successfully growing on these plates should theoretically contain the pEF6 plasmid (refer to Figure 4.3).

The pEF6 plasmid contains a number

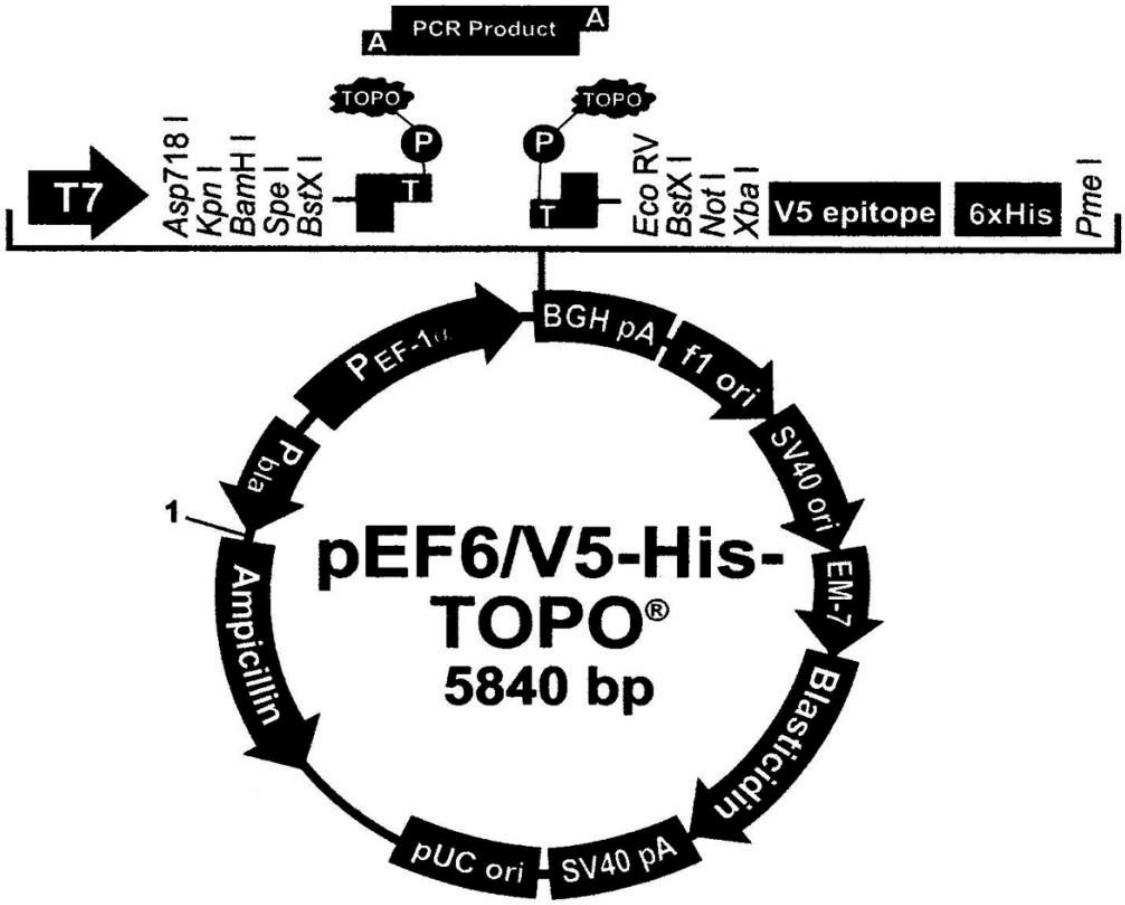


Figure 4.3 Schematic diagram of the pEF6 plasmid used during cell transfection. Figure was taken from the pEF6/V5-His TOPO TA Expression Kit protocol (Invitrogen, Life Technologies Ltd, UK)

Human elongation factor-1α (hEF-1α) promoter: a constitutive promoter of human origin that can be used to drive ectopic gene expression in various *in vitro* and *in vivo* contexts

T7 promoter/priming site: Allows for in vitro transcription in the sense orientation and sequencing through the insert

TOPO® Cloning site: Allows insertion of the PCR product in frame with the C-terminal V5 epitope and polyhistidine (6×His) tag

V5 epitope: Allows detection of the fusion protein with the Anti-V5 Antibody or the Anti-V5-HRP Antibody

C-terminal Polyhistidine (6xHis) tag: Permits purification of your fusion protein on metal-chelating resins. In addition, the C-terminal polyhistidine tag is the epitope for the Anti-His(C-term) Antibody and the Anti-His(C-term)-HRP Antibody

Bovine Growth Hormone (BGH) reverse priming site: Permits sequencing through the insert

BGH polyadenylation signal: Efficient transcription termination and polyadenylation of mRNA

F1 origin: Allows rescue of single-stranded DNA

SV40 early promoter and origin: Allows efficient, high-level expression of the blasticidin resistance gene and episomal replication in cells expressing the SV40 large T antigen

EM-7 promoter: For expression of the blasticidin resistance gene in E. coli

Blasticidin Resistance gene – Enables selection of stable transfectants in mammalian cells

SV40 polyadenylation signal: Efficient transcription termination and polyadenylation of mRNA

pUC origin: High-copy number replication and growth in E. coli

***bla* promoter**: Allows expression of the ampicillin (bla) resistance gene

Ampicillin resistance gene (β-lactamase): Selection of transformants in E. coli

4.3.4 Selection and orientation analysis of positive colonies

Confirmation of correct insertion and orientation of the ribozyme sequence in the pEF6 plasmid was analysed to ensure whether transcription of the sequence would generate the transcript of interest. The colonies were tested using polymerase chain reaction (PCR) using primers specific to either the plasmid or the ribozyme sequence. To check the orientation of the ribozyme sequences a combination of T7F vs Ribozyme specific forward primer (RbToP) and T7F vs Ribozyme specific reverse primer (RbBMR) were used (refer to Table 4.2). RbToP and RbBMR recognise and bind to sequences within the ribozyme transgene that are common to all of the ribozymes used. There are approximately 90bp between the T7F promoter and the beginning of the insert. Thus, correct orientation and ribozyme size (based on approximate ribozyme size of 50bp), would be confirmed by a band of approximately 140bp in the T7F vs RbBMR reaction. Likewise, a band of approximately 140bp in the T7F vs RbToP would indicate incorrect orientation of the sequence.

Primer name	Primer sequence
T7F	TAATACGACTCACTATAGGG
RbBMR	TTCGTCCTCACGGACTCATCAG
RbToP	CTGATGAGTCCGTGAGGACGA

Table 4.2 Plasmid/ribozyme specific primers

Following overnight incubation, the plates were examined for colony growth. Colonies were selected for orientation analysis and labelled on the Petri dishes. Two PCR reactions were carried out for each selected colony using the following parameters (full primer sequences are given in Table 4.3 shown in the Reverse transcription-Polymerase chain reaction (RT-PCR) Section 4.4.4):

Ribozyme orientation reaction 1

• 8µl – 2x GoTaq Green Master mix (Promega, Dorset, UK)

• 1µl – T7F plasmid specific forward primer

• 1µl – RbToP

• 6µl – PCR water

Ribozyme orientation reaction 2

• 8µl – 2x GoTaq Green Master mix (Promega, Dorset, UK)

• 1µl – T7F plasmid specific primer

• 1µl – RbBMR

• 6µl – PCR water

In order to test the orientation of the inserted ribozyme sequence present in the colonies, a sample was picked from the plate using a sterile pipette tip and inoculated into both mixes before the addition of the specific primers. Each reaction mix was then placed in a thermal cycler and subjected to the following conditions:

• Step 1: Initial denaturing period – 95˚C for 10 minutes

• Step 2: Denaturing step – 94˚C for 1 minute

• Step 3: Annealing step – 55˚C for 1 minute 34 cycles

• Step 4: Extension step – 72˚C for 1 minute

• Step 5: Final extension period – 72˚C for 10 minutes 105

The mixture was run on a 2% agarose gel and visualized under ultra violet light. Colonies showing correct orientation of the insert were picked off the plate, used to inoculate 10ml of ampicillin selective LB broth and incubated overnight.

4.3.5 Plasmid extraction, purification and quantification

Plasmid extraction was undertaken using the Sigma GenElute Plasmid MiniPrep Kit according to the manufacturer's protocol. Five millilitres of the LB broth, previously inoculated with the correct colony and cultured overnight, were centrifuged at approximately 1062g (3,000 RPM) for 10 minutes to obtain a pellet of bacteria. The supernatant was discarded and the bacterial pellet was re-suspended in 200µl of resuspension solution (containing RNase A) and mixed through repetitive pipetting. Two hundred microlitres of lysis solution were then added to the container and inverted 5 - 6 times. This stage was completed within 5 minutes before adding 350µl of the neutralisation solution, inverting 4 – 6 times and centrifuging at approximately 16099g (12,000 RPM) in a microcentrifuge. Plasmid DNA was bound to the column by transferring the cleared lysate to a Mini Spin Column placed inside a collection tube, spinning at approximately 16099g (12,000 RPM) for 30 seconds to 1 minute and discarding the flow through. Seven hundred and fifty microlitres of wash solution (containing ethanol) were added to the column before spinning at approximately 16099g (12,000 RPM) for 30 seconds to 1 minute and again discarding flow through. The column was spun at approximately 16099g (12,000 RPM) for 30 seconds – 1 minute to remove any remaining flow through before transferring the Mini Spin Column to a fresh collection tube.

Plasmid DNA was eluted by the addition of 100µl of elution solution and spinning the column at approximately 16099g (12,000 RPM) for 1 minute. The eluted plasmid solution was then electrophoresed on a 0.8% agarose gel to confirm presence and correct size of the plasmid.

4.3.6 Transfection of mammalian cells using electroporation

Following plasmid purification and quantification (quantification carried out utilising protocol as described for RNA quantification in Section 2.4.2 with a configuration to detect double stranded DNA and DEPC water substituted for elution solution) 1-10µg of the extracted plasmid was used to transform the colorectal cancer cell lines; RKO, CaCo2 and HRT-18. Confluent wild type cells were detached from tissue culture flasks using trypsin/EDTA, pelleted and resuspensed in the required volume of medium. Six hundred microlitres of this cell suspension was added to an electroporation cuvette (Eurgenetech, Southampton, UK) together with the purified plasmid. This was mixed briefly before being subjected to an electrical pulse of 290V and 1500 capacitance from an electroporator (Easyject, Flowgene, Surrey, UK). Following this pulse, the cell and plasmid suspension was quickly transferred into 10ml of pre-warmed medium and placed in an incubator to allow any surviving cells to fully recover from the electroporation process.

4.3.7 Establishment of stably transformed RKO, CaCo2 and HRT-18 cancer cell lines

The pEF6 plasmid used to transform the cells, encodes two antibiotic resistance genes. As previously described, the ampicillin resistance gene allows initial selection of bacterial cells containing the plasmid. The plasmid also contains a blasticidin S resistance gene. Blasticidin S is a potent microbial antibiotic that inhibits protein synthesis in both prokaryotes and eukaryotes and is used to specifically select for mammalian cells containing the pEF6 plasmid. The use of two antibiotic resistance genes allows an accurate selection of plasmid containing cells throughout the cloning process. Following overnight incubation, the cells were subjected to an initial intense selection period of 7 days. During this 7 day period, the cells were incubated in medium that had been supplemented with 5µg/ml of the blasticidin S antibiotic (Melford Laboratories Ltd., Suffolk, UK) to kill all cells that did not contain the pEF6 plasmid. After this initial intense selection the cells were maintained in maintenance medium containing 0.5µg/ml of blasticidin S, this maintains a selection pressure on the cells to retain the plasmid and results in long term transformation of the cells.

All cells were tested initially and following routine use, to estimate the efficacy and stability of both the transformation and the ribozyme transgene or expression sequence using RT-PCR and Q-PCR analysis. This methodology for altering the expression levels of various proteins within mammalian cells is well established within our research group.

4.4 Synthesis of complementary DNA for use in PCR analysis

4.4.1 Total RNA isolation

RNA isolation was completed using the TRI Reagent RNA Isolation Reagent and protocol. Cells were cultured until 70-90% confluent; medium was aspirated prior to the addition of 1ml TRI Reagent which aids detachment of the cell monolayer and induces lysis. After approximately 5 minutes at room temperature, a cell scraper was used to scrape the cell monolayer into the TRI Reagent. The cell lysate was then transferred into an Eppendorf tube. 200µl of chloroform was added to the cell lysate, the Eppendorf was shaken vigorously, inverted repeatedly for 15 seconds and then centrifuged in a refrigerated centrifuge at 4-5°C (Boeco, Germany) for 15 minutes at approximately 16099g (12,000 RPM). After centrifugation, the homogenate separates into three phases: a colourless upper aqueous phase containing the RNA molecules, a white interphase containing the DNA molecules and a red organic lower phase containing the protein molecules. The upper aqueous layer was transferred to a fresh Eppendorf tube containing 500µl isopropanol and left at room temperature for 10 minutes before centrifugation at approximately 16099g (12,000 RPM) for 10 minutes at 4˚C. RNA is insoluble in isopropanol so this step precipitates RNA out of the solution and forms a visible pellet at the bottom of the Eppendorf tube. The supernatant was discarded, 1ml 75% ethanol (3:1 ratio of ethanol to DEPC water) added to the pellet and the mixture was then centrifuged at approximately 6289g (7,500 RPM) for 5 minutes at 4˚C. The ethanol was removed leaving the pellet which was dried in a drying oven (Techne Hybridiser, UK) at 55˚C for 5-10 minutes. The remaining RNA pellet was dissolved in 40-60µl DEPC water (dependent on pellet size) via repetitive pipetting and vortexing for subsequent RNA quantification. DEPC

water was used in RNA isolation to reduce the effects of any RNases that may be present. DEPC is a histidine specific alkylating agent and inhibits the action of RNases which rely on histidine active sites for their activity

4.4.2 RNA quantification

Following isolation, RNA was quantified using a UV1101 Biotech Photometer (WPA, Cambridge, UK) that was configured to detect single stranded RNA (µg/µl) in a 1:10 dilution based on the difference in absorbance at 260nm wavelength to a DEPC blank that had been used to normalise the photometer. All samples and blanks were pipetted into a glass cuvette (StamaBrand, Optiglass Limited, UK).

4.4.3 Reverse transcription-polymerase chain reaction (RT-PCR) of RNA

The newly quantified RNA was used as template for reverse transcription to complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies Ltd, UK) following the manufacturer's protocol as described here. The RNA was prepared with PCR water to provide a final concentration of 250ng in 10µl which was added to 10µl of 2xRT master mix in a thin-walled 200µl PCR tube (ABgene, Surrey, UK). This was placed into an ABi 2720 Thermal cycler (Applied Biosystems, Life Technologies Ltd, UK) and the following parameters applied:

- **25°C for 10 minutes**
- **37°C for 120 minutes**

• 85°C for 5 minutes

The newly generated cDNA was diluted 1:4 with PCR water and stored at -20°C until required.

4.4.4 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is used for detecting and amplifying a specific target DNA sequence. PCR was carried out using GoTaq Green Master mix (Promega, Dorset, UK). PCR reactions were set up for each cDNA sample with a total reaction volume of 16µl containing the following reagents:

• 8µl – 2x GreenTaq ReadyMix PCR Reaction mix
• 1µl – Specific forward primer
• 1µl – Specific reverse primer
• 5µl – PCR water
• 1µl – cDNA

Primers were designed using the Beacon Designer programme (Palo Alto, California, USA) and were synthesised by Invitrogen (Paisley, UK). These are listed in Table 4.3. Primers were diluted to a concentration of 10pM before being used in the PCR reaction. The PCR reaction was set up in a 200µl PCR tube (ABgene, Surrey, UK), mixed briefly and centrifuged before being placed in an ABi 2720 Thermocycler (Applied Biosystems; Life Technologies Ltd, Paiseley, UK) and subjected to the following temperature programme:

• Step 1: Initial denaturing period – 94°C for 5 minutes

• Step 2: Denaturing step – 94°C for 40 seconds

• Step 3: Annealing step – 55°C for 40 seconds

• Step 4: Extension step – 72°C for 40 seconds

• Step 5: Final extension period – 72°C for 10 minutes

Steps 2 – 4 were repeated for typically 34 cycles. Primer binding sites and predicted product sizes were verified using the Primer3 (v.0.4.0) software available online (http://frodo.wi.mit.edu/). RT-PCR products which corresponded with this predicted size following electrophoresis (refer to Section 4.4.5) and staining were taken as being accurate. A negative control which replaced cDNA with PCR water was also included to assess any contamination.

Gene	Primer	Primer sequence 5`-3`
WAVE 1	**WAVE 1F11**	CCTCCTCCACCACCTCTTC
	WAVE 1R11	GCACACTCCTGGCATCAC
WAVE 2	**WAVE 2F11**	ATGCCGTTAGTAACGAGGAACATCG
	WAVE 2R11	TTAATCGGACCAGTCGTC
WAVE 3	**WAVE 3F11**	TACTCTTGCCGCTATCATACG
	WAVE 3R11	TGCCATCATATTCCACTCCTG
GAPDH	**GAPDHF8**	GGCTGCTTTTAACTCTGGTA
	GAPDHR8	GACTGTGGTCATGAGTCCTT
PDPN	**PDPNF8**	GAATCATCGTTGTGGTTATG
	PDPNZR	ACTGAACCTGACCGTACACTTTCATTTGCCTATCACAT

Table 4.3 Primer used for polymerase chain reaction. Primers listed 'ZR' include Z sequences designed for quantitative PCR WAVE1 and 3 primers were previously published (Fernando et al 2008; 2010).

4.4.5 Agarose gel electrophoresis

Amplified PCR products were separated according to product size using agarose gel electrophoresis. Generally, PCR product sizes were approximately 500bp, thus a 0.8% agarose gel was used. For PCR products amplified using primers designed for quantitative PCR (Q-PCR), these were typically 100-200bp and therefore a 2% agarose gel was used. Powdered agarose (Melford Laboratories Ltd., Suffolk, UK) was added to 1xTBE solution and heated to fully dissolve the agarose. The molten solution was poured into an electrophoresis cassette (Scie-Plas Ltd., Cambridge, UK) and prepared with plastic combs that would create loading wells once the gel had set. The set agarose gel was placed into the electrophoresis tank and submerged in 1xTBE buffer before the loading of 8µl PCR product and a 100bp DNA ladder (GenScript, New Jersey, USA) after the removal of the combs. The samples were electrophoretically separated at 95 volts for approximately 30 minutes (depending on the degree of separation required) by connecting a power pack to the electrophoretic tank (Gibco BRL, Life Technologies Inc.).

4.4.6 DNA staining and visualisation

Following successful electrophoresis, the gel was placed in ethidum bromide stain diluted in the TBE buffer used in the run. The gel was left to stain for 15 minutes before being visualised under ultra violet light using a UV illuminator (UVitech, Cambridge, UK) and capturing images using a UV camera imaging system (UVitech, Cambridge, UK). If necessary, the gel can be returned to the ethidum bromide stain for additional staining or to a container of distilled water for destaining to remove excessive background staining.

4.4.7 Quantitative RT-PCR (Q-RT-PCR)

The cDNA for use in Q-RT-PCR was generated as previously described. It was then used in the following Q-PCR reaction mix:

- Forward Z primer – 0. 3µl (1pmol/µl)
- Reverse primer - 0.3µl (10pmol/µl)
- iQSupermix (Bio-Rad, UK) - 5µl
- Probe Ampiflour – 0.3µl (10pmol/µl)
- PCR water – 2.1µl
- cDNA - 2µl

Q-RT-PCR is a sensitive technique capable of detecting very small quantities of cDNA within a sample. It allows an accurate determination of template copy number or gene expression. This technique is based on the principle of a sequence-specific DNA based fluorescent reporter probe which allows the quantification of DNA templates containing the probe sequence (Figure 4.4).

The Q-PCR protocol used in this study utilised the Amplifluor™ Uniprimer™ Universal system (Intergen company®, New York, USA) to quantify transcript copy number. The amplifluor probe carries a 3'region which is complementary to the Z-sequence (ACTGAACCTGACCGTACA) which has been incorporated into one of the primers included in the Q-PCR reaction. This is used at a 1/10 concentration of the other primer and the amplifluor probe. In addition to the Z sequence specific region found at the 3' end of the probe is the presence of a 5'hairpin structure labelled with a fluorophore tag (FAM). In this hairpin structure the fluorophore tag associates with an acceptor moiety (DABSYL) which quenches fluorescence and therefore produces no signal. During PCR, the specificity of the amplifluor's 3' region to the Z sequence present in the PCR primer generates PCR products with an incorporation of the amplifluor. This sequence itself acts as a template for subsequent steps in DNA polymerisation resulting in the disruption of the hairpin structure causing fluorescence which can be detected and quantified. The fluorescent signal emitted during Q-PCR reaction is compared to a range of standards of known transcript copy number thus allowing the calculation of transcript copy number within each sample. The same samples are also run in parallel using primers specific for the gene *GAPDH* whose transcript copy numbers are used to standardise and normalise the calculation of transcript copy number for the gene of interest in the samples.

Each sample was placed into a 96-well plate (BioRad laboratories, Hemel Hampstead, UK) in parallel with the standards mentioned previously (copy numbers ranging from 10^1 to 10^8) which would permit quantification of samples (refer to Figure 4.5). The standards used for this purpose were amplified using primers targeting the *PDPN* gene (Podoplanin). Sample cDNA was amplified and quantified over a large number of shorter cycles using an iCycler IQ thermal cycler and detection software (Bio-Rad, UK) and experimental conditions as outlined below:

- Step 1: Initial denaturing period – 95°C for 7 minutes
- Step 2: Denaturing step – 95°C for 10 seconds
- Step 3: Annealing step – 55°C for 35 seconds
- Step 4: Extension step – 72°C for 20 seconds

Step 2 – 4 was repeated over 90 cycles. The camera used in this system was set to detect fluorescent signals during the annealing stage. The calculation of sample copy number depends on the point at which the sample crosses threshold (CT) in comparison to the standards, automatically generated by the instrument software.

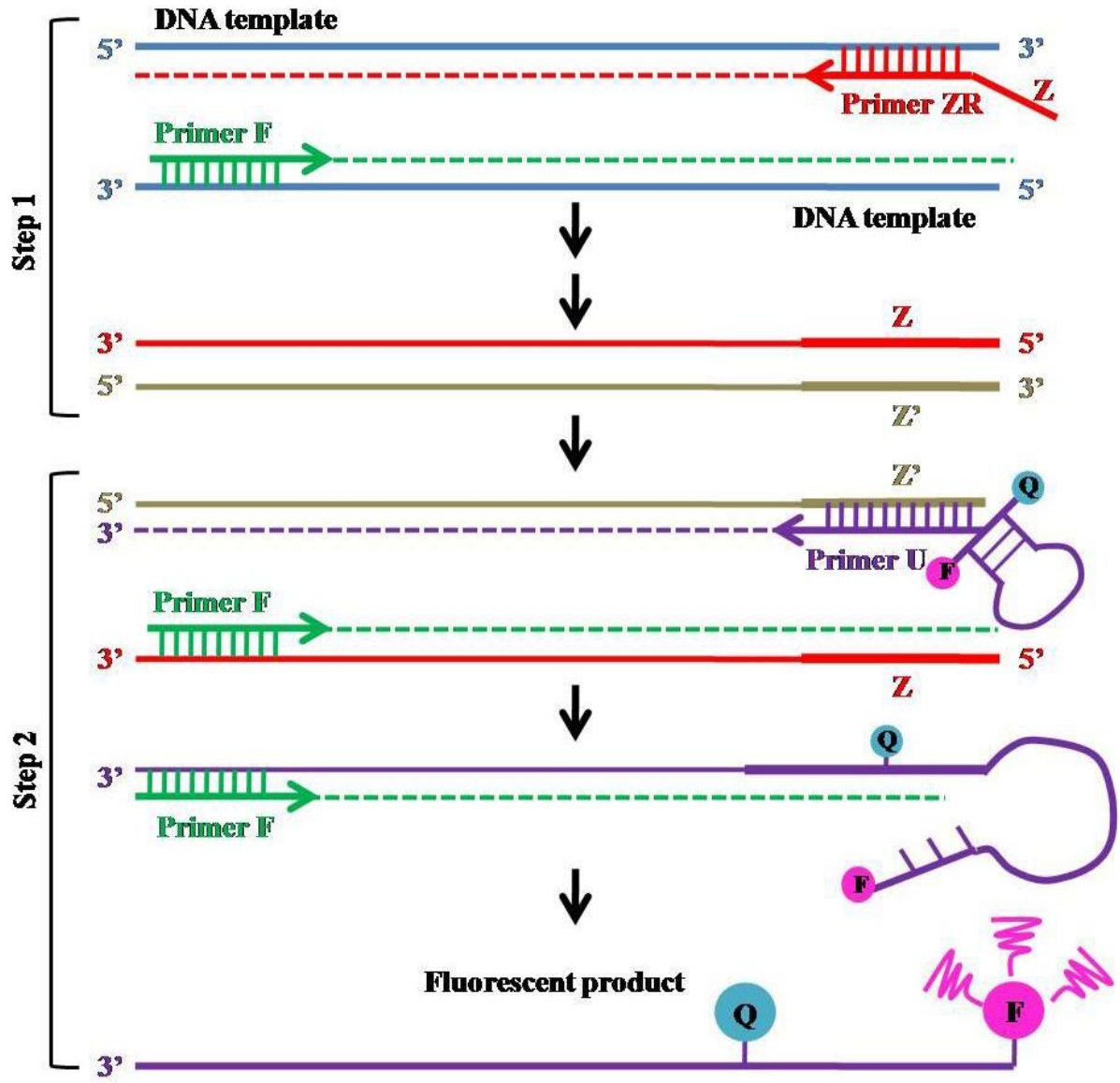


Figure 4.4 Diagram depicting the steps underlying Q-PCR when using the uniprimer fluorescent probe. In step 1 of the reaction, the extension of the target-specific primer carrying the Z-sequence addition (Primer ZR) yields a product that contains the Z sequence. When the unmodified target-specific primer (Primer F) anneals to this template and is extended, the product contains the complement of the Z-sequence (Z`-sequence). In Step 2 of the reaction, UniPrimer™ anneals to the template containing the complimentary Z` sequence. During the polymerisation reaction, the reporter [F] (Fluorescein) and the quencher [Q] (DABSYL) are incorporated into the product. This product in turn serves as a template for Primer F. As the primer is extended, the hairpin conformation of the template is unfolded. The fluorescein and DABSYL are no longer physically close enough to permit quenching and instead a fluorescent signal is emitted. Taken from (Intergen Company, 2000).

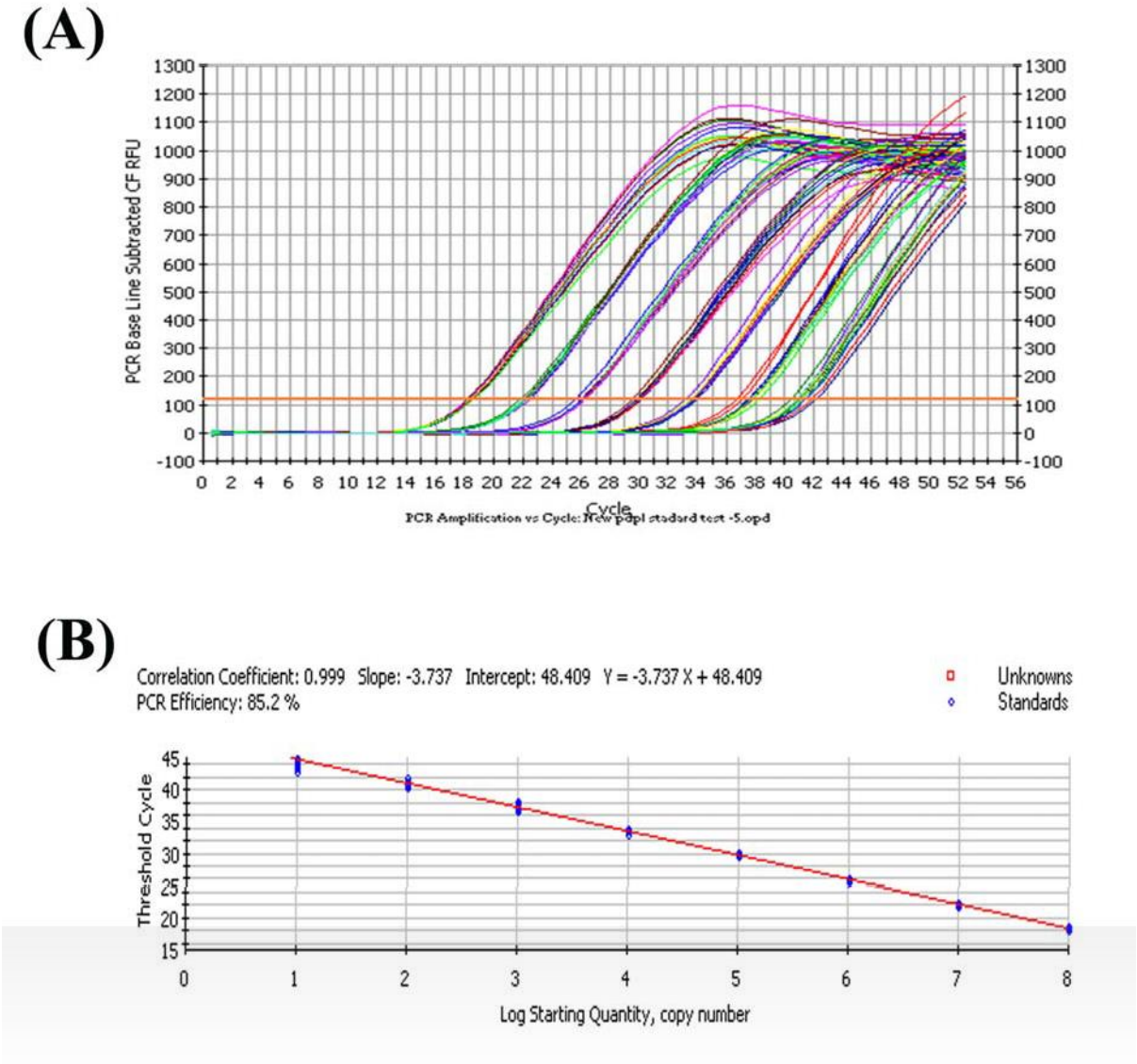


Figure 4.5 Standardisation of transcript copy number (A) Quantitative PCR was carried out on a series of standard samples ranging from 101 to 108 (B) A standard curve was generated from the standard samples and was used to determine copy number in tested samples

4.5 Tumour cell functional assays

4.5.1 *In vitro* tumour cell growth assay

Cells were detached from the culture flask and cell density (per millilitre) was established as described previously. Cells were then seeded into a 96 well plate (Nunc, Fisher Scientific, Leicestershire, UK) at a seeding density of 3,000 cells in 200µl of normal medium. Triplicate plates were set up to obtain a cell density reading following 1, 3 and 5 day incubation periods. Following the appropriate incubation period, the medium was removed and cells were fixed in 4% formaldehyde in BSS for at least 5 minutes before rinsing and staining in 0.5% (w/v) crystal violet in distilled water, for 5 minutes. The stain was then extracted from the cells using 10% acetic acid and cell density determined by measuring the absorbance at 540nm on a plate reading spectrophotometer (ELx800, Bio-Tek, Wolf laboratories, York, UK). Cell growth was presented as percentage increase and calculated by comparing the absorbances obtained for each incubation period using the following equation:

Percentage increase = ((day 3 or 5 absorbance) – day 1 absorbance / day 1 absorbance) X 100

Six replicate wells were set up for each experiment and the entire experiment was repeated at least three times. The *in vitro* cell growth assay outlined here has previously been described and is well established in our research group (Fernando *et al*., 2008).

4.5.2 *In vitro* tumour cell Matrigel invasion assay

The invasive capacity of the cells used in this study was determined using an *in vitro* Matrigel invasion assay. This assay measures the cells ability to degrade and invade through an artificial basement membrane and migrate through 8μm pores. Cell culture plate inserts (BD Biosciences, Oxford, UK) containing 8.0μm pores were coated in 50μg of Matrigel (BD Biosciences, Oxford, UK). The working concentration of Matrigel at 500μg/ml was made up in serum free medium where 100μl was added to each insert and allowed to set in a HB-1D Techne Hybridiser drying oven (Techne, Staffordshire, UK). Once dried, these inserts were placed into sterile 24 well plates and the artificial membrane was rehydrated in 200μl of serum free medium for approximately 40 minutes. Once rehydrated, the serum free medium was aspirated and 1ml of normal medium was added to the well containing the insert in order to sustain any cells that may have invaded through the insert. Twenty thousand cells in 200μl of normal medium were then added to the insert over the top of the artificial basement membrane. The plate was then incubated for 72 hours at 37˚C, 5% CO2 and 95% humidity. After 72 hours, the inserts were removed from the plate and the inside of the insert (which was initially seeded with cells) was cleaned thoroughly with tissue paper to remove Matrigel and non-invaded cells. Any cells which had invaded through the membrane and passed to the underside of the insert were fixed with 4% formaldehyde (v/v) in BSS for 5 minutes before being stained with 0.5% crystal violet solution (w/v) in distilled water. Excess crystal violet was washed away and the inserts were left to dry. These cells were then visualised under the microscope under x20 objective magnification and the random fields captured using Motic Plus 2.0 imaging software (Motic, Wetzlar, Germany). Three random fields per insert were counted and the experimental procedure was repeated a minimum of

three times. The *in vitro* cell invasion assay outlined here has previously been described and is well established in our research group (Fernando *et al*., 2008).

4.5.3 In vitro tumour cell Matrigel adhesion assay

The ability of tumour cells to adhere to an artificial Matrigel basement membrane was examined using an *in vitro* Matrigel adhesion assay. A working concentration of Matrigel at 50µg/ml was made in serum free medium whereby 100µl were pipetted into each well of a 96-well plate and placed into an oven to dry to form an artificial basement membrane. This membrane was then rehydrated in 100µl of serum free medium for 40 minutes before cell seeding. Forty five thousand cells were seeded onto the Matrigel basement membrane in 200µl of normal medium and incubated for 45 minutes. Following this incubation period, the medium was removed and the membrane washed five times with BSS to remove non- and loosely attached cells. Adherent cells were then fixed with 4% formaldehyde (v/v) in BSS for 5 minutes before being stained with 0.5% crystal violet solution (w/v) in distilled water. Adherent cells were then visualised under the microscope under x20 objective magnification and random fields captured using Motic Plus 2.0 imaging software (Motic, Wetzlar, Germany). Three random fields per insert were counted, with 6 replicate wells each run and the experimental procedure was repeated a minimum of three times. The *in vitro* cell adhesion assay outlined here has previously been described and is well established in our research group (Fernando *et al*., 2008).

4.5.4 In vitro tumour cell motility assay

A wounding/migration assay was used to assess the migratory/motility properties of the tumour cells. This technique has been modified from a previously described method (Jiang *et al*., 1999). Cells were grown in a 24 well plate and, upon reaching confluence, the monolayer of cells was scraped with a 21G needle. After wounding the cells were given 15 minutes to recover. Following this the closure of the wound via the migration of cells was captured using a CCD camera attached to a Lecia DM IRB microscope (Lecia GmbH, Bristol, UK). Images were taken at 0, 30,60,90 and 120 minute time intervals. The 24 well plate was placed on a heated plate (Lecia GmbH, Bristol, UK) to maintain a constant temperature of 37^{o}C. Cell migration was measured using Image J software. The distance between the two wound fronts at 5 random points per incubation time was calculated using the Image J software; the arbitrary values obtained were converted into µm by multiplying the value by 0.88 as previously calibrated using a calibration grid. The distance that the wound fronts had migrated into the wound at each time point could then be determined by subtracting the distance between the two fronts at any given time point from that at the initial 0 minute experimental start point. The experimental procedure was repeated at least three independent times.

4.6 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a pathological technique used to stain tissue sections for the presence of specific proteins. The technique involves the use of anti-human protein antibodies developed for use in the technique and are specific to the protein being investigated. Secondary antibodies are then used to link the primary antibody to the Avidin-Biotin staining complex (ABC) which in turn allows the binding of the staining agent 3,3`-diaminobenzidine (DAB) which indicates the location of primary antibody binding (Kroese, 2001).

4.6.1 Immuno-histochemical (IHC) staining of frozen colorectal tissues

Frozen colorectal specimens were cut into 5µm sections using a cryostat (Leica Microsystems (UK) Ltd., Bucks, UK) and were left at room temperature for 30 minutes to dry. Once dried the sections were fixed in a 1:1 mixture of acetone and methanol for 20 minutes before either storing the sections at -20°C, or staining the sections for specific protein expression.

If previously stored, the sections were allowed to reach room temperature before carrying out the staining procedure. Once sections were at room temperature a reservoir was created around the individual sections (to contain the various staining solutions) using a Dakopen^{TM} water proof marker pen, the sections were placed in phosphate buffered saline (PBS) for 5 minutes. Non-specific binding was blocked through the addition of Optimax wash buffer containing horse serum (1 drop of horse serum per 5ml of wash buffer) to the sections for 20 minutes. The sections

were washed in wash buffer 4 times before the addition of the primary antibody for 1 hour at room temperature. The primary antibodies in this method are used at their optimal dilutions determined within the host department using a standardization technique. Unbound primary antibody was removed by washing the sections 4 times in wash buffer before adding a universal secondary antibody (Vectorstain ABC Kit, Vector Laboratories Inc., Burlingame, USA) for 30 minutes at room temperature (4 drops of universal secondary per 5ml wash buffer). Again, unbound secondary antibody was removed by washing the sections in wash buffer 4 times. The sections were incubated with the ABC complex for 30 minutes at room temperature, washed 4 times in wash buffer and incubated with the DAB chromogen for 5 minutes in the dark. The DAB was removed by placing the sections in running tap water for 2 minutes before finally counterstaining the sections with Mayer's Haemotoxylin for 1 minute and placing the sections in running water for 5 minutes.

The slides are dehydrated in progressively increasing concentrations of alcohol; the alcohol is subsequently cleared from the slides by immersion in xylene and mounted with glass cover-slips.

The slides are examined under the microscope and photographed at varying magnifications and a comparison made between the carcinoma tissues and the normal colorectal tissues. An assessment of location of the subject protein and its degree of staining is made and documented.

4.7 Data collection and colorectal tissue processing

4.7.1 Colorectal tissue processing

Historical samples from deep freeze storage were retrieved that had been collected for previous studies performed in the host laboratory. A total of 174 samples had previously been collected, 80 normal colorectal tissue samples and 94 colorectal carcinoma samples with 68 of those being paired samples. These samples had been collected from a cohort of patients who had presented to local colorectal surgeons for surgical treatment of their diagnosed colorectal carcinomas. Following surgical excision, samples were retrieved and `snap' frozen in liquid nitrogen before being stored at -80°C. Median age of contributors at the time of their operation was 73 for male subjects and 74 for females with a gender distribution of 43 females and 46 males.

Tissues were sectioned on a Leica cryostat (Leica Microsystems (UK) Ltd., Bucks, UK) at a thickness of 5-10µm and mounted on electro-statically charged glass slides. Three sections were mounted per slide. Following mounting the slides were allowed to defrost and air dried for up to 30 minutes following which they were fixed in a solution of 50% Ethanol and 50% Acetone for a period of 15 minutes. The slides were air dried for approximately 30 minutes and then packaged in foil and frozen in a -20°C freezer until required for further analysis.

Tissues samples had previously been subjected to triple extraction of RNA, DNA and protein. This was achieved by taking 20 to 30 thick sections of tissue on a Leica cryostat. These were homogenized in ice cold TRI REAGENT^{TM} (Sigma Aldrich Inc.,

Saint Louis, USA) (1ml per 50-100mg of tissue) in an appropriate homogenizer. The following process was then identical to that outlined in section 4.4.1.

4.7.2 Data collection

Data sets with clinical information pertinent to patients at the time of surgical resection were available for 77 of the paired tissue samples. As part of this study, the clinical information was updated where possible. Due to the age of some of the medical records, follow-up data could only be found for 61 (79%) of the 77. Clinical data collected consisted of a number of outcomes which included patient survival, disease free time periods, development of metastatic disease and recurrence of local disease. This data looked at a follow-up time period of between 6 and 18 years, depending on when the original tissue sample was obtained.

4.8 Statistical analysis

For statistical analysis experiments were repeated at least three independent times. Statistical analysis was performed using SigmaPlot 11.0 statistical software (Systat Software Inc, London, UK). Data was stored prior to analysis and collated with clinical and demographic data using Microsoft® Office Excel 2003-2013 (Microsoft Corporation, USA).

Data was analysed using a Students two-tailed t-test and one way analysis of variance (ANOVA) if the data was found to be normalised. Normality of data to perform these parametric tests was assessed by the Sigmaplot software and if deemed non-parametric, Mann-Whitney was performed. Where the median value of a group was not informative, the inter-quartile range and mean values of the group were provided as a reference point. In all cases P-values $<$ 0.05 were considered statistically significant.

CHAPTER 5

CLINICAL SIGNIFICANCE OF WAVE IN COLORECTAL CARCINOMA:

Expression of WAVE 1, 2 and 3 in Colorectal cancer tissues

5.1 Introduction

As already discussed in chapter 2, the WASP family of proteins contain five members of which the WAVE protein family is a subgroup consisting of three of these members (WAVE 1, 2 and 3). There is evidence that the WASP family, particularly WAVE proteins, are associated with migration of a range of tumour cells, and are implicated in tumour cell invasion and metastasis (Lane et al, 2014; Fernando et al, 2009), with an aberrant WAVE expression associated with several metastatic cancers (Kurisu et al., 2005; Iwaya et al., 2007; Sossey-Alaoui et al., 2007).

This chapter aims to present the analysis of results from the quantitative gene transcript analysis (Q-PCR) experiments on human colorectal tissue samples previously collected from surgical specimens and the result of the immunohistochemistry staining experiments that looked at WAVE 1, 2 and 3 expression.

5.2 Clinical Cohort demographics

5.2.1 Patient Demographics

Historical samples from deep freeze storage were used that had been collected for previous studies performed in the host laboratory. A total of 174 samples had previously been collected, 80 normal colorectal tissue samples and 94 colorectal carcinoma samples with 68 of those being paired samples. These samples had been collected from a cohort of patients who had presented to local colorectal surgeons for surgical treatment of their diagnosed colorectal carcinomas. Median age of contributors at the time of their operation was 73 for male subjects and 74 for females with a gender distribution of 43 females and 46 males.

5.2.2 Clinico-Pathological data

Of the colorectal carcinoma samples used, 77 (82%) had complete clinico-pathological data from the time of collection (Tables 5.1 to 5.4). Metastatic disease was found in 19 out of 77 samples (24.7%).

Table 5.1	**Dukes stage data**		
	Dukes A	**Dukes B**	**Dukes C**
Sample No.'s (%)	15 (19.5%)	32 (41.6%)	30 (30.9%)

Table 5.2	**Disease T-stage distribution**			
	T1	**T2**	**T3**	**T4**
Sample No.'s (%)	8 (10.4%)	11 (14.3%)	39 (50.6%)	19 (24.7%)

Table 5.3	Nodal disease distribution			
	N0 (Node negative)	**N1**	**N2**	**Node positive (N1 & N2)**
Sample No.'s (%)	46 (59.7%)	15 (19.5%)	16 (20.8%)	31 (33%)

Table 5.4	Overall disease stage			
	Stage I	**Stage II**	**Stage III**	**Stage IV**
Sample No.'s (%)	16 (20.8%)	30 (39%)	25 (32.5%)	6 (7.8%)

During the period of this study, further clinical data was collected for those patients whose colorectal carcinoma samples were used. Due to the age of some of the medical records, follow-up data could only be found for 61 (79%) of the 77.

Clinical data collected consisted of a number of outcomes which included patient survival, disease free time periods and development of metastatic disease. This information allowed us to analyse and correlate the relationship between WAVE 1, 2 and 3 expression and disease severity. This data looked at a follow-up time period of between 6 and 18 years, depending on when the original tissue sample was obtained.

5.2.3 Histology and grade

Upon histopathological assessment, all tumour samples were found to be adenocarcinomas and the tumour grade (Well/moderately/poorly differentiated) was recorded (Table 5.5).

Table 5.5	**Histological tumour grading**		
	Well-differentiated	**Moderately-differentiated**	**Poorly-differentiated**
Sample No.'s (%)	10 (13%)	53 (68.8%)	14 (18.2%)

5.2.4 Anatomical Distribution

Analysis of the anatomical distribution of the colorectal carcinomas can be seen in table 5.6.

Table 5.6 ***Anatomical distribution of tumours***	**Sample No.'s (%)**
Rectum	22 (28.6%)
Sigmoid colon	20 (26%)
Caecum	19 (24.7%)
Descending colon	9 (11.7%)
Ascending colon	2 (2.6%)
Transverse colon	2 (2.6%)
Splenic flexure	1 (1.3%)
Hepatic flexure	1 (1.3%)

5.3 Quantitative PCR analysis of gene transcripts in colorectal samples

5.3.1 WAVE 1

Distribution of the WAVE 1 data was identified as not being normally distributed. Because of this the non-parametric Mann-Whitney test has been used for the analysis of this data. This holds true for the analysis of the WAVE 2 and 3 data that is presented later in this chapter.

Expression of WAVE 1 shows no statistically significant difference between that in normal colorectal tissues and that in carcinoma tissues (p=0.6284).

5.3.1.1 WAVE 1 expression and T-stage

There was no statistically significant difference found in the expression of WAVE 1 in normal colorectal tissues when compared with T2 to T4 stage tumours and there was no statistically significant difference in WAVE 1 expression when further comparing between T-stages (Table 5.7). T1 had a very low sample number of 2 and the statistical analysis of it was not included.

Table 5.7 Median values and significance levels of WAVE 1 expression of Normal versus T-Stage samples.

T-Stage	Median copy number per 50µg RNA (Interquartile range; Mean copy no. as reference)	Sample No.	p-Value
2	0.0000 (0.007; 0.0177)	10	0.39
3	0.00000 (0.00442; 0.00014)	40	0.13
4	0.000 (4.41; 0.245)	18	0.44

5.3.1.2 WAVE 1 expression and nodal disease

There was no statistically significant difference in the expression of WAVE 1 in normal colorectal tissues compared to those carcinoma tissues with nodal disease or without nodal disease (Table 5.8). No significant differences were found between nodal stages either.

Table 5.8 Median values and significance levels of WAVE 1 expression of Normal versus N-Stage samples.

N-Stage	Median copy number per 50µg RNA (Interquartile range; Mean copy no. as reference)	Sample No.	p-Value
N0 (Node negative)	0.00000 (0.1755; 0.00453)	39	0.17
Node positive (N1 & N2)	0.000 (4.41; 0.142)	31	0.34

5.3.1.3 WAVE 1 expression and tumour grade/differentiation

Carcinoma tissue samples graded as well, moderate and poorly differentiated show no significant difference in their WAVE 1 expression when compared to normal tissue samples (Table 5.9). There was no statistically significant difference demonstrated between different grades of tumour samples either. The sample number for the well differentiated tissue samples was only 2 and so the statistical analysis was not included.

Table 5.9 Median values and significance levels of WAVE 1 expression of Normal versus grade/differentiation of tumour samples.

Histological grade	Median copy number per 50µg RNA (Interquartile range; Mean copy no. as reference)	Sample No.	p-Value
Mod-Diff	0.00000 (0.1755; 0.00336)	54	0.16
Poorly-Diff	0.000 (4.410; 0.315)	14	0.42

5.3.1.4 WAVE 1 expression and presence of distant metastases

WAVE 1 expression showed no statistically significant differences between those tumours with metastatic disease and those without.

5.3.1.5 WAVE 1 expression and overall disease Stage

There was no statistically significant difference in WAVE 1 expression between normal colorectal tissue samples and any of the overall disease staged carcinoma tissue samples (Table 5.10).

Table 5.10 Median values and significance levels of WAVE 1 expression of Normal versus Overall Disease Stage samples.

Stage	Median copy number per 50µg RNA (Interquartile range; Mean copy no. as reference)	Sample No.	p-Value
I	0.0000 (0.1755; 0.0195)	9	0.43
II	0.00000 (0.00043; 0.00003)	30	0.13
III	0.00000 (0.00442; 0.00023)	26	0.13
IV	0.000 (4.410; 0.735)	6	0.39

There was no significant difference in WAVE 1 expression between normal colorectal tissues and any of the Dukes staged tumour tissue samples. Again, no significant difference was found between the different stages of Dukes classification.

5.3.1.6 WAVE 1 expression and Survival

There was no statistically significant correlation between WAVE 1 expression in carcinoma tissues and patient outcomes in terms of overall survival or disease free survival.

5.3.2 WAVE 2

WAVE 2 was expressed in both normal colorectal tissue samples and carcinoma tissue samples. Normal colorectal tissue samples had a median value for WAVE 2 mRNA expression of 0.000 copies per 50µg total cellular RNA. This is compared to the value for carcinoma tissues with a median value of 0.3 copies per 50µg total cellular RNA. Expression in colorectal carcinoma tissue samples was significantly higher than in normal colorectal tissues ($p<0.00005$).

This finding is echoed in analysing WAVE 2 expression in the paired tissue samples. There is a significantly higher WAVE 2 expression in the carcinoma tissue samples of a patient (median value of 0.343 copies per 50µg total cellular RNA) compared to the normal tissue samples (median value of 0.000 copies per 50µg total cellular RNA) from the same patient ($p=0.0001$).

5.3.2.1 WAVE 2 expression and T-stage

Expression of WAVE 2 was significantly higher in T-stage 3 and 4 tumours compared to normal colorectal tissues (Table 5.11).

There was no significant difference found in the expression of WAVE 2 in normal colorectal tissues when compared with T2 stage tumours. T1 stage tumours had a very small sample number of 2 and so the statistical analysis has not been included.

Table 5.11 Median values and significance levels of WAVE 2 expression of Normal versus T-Stage samples.

T-Stage	Median copy number per 50µg RNA	Sample No.	p-Value
2	0.022	10	0.5058
3	0.205	40	0.0003
4	5.09	18	<0.00005

There is an increasing difference in WAVE 2 expression as the primary tumour tissues T stage increases (p=0.007; Kruskal-Wallis Test).

5.3.2.2 WAVE 2 expression and nodal disease

WAVE 2 was expressed at significantly higher levels in all carcinoma tissues, regardless of the nodal status (Table 5.12). Despite the median copy number of WAVE 2 being lower in the primary tumours of node positive patients compared to the node negative patients, there was no statistically significant difference in WAVE 2 expression found between the nodal stages.

Table 5.12 Median values and significance levels of WAVE 2 expression of Normal versus N-Stage samples.

N-Stage	Median copy number per 50µg RNA	Sample No.	p-Value
N0 (Node negative)	0.39	39	0.0001
Node positive (N1 & N2)	0.20	31	0.0001

5.3.2.3 WAVE 2 expression and tumour grade/differentiation

Moderately and poorly differentiated colorectal carcinoma tissues express WAVE 2 at a significantly higher level than normal colorectal tissue samples (Table 5.13). Those carcinoma tissue samples graded as well differentiated had only a sample size of 2 and the statistical analysis has not been included due to the potential for them to not be representative. There was no statistically significant difference demonstrated between different grades of tumour samples.

Table 5.13 Median values and significance levels of WAVE 2 expression of Normal versus grade/differentiation of tumour samples.			
Histological grade	Median copy number per 50µg RNA	Sample No.	p-Value
Mod-Diff	0.21	54	0.0002
Poorly-Diff	0.49	14	0.0001

5.3.2.4 WAVE 2 expression and presence of distant metastases

WAVE 2 expression was significantly higher in tumours both with and without metastatic disease either at the time of surgery or having developed in the follow-up time period (Table 5.14). However, No statistically significant differences in WAVE 2 expression were found between those tumours with metastatic disease and those without.

Table 5.14 Median values and significance levels of WAVE 2 expression of Normal versus Tumour with and without the presence of distant metastatses.			
Metastases	Median copy number per 50µg RNA	Sample No.	p-Value
No distant mets	0.272	50	<0.00005
Distant mets present	1.75	19	0.0006

5.3.2.5 WAVE 2 expression and overall disease Stage

Expression of WAVE 2 was significantly higher in disease Stages II, III and IV tumours compared to normal colorectal tissues (Table 5.15).

There was no significant difference found in the expression of WAVE 2 in normal colorectal tissues when compared with Stage I tumours.

There was a statistically significant difference in WAVE 2 expression when comparing Stage I tumours to Stage II and IV tumours. The low copy number of the stage III tumours means that there is no significant difference in the WAVE 2 expression when comparing them and stage I tumours. The reason for the low Stage III copy number is unclear and may be attributable to a random error.

Table 5.15 Median values and significance levels of WAVE 2 expression of Normal versus Overall Disease Stage samples.

Stage	Median copy number per 50µg RNA	Sample No.	p-Value
I	0.002	9	0.5975
II	0.53	30	<0.00005
III	0.05	26	0.0031
IV	1.83	6	0.0009

Expression of WAVE 2 was significantly higher in carcinoma tissue samples from Dukes stage B and C tumours compared to normal colorectal tissues (Table 5.16). There was no significant difference in WAVE 2 expression between normal colorectal tissues and Dukes stage A tumour tissue samples. No statistically significant difference was found in the WAVE 2 expression between Dukes stages B and C, however, there was a significant difference between Dukes stage A when compared to stages B and C (p=0.0062).

Table 5.16 Median values and significance levels of WAVE 2 expression of Normal versus Dukes-Stage samples.

Dukes-Stage	Median copy number per 50µg RNA	Sample No.	p-Value
A	0.000	7	0.8755
B	0.49	33	<0.00005
C	0.229	32	0.0001

5.3.2.6 WAVE 2 expression and Survival

WAVE 2 expression correlated with survival (Figure 5.1). Those with high expression in the tumour tissue had a significantly lower mean survival period (33 months) than those with a low expression (136 months) (p=0.004).

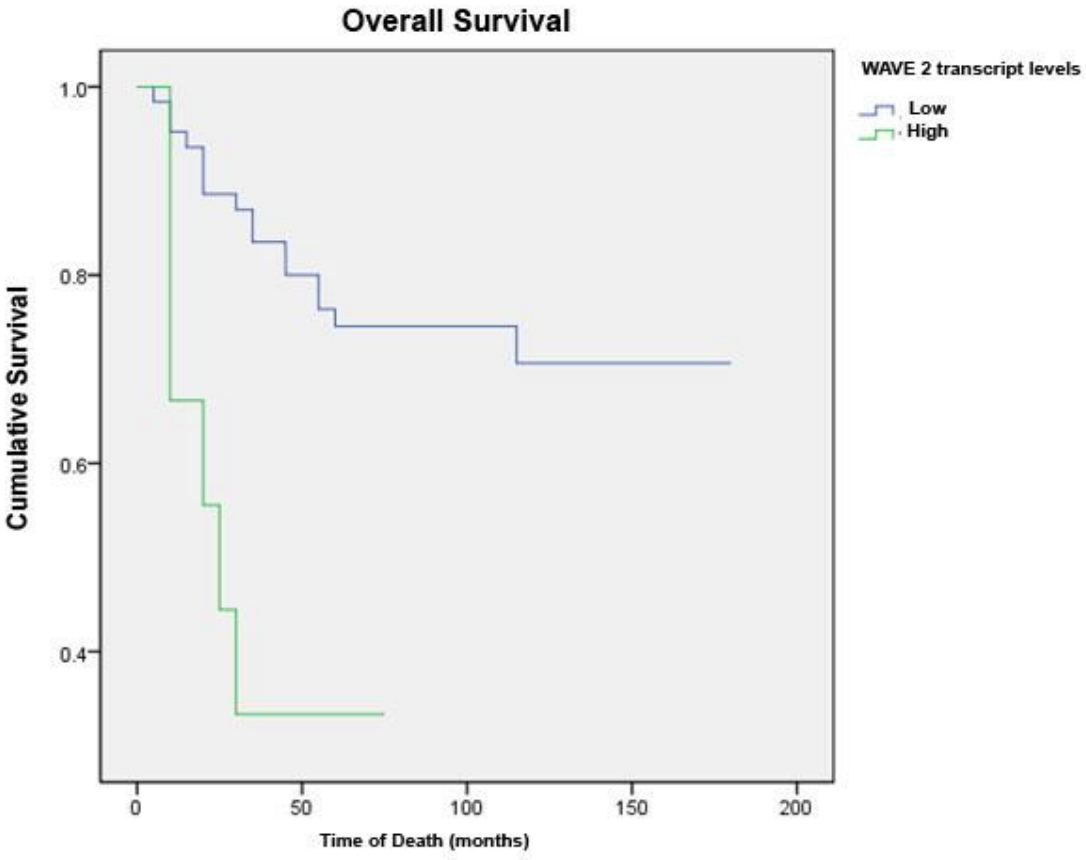


Figure 5.1 – Overall Survival when correlated with WAVE 2 expression

WAVE 2 expression correlated with Disease Free Survival (Figure 5.2). Those with high expression had a significantly shorter disease Free survival period (30 months) than those with low expression (131 months) (p=0.003).

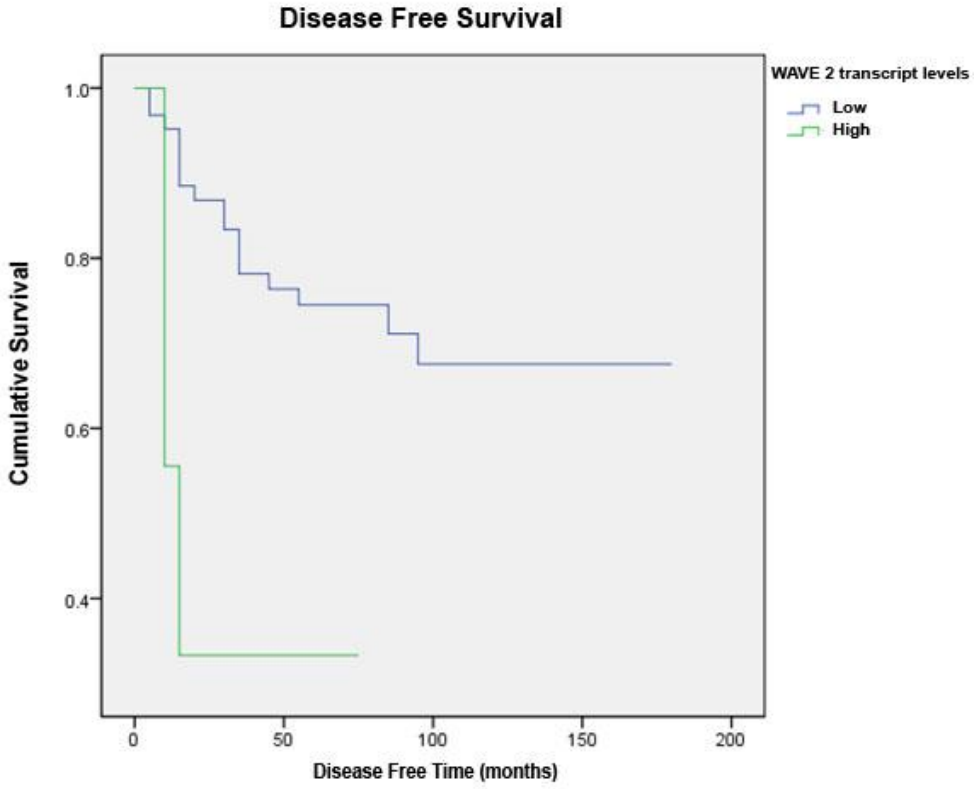


Figure 5.2 – Disease Free Survival when correlated with WAVE 2 expression

5.3.4 WAVE 3

WAVE 3 was expressed in both normal colorectal tissue samples and carcinoma tissue samples. Expression in colorectal carcinoma tissue samples was significantly higher than in normal colorectal tissues ($p<0.00005$). The median values for both the normal and tumour tissues are unhelpful in this situation and so the mean expression in normal tissues is 678 copies with an interquartile range of 0. In contrast the mean expression in tumour tissues is 46336 copies with an interquartile range of 5.

This finding is echoed in analysing WAVE 3 expression in the paired tissue samples. There is a significantly higher WAVE 3 expression in the carcinoma tissue samples of a patient compared to the normal tissue samples from the same patient ($p<0.00005$).

5.3.3.1 WAVE 3 expression and T-stage

Expression of WAVE 3 was significantly higher in T-stage 2, 3 and 4 tumours compared to normal colorectal tissues (Table 5.17). T1 stage tumours had a very small sample number of 2 and so the statistical analysis has not been included.

There is no significant difference in WAVE 3 expression as the primary tumour tissues T-stage increases (p=0.557).

Table 5.17 Median values and significance levels of WAVE 3 expression of Normal versus T-Stage samples.

T-Stage	Median copy number per 50µg RNA (Interquartile range; Mean copy no. as reference)	Sample No.	p-Value
2	0.03 (27.2; 13.96)	10	0.0006
3	0 (1; 106590)	40	<0.00005
4	0.0 (2410; 136)	18	0.0005

5.3.3.2 WAVE 3 expression and nodal disease

WAVE 3 was expressed at significantly higher levels in all carcinoma tissues, regardless of the nodal status (Table 5.18). No significant differences were found between nodal stages.

Table 5.18 Median values and significance levels of WAVE 3 expression of Normal versus N-Stage samples.

N-Stage	Median copy number per 50µg RNA (Interquartile range; Mean copy no. as reference)	Sample No.	p-Value
N0 (Node negative)	0 (1; 109183)	39	<0.00005
Node positive (N1 & N2)	0 (2; 260)	31	<0.00005

5.3.3.3 WAVE 3 expression and tumour grade/differentiation

Moderately and poorly differentiated colorectal carcinoma tissues express WAVE 3 at a significantly higher level than normal colorectal tissue samples (Table 5.19). Those carcinoma tissue samples graded as well differentiated had a very small sample size of 2 and so the statistical analysis was not included. There was no significant difference in WAVE 3 expression demonstrated between different grades of tumours.

Table 5.19 Median values and significance levels of WAVE 3 expression of Normal versus grade/differentiation of tumour samples.

Histological grade	Median copy number per 50µg RNA (Interquartile range; Mean copy no. as reference)	Sample No.	p-Value
Mod-Diff	0 (1; 41965)	54	<0.00005
Poorly-Diff	0 (2; 142860)	14	<0.00005

5.3.3.4 WAVE 3 expression and presence of distant metastases

WAVE 3 expression was significantly higher in tumours both with and without metastatic disease either at the time of surgery or having developed in the follow-up time period (Table 5.20). However, No significant differences in WAVE 3 expression were found between those tumours with metastatic disease and those without (p=0.3933).

Table 5.20 Median values and significance levels of WAVE 3 expression of Normal versus presence of distant metastatses.

Metastases	Median copy number per 50µg RNA (Interquartile range; Mean copy no. as reference)	Sample No.	p-Value
No distant mets	0 (7; 85716)	50	<0.00005
Distant mets present	0.0 (4820; 259)	19	0.0005

5.3.3.5 WAVE 3 expression and overall disease Stage

Expression of WAVE 3 was significantly higher in all disease stages compared to normal colorectal tissues (Table 5.21).

There was no statistically significant difference found in the expression of WAVE 3 when comparing between the different stages.

Table 5.21 Median values and significance levels of WAVE 3 expression of Normal versus Overall Disease Stage samples.

Stage	Median copy number per 50µg RNA (Interquartile range; Mean copy no. as reference)	Sample No.	p-Value
I	0.00 (7.19; 6.79)	9	0.0449
II	0 (2; 141936)	30	<0.00005
III	0.0 (1.2; 124.6)	26	<0.00005
IV	2.6 (2255; 1038)	6	0.0006

Expression of WAVE 3 was significantly higher in carcinoma tissue samples from all Dukes stage tumours compared to normal colorectal tissues (Table 5.22). Again, no significant difference was found between the different stages of Dukes classification (p=0.557).

Table 5.22 Median values and significance levels of WAVE 3 expression of Normal versus Dukes-Stage samples.

Dukes-Stage	Median copy number per 50µg RNA (Interquartile range; Mean copy no. as reference)	Sample No.	p-Value
A	0.00 (14.2; 8.72)	7	0.012
B	0 (1; 129033)	33	<0.00005
C	0.0 (4; 296)	32	<0.00005

5.3.3.6 WAVE 3 expression and Survival

There was no statistically significant correlation between WAVE 3 expression in carcinoma tissues and patient outcomes in terms of overall survival or disease free survival.

5.4 Immunohistochemistry Results

Immunohistochemistry (IHC) results are presented with a descriptive analysis. This is because the quantitative analysis has been performed using qPCR methods and the results are as already shown. This method of analysis allows the distribution of the specified molecules through the cells and the cell structures to be studied rather than just how much of the molecule is present.

5.4.1 WAVE 1

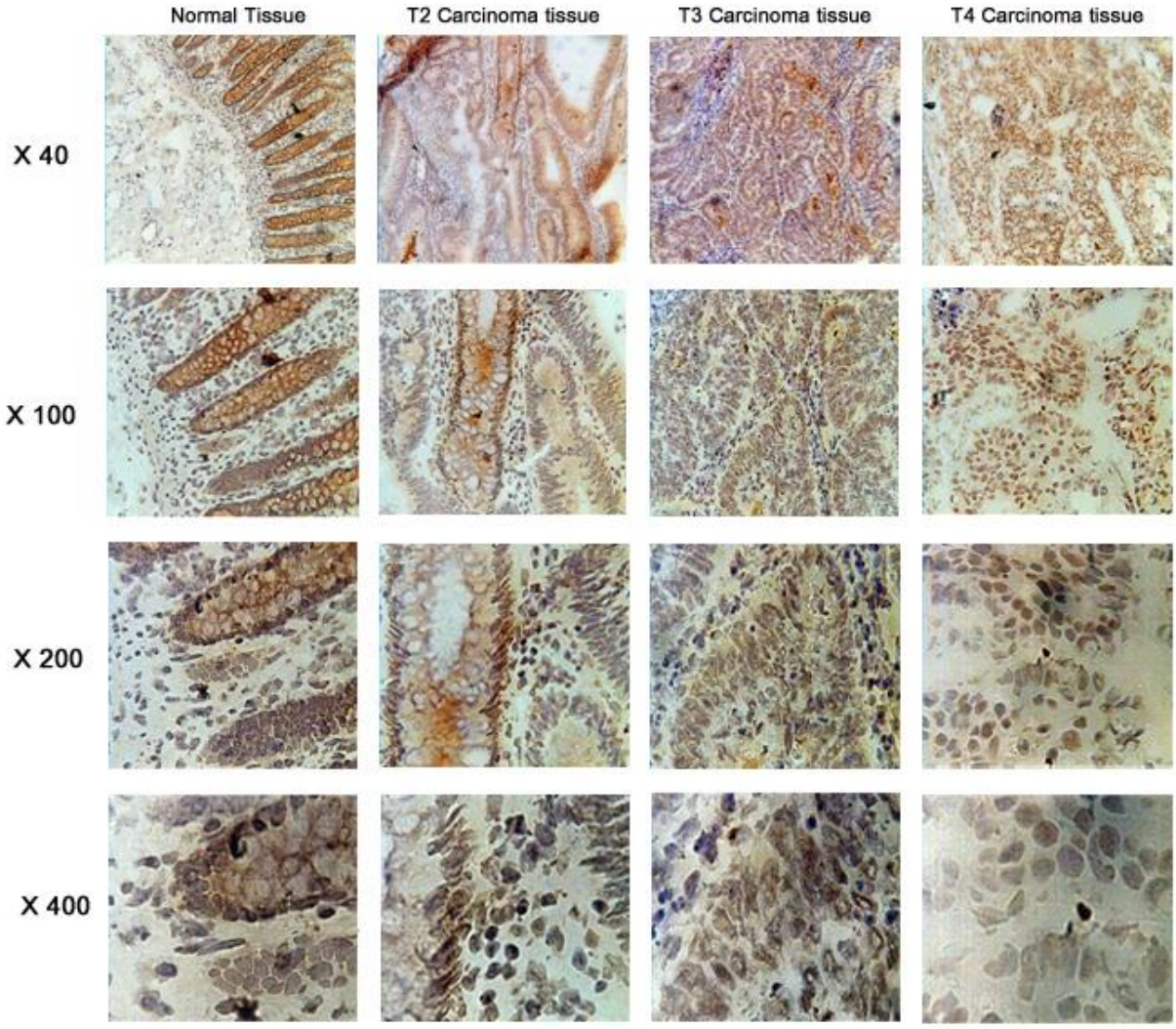
5.4.1.1 WAVE 1 in normal colorectal tissues

In the normal colorectal tissues, the distribution of WAVE 1 on IHC staining is shown to be distributed within the cytoplasm of the epithelial cells that line the glands and crypts. This is along the luminal surfaces and would fit with the normal physiology of colorectal mucosa as the luminal epithelial cells within the crypts migrate towards the surface mucosa as they mature until they are eventually shed. There is no staining found in the stromal areas (Figure 5.3).

5.4.1.2 WAVE 1 in colorectal carcinoma tissues

The colorectal carcinoma tissues demonstrated a subtle difference in the staining of WAVE 1 compared to the normal colorectal tissues. In the carcinoma tissues there was still the cytoplasmic staining along the luminal surfaces of the crypts epithelial cells but this became less intense as the T- stage increased. However, there was a change in the staining distribution within the tissues with an increasing cell membrane distribution in the more basal cell layers of the crypts that border the stroma. This appearance became more pronounced the higher the T-stage of the tumour tissue. In addition, with the higher T-stages came a very small amount of staining found in the stromal areas (Figure 5.3).

Figure 5.3 - WAVE 1 immunohistochemistry of normal colorectal tissue and colorectal carcinoma tissue of varying T stages at a selection of magnifications



5.4.2 WAVE 2

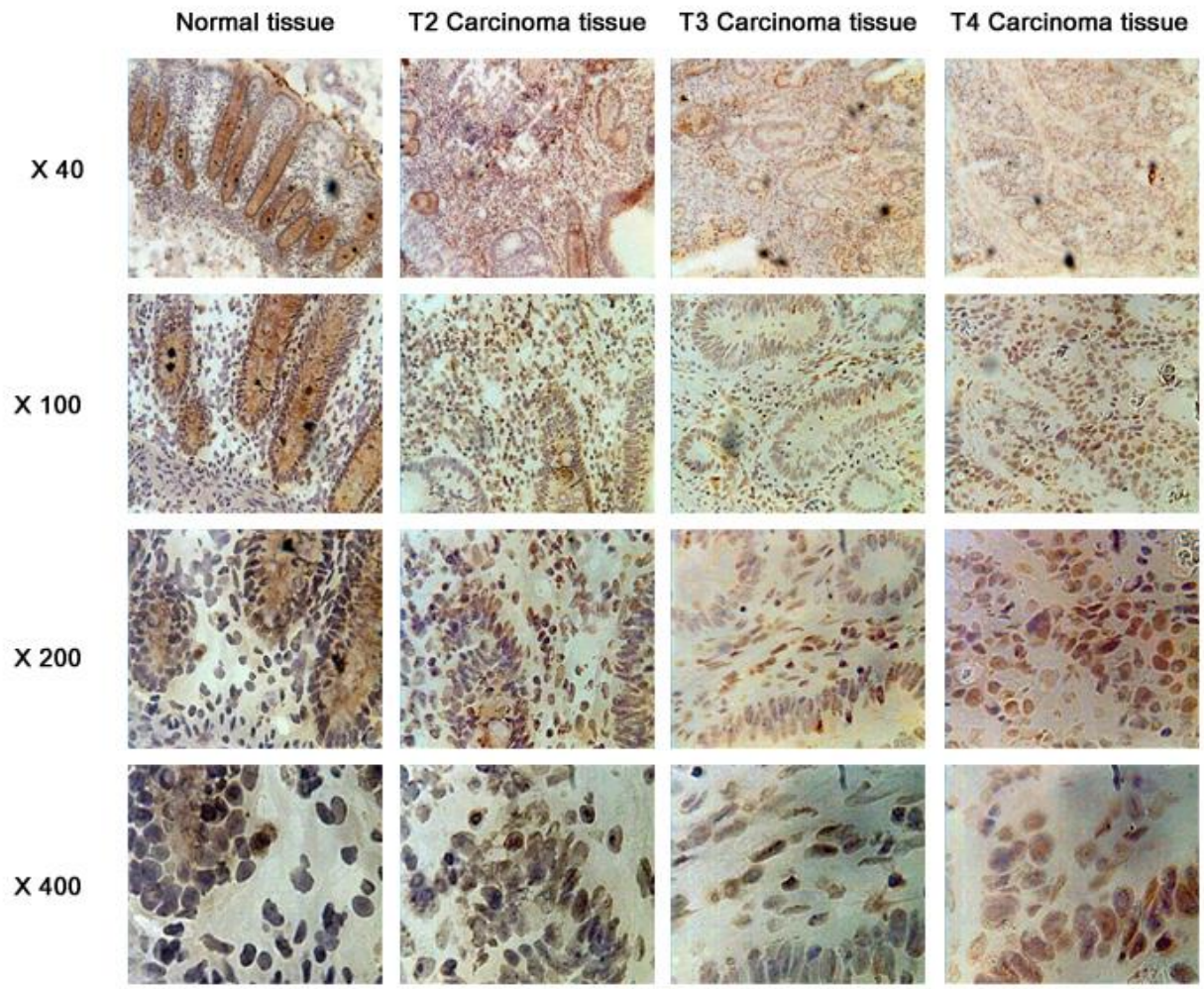
5.4.2.1 WAVE 2 in normal colorectal tissues

In the normal colorectal tissues, the distribution of WAVE 2 on IHC staining is again shown to be distributed within the luminal cytoplasm of the epithelial cells that line the glands and crypts and along the mucosal surface. There is very little to no staining found in the stromal areas (Figure 5.4). On higher power it is possible to see darker staining at the polar membrane surfaces of some cells which would be in keeping with the distribution required for cell motility and migration as described in chapter 2.

5.4.2.2 WAVE 2 in colorectal carcinoma tissues

The colorectal carcinoma tissues demonstrated a more substantial difference in the staining of WAVE 2 distribution compared to the normal colorectal tissues. In the carcinoma tissues there was still the cytoplasmic staining along the luminal surfaces of the crypts epithelial cells. However, there was an increase in the depth of staining present suggesting larger amounts of the WAVE 2 molecule being present and there also appeared to be a significant increase in cell membrane distribution along the more basal layers of the crypts, bordering the stroma. This appearance became significantly more pronounced the higher the T-stage of the tumour tissue. With the higher T-stages came a larger amount of cytoplasmic and membrane staining within the stromal areas of these tissues (Figure 5.4).

Figure 5.4 - WAVE 2 immunohistochemistry of normal colorectal tissue and colorectal carcinoma tissue at varying T-stages at a selection of magnifications



5.4.3 WAVE 3

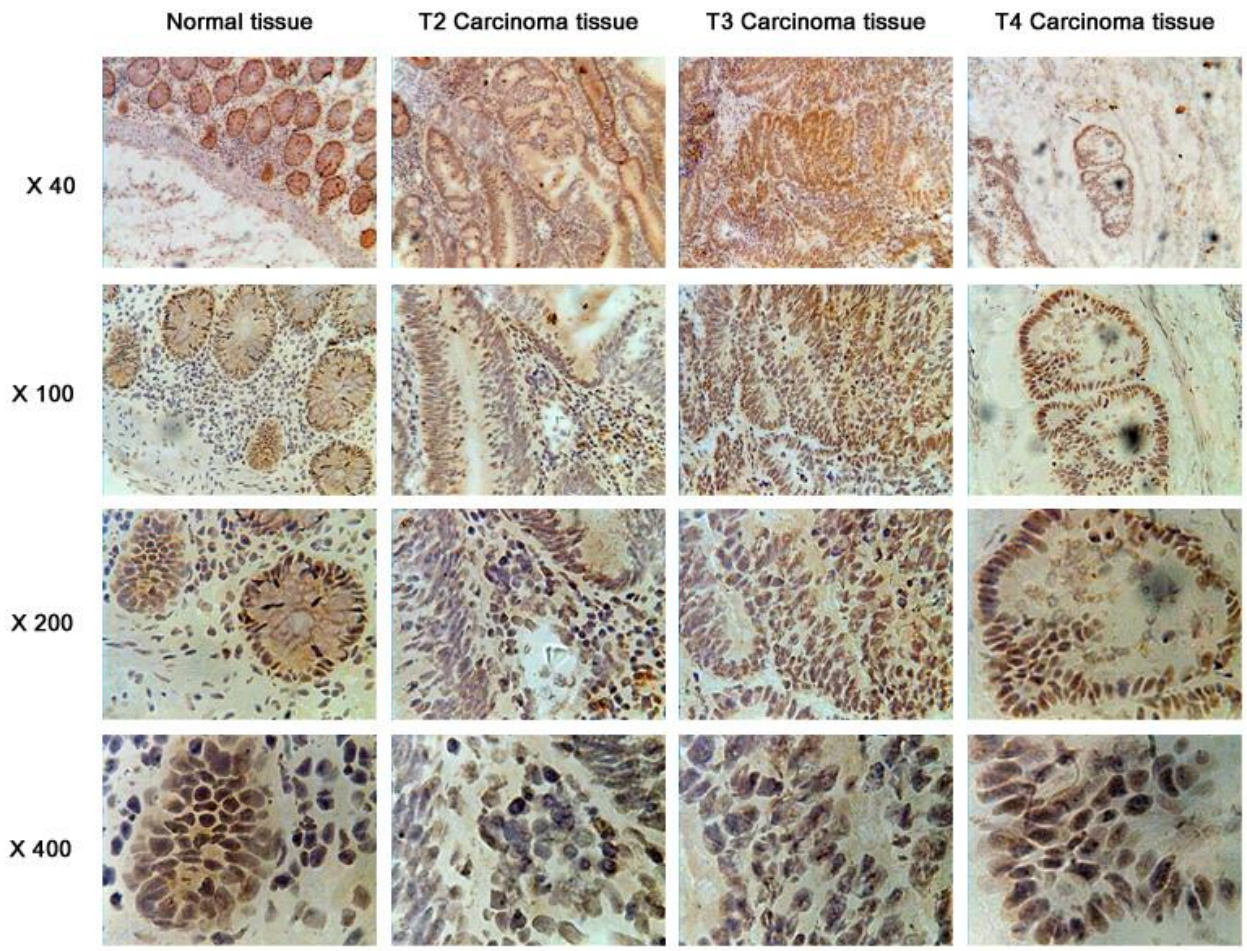
5.4.3.1 WAVE 3 in normal colorectal tissues

In the normal colorectal tissues, the distribution of WAVE 3 on IHC staining is again shown to be distributed within the luminal cytoplasm of the epithelial cells that line the glands and crypts and along the mucosal surface. There is a little staining found in the cytoplasm of some cells within the stromal areas (Figure 5.5).

5.4.3.2 WAVE 3 in colorectal carcinoma tissues

Again, the colorectal carcinoma tissues demonstrated a more substantial difference in the staining of WAVE 3 distribution compared to the normal colorectal tissues. The appearances were very similar to WAVE 2. In the carcinoma tissues there was cytoplasmic staining along the luminal surfaces of the crypts epithelial cells with a significant increase in cell membrane distribution along the basal cell layers of the crypts. This was associated with an increase in the depth of staining present suggesting larger amounts of the WAVE 3 molecule being present. This appearance became significantly more pronounced the higher the T-stage of the tumour tissue. With the higher T-stages came a larger amount of cytoplasmic and membrane staining within the stromal areas of these tissues (Figure 5.5).

Figure 5.5 - WAVE 3 immunohistochemistry of normal colorectal tissue and colorectal carcinoma tissue at varying T-stages at a selection of magnifications



5.5 Discussion

The expression of WAVE 1, 2 and 3 was examined in a number of available clinical colorectal samples. They were examined using Q-PCR techniques and frozen IHC staining.

For WAVEs 2 and 3, their respective protein expression was at its lowest in the normal colorectal tissues as shown by Q-PCR and as seen on the frozen sections. The IHC stained sections showed that this expression was very focally located within the luminal cytoplasm of the epithelial cells lining the colonic crypts and glands, with some minimal expression in the cytoplasm of occasional stromal cells.

Within the colorectal carcinoma tissues, the expression profiles varied between the WAVE proteins.

WAVE 1 expression stained for on the frozen sections showed only a subtle change between normal colorectal tissues and colorectal carcinoma tissues. These changes were mainly in distribution of expression rather than in amount of expression. The staining pattern that was present moved to involve the cytoplasm of the basal cell layers of the crypts, rather than the luminal, and a very small amount was seen within the cytoplasm of some stromal cells. This is potentially in keeping with the Q-PCR results which actually showed that there was no statistically significant change in overall WAVE 1 expression between the normal colorectal tissues and the colorectal carcinoma tissues. As the IHC results show, it may be that the expression levels remained the same but the distribution of that expression changed.

These findings are in contrast to other studies which have shown an increase in the expression of WAVE 1 in breast and prostate cancer (Fernando et al., 2007; 2008)

WAVE 2 expression stained for on the frozen sections demonstrated a more substantial difference when compared to the normal colorectal tissue sections and indeed in comparison to WAVE 1 staining. Within the T2 stage tumours there was still the luminal cytoplasmic staining of the epithelial cells along the crypts but there was an increase in the intensity of the staining which suggested an increase in WAVE 2 expression. In addition, this intense staining now also included a cytoplasmic and cell membrane distribution within the basal cell layers of the crypts. Although not as intense, a cytoplasmic distribution was also seen within the stromal cells. These appearances became more pronounced with deeper staining, the higher the T-stage of the tumour tissue.

The Q-PCR results were somewhat in keeping with the IHC staining with a significantly higher WAVE 2 expression level found in carcinoma tissues compared to normal colorectal tissues. On analysis of WAVE 2 expression between the different T-stages of tumour tissues, it was found that there was a significant difference in the expression levels between the stages and there was an increase in WAVE 2 expression as the carcinoma T-stage increased (T3 and T4). In the lower, T2, stage there was no significant difference between WAVE 2 expression in the carcinoma tissues compared to normal tissues.

There was a higher WAVE 2 expression in the less histologically differentiated tumours (moderately differentiated and poorly differentiated) than compared to

normal tissues. WAVE 2 expression is significantly higher in the tumours of patients with a higher overall disease stage (stage II, III and IV) compared to normal colorectal tissues and stage I carcinoma tissues. Additionally, WAVE 2 expression is significantly higher in the carcinoma tissues of those patients classified as having Dukes B and C stage disease compared to normal colorectal tissue expression and Dukes A stage carcinoma tissues.

The WAVE 2 expression showed no difference in tissues from tumours that had nodal disease compared to those that did not have nodal disease. In addition, there was no difference in WAVE 2 expression from tumour tissues that had metastatic disease compared to those that did not have metastatic disease. The possibility here is that the WAVE 2 expression being analysed is from the primary tumour site and may not be representative of the cell phenotypes which have actually metastasised via the lymphatics and vasculature. Or in addition it could be that low numbers in certain categories may have impacted the statistical analysis.

The final point for WAVE 2 expression is that in those colorectal carcinoma tissues analysed, a higher WAVE 2 expression was associated with a significantly lower mean survival period (33 months) and a shorter disease free survival period (30 months) compared to those with a low WAVE 2 expression who had a longer mean survival period (136 months) and disease free survival period (131 months).

These findings for WAVE 2 fit with earlier studies, whereby there is increased WAVE 2 expression in hepatocellular and breast cancer (Yang et al., 2006; Fernando et al., 2007) associated with shorter survival times and a higher WAVE 2 co-localisation level and co-expression level with Arp 2 in colorectal cancer and lung

adenocarcinoma when associated with metastases (Iwaya et al., 2007; Semba et al., 2006).

WAVE 3 expression stained for on the frozen section again demonstrated a more substantial difference when compared to the normal colorectal tissue sections. The appearances were very similar to the WAVE 2 distribution and intensity. In the carcinoma tissues there was cytoplasmic staining along the luminal surfaces of the crypts epithelial cells with a significant increase in cell membrane distribution along the basal cell layers of the crypts. This was associated with an increase in the depth of staining present suggesting an increase in WAVE 3 expression. This appearance became significantly more pronounced the higher the T-stage of the tumour tissue. With the higher T-stages also came a larger amount of cytoplasmic and membrane staining within the stromal areas of these tissues.

The Q-PCR results were somewhat in keeping with the IHC staining with significant WAVE 3 expression levels found in carcinoma tissues compared to normal colorectal tissues. On analysis of WAVE 3 expression between the different T-stages of tumour tissues, there was higher expression in T2, 3 and 4 tumours compared to normal colorectal tissue.

There was a higher WAVE 3 expression in the less histologically differentiated tumours (moderately differentiated and poorly differentiated) than compared to normal colorectal tissues. WAVE 3 expression is significantly higher in the tumours of patients with a higher overall disease stage (stage II, III and IV) compared to normal colorectal tissues and stage I carcinoma tissues. Additionally, WAVE 3 expression is significantly higher in the carcinoma tissues of those patients classified

as having Dukes B and C stage disease compared to normal colorectal tissue expression and Dukes A stage carcinoma tissues.

Finally, WAVE 3 expression showed no difference in tissues from tumours that had nodal disease compared to those that did not have nodal disease. In addition, there was no difference in WAVE 3 expression from tumour tissues that had metastatic disease compared to those that did not have metastatic disease.

A similar pattern of WAVE 3 expression has been found in breast and prostate cancer, with high WAVE 3 levels being expressed in advanced breast cancer tissues (Sossey-Alaoui et al., 2007) and high WAVE 3 expression levels found in prostate cancer tissues compared to normal prostate tissues along with stronger IHC staining in the cancer tissues (Fernando et al., 2008; 2010).

CHAPTER 6

RESULTS

CELL CULTURE EXPERIMENTS

6.1 Introduction

As discussed in chapter 2, the WAVE proteins have been linked with the aggressiveness and invasiveness of cancer cells, in relation to their function as nucleators of actin through their association with the Arp2/3 complex, driving lamellipodia formation and cell motility (Fernando et al., 2009).

Cell motility is an essential mechanism that underlies numerous cellular processes and is physiologically important in normal, healthy cells. However, it also has the ability to drive the migration of abnormal cells and when unregulated contributes to the aggressive spread of tumour cells and is acknowledged as a promoting aspect of cancer metastasis (Wang et al., 2007; Liotta, 1986). The gene expression profiles of invasive tumour cells were compared to those of the primary tumour cell population in an *in vivo* study that utilised a xenograft tumour model. The results showed an up-regulation of genes involved in cell motility pathways including those of actin polymerisation. These included the Arp2/3 complex as well as Cdc42, an upstream stimulator of Arp2/3 found to regulate N-WASP (Wang et al., 2007).

A large amount of the work in the cancer metastasis field has focused on the importance of the Rho GTPases and the part they play in cellular motility (Chapter 2). The role played by proteins further down in the motility pathway have been investigated to a lesser extent. However, as more evidence emerges to link the WASP family proteins with cancer cell motility and the findings of their upregulated expression in aggressive and metastatic cancers, it is important to explore the potential contribution of WAVE proteins in cancer metastasis (Lane et al, 2014; Fernando et al, 2009; Kurisu et al., 2005; Iwaya et al., 2007; Sossey-Alaoui et al., 2007).

6.2 Methods and materials

6.2.1 Cell lines

RKO, HRT-18 and CaCo2 cell lines were cultured and maintained as described in section 4.2.4.

6.2.2 Generation of WAVE 1 and 3 knockdown colorectal cancer cell lines

Hammerhead ribozyme transgenes that specifically target and cleave WAVE 1 or 3 mRNA transcripts were generated. The WAVE 1 targeted ribozyme was cloned into the RKO, CaCo2 and HRT-18 colorectal cancer (CRC) cell lines and the WAVE 3 targeted ribozyme was cloned into the CaCo2 CRC cell line.

The protocols followed to generate ribozymes, for insertion into the plasmid vector, to amplify the plasmid in *E.coli*, for plasmid extraction and for electroporation to introduce the plasmids into the cell lines are outlined in section 4.3.

6.2.3 Synthesis of complementary DNA and reverse transcription polymerase chain reaction

See section 4.4.3. RNA was isolated from wild type RKO, HRT-18 and CacCo2 cells and their plasmid transfected equivalents. Complementary DNA (cDNA) was generated from the standardised RNA extractions using reverse transcription polymerase chain reaction (RT-PCR). This then enabled subsequent analysis of the degree of WAVE 1, 2 and 3 expression in the three CRC cell lines, using conventional and quantitative PCR techniques and primer sequences as shown in

table 4.3 in the general methods section. GAPDH expression was also examined in the cell lines to validate the cDNA quality and to demonstrate normalised levels of cDNA within the separate cell line samples. All primers were synthesised and provided by Invitrogen (Paisley, UK).

6.3 Results

6.3.1 Expression of WAVE 1, 2 and 3 at the mRNA level in colorectal cancer cell lines

The results of the PCR screen demonstrating the expression of WAVEs 1, 2 and 3 are shown in figure 6.1. WAVE 1 expression was seen in all of the CRC cell lines with the CaCo2 and RKO cell lines showing the strongest expression of this transcript. WAVE 3 expression was only demonstrated in the CaCo2 cell line. Unfortunately, the WAVE 2 primers consistently failed and so an expression profile was unable to be obtained for the CRC cell lines. It was decided to proceed with the knockdown of WAVE 1 and 3 expression in the relevant CRC cell lines with the option to attempt knockdown of WAVE 2 at a later date should a successful primer be designed within the duration of this study.

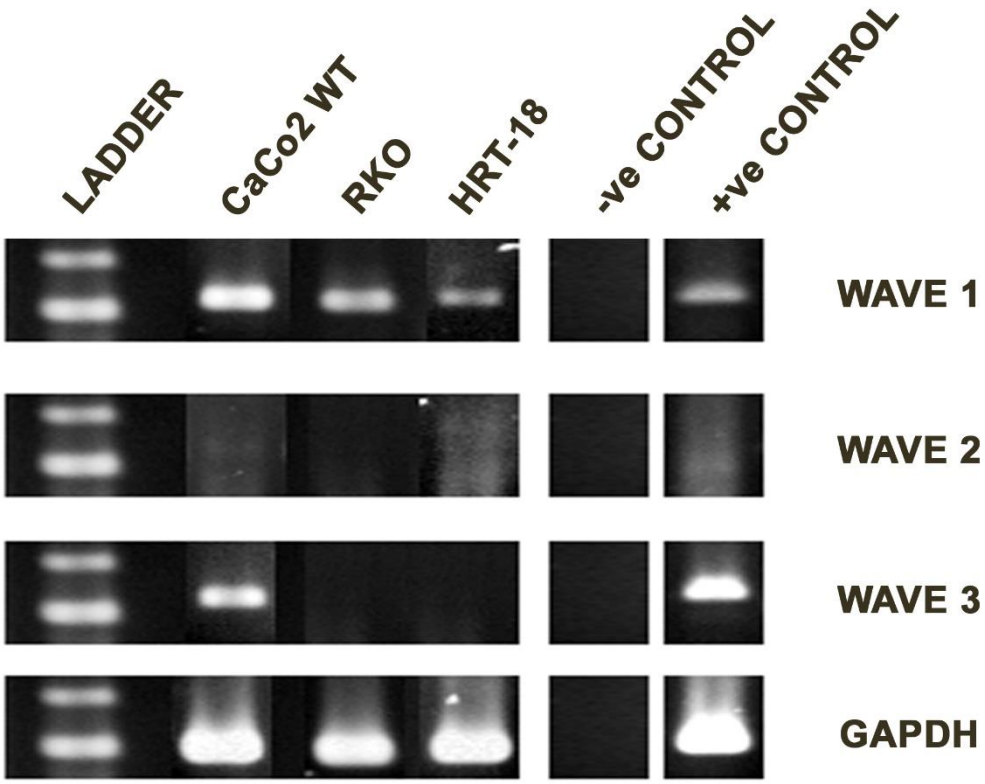


Figure 6.1 - The expression of mRNA encoding for WAVE 1, 2 and 3 in colorectal cancer cells.

GAPDH was used as an internal standard. A mixture of cDNA extracted from breast cancer tissues was included as a positive control for all molecules. A negative control containing no cDNA was also included.

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6.3.2 Generation of WAVE 1 and WAVE 3 ribozyme transgene pEF6 plasmids

Firstly, in order to create the WAVE 1 and WAVE 3 ribozyme transgenes that specifically target and cleave WAVE 1 and 3 mRNA transcripts, a suitable targeting site for the hammerhead ribozymes was identified on the predicted secondary structure of the WAVE 1 and 3 mRNA transcripts. This was achieved using a software programme (described in section 4.3.1) that predicts the way the mRNA transcripts fold.

Initial synthesis of the WAVE 1 and 3 ribozyme transgenes was carried out following the touchdown PCR parameters as outlined in section 4.3.1. Following the plasmid integration and consequent amplification in *E.coli*, the plasmids were extracted for analysis. It is crucial for the ribozyme transgenes to be integrated into the plasmid vector in the correct orientation and so orientation checks were performed with PCR and electrophoresis. Bands of approximately 140bp resulting from a T7F vs RbBMR reaction indicated correct orientation. However, a 140bp band from a T7F vs RbToP reaction was taken as indicating incorrect orientation of the ribozyme transgene insert. This is demonstrated in Figure 6.2. As can be seen, colonies 4 and 7 display bands of 140bp for T7F and RbBMR PCR reactions for WAVE 1 ribozyme 1 (W1R1) samples which indicates correct orientation of the ribozyme transgene within the plasmid vector. These colonies were therefore chosen to be used for plasmid amplification, purification and transfection into the RKO, HRT-18 and CaCo2 cell lines. Additionally, colonies 2 and 6 were identified as carrying WAVE 1 ribozyme 2 (W1R2), colonies 1 and 4 were selected for WAVE 3 ribozyme 1 (W3R1) and colony 8 was selected for WAVE 3 ribozyme 2 (W3R2).

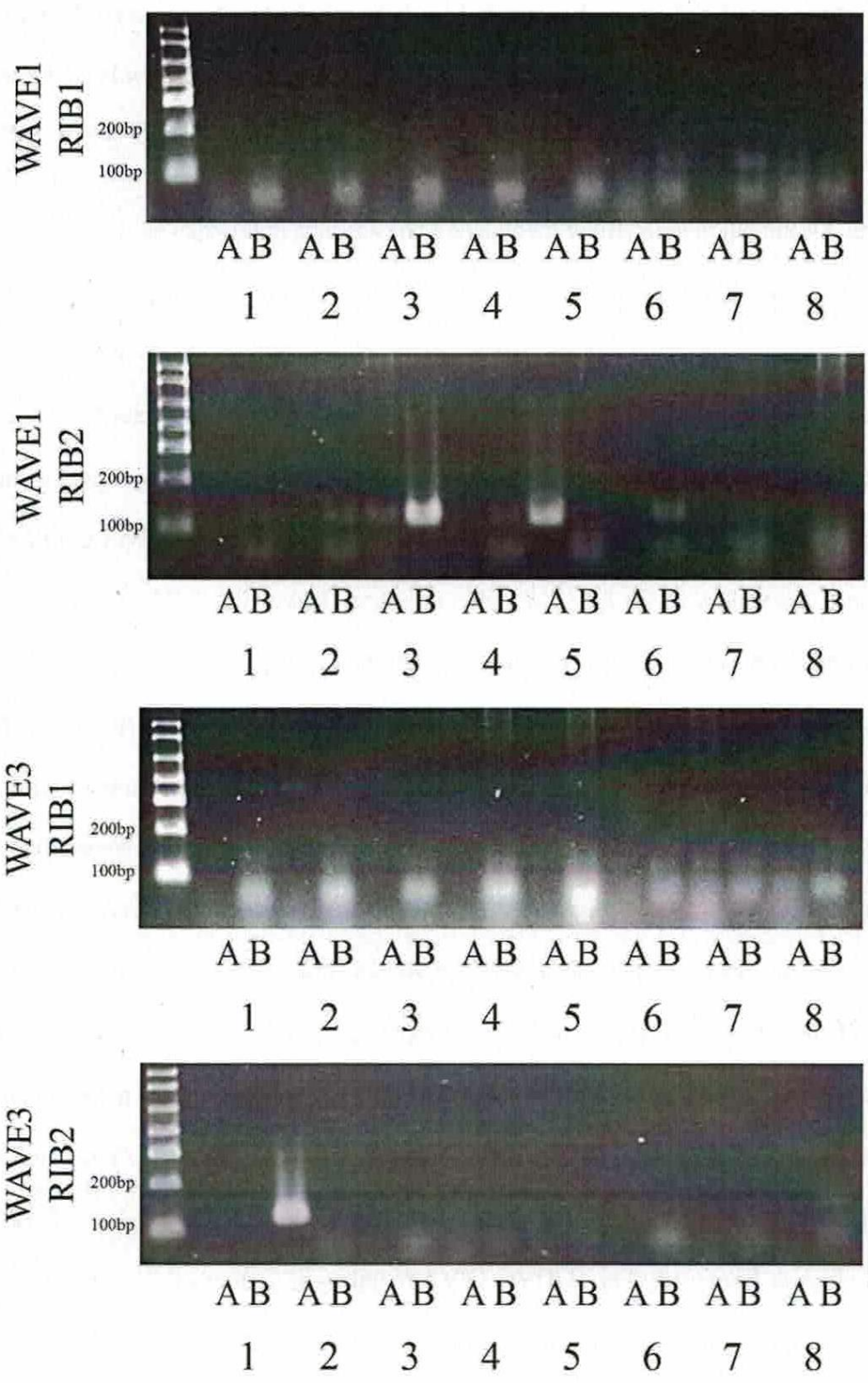


Figure 6.2 - Plasmid insertion and orientation analysis of the ribozyme transgenes into the pEF6 plasmd vector. Colonies picked for orientation analysis are labelled 1 to 8. Correct orientation checks with a T7F and RbBMR reaction are labelled B whilst a T7F and RbToP reaction tested for incorrect orientation and is labelled A. A 140bp band present for either reaction A or B provides an indication of insert orientation into the plasmid vector.

6.3.3 Confirmation of successful WAVE 1 and WAVE 3 knockdown in RKO and CaCo2 cell lines at the mRNA level with polymerase chain reaction (PCR) and quantitative PCR (Q-PCR).

Conventional and quantitative/real time PCR (Q-PCR) were used to test for successful knockdown of WAVE 1 and 3 expression following the transfection of RKO, HRT-18 and CaCo2 wild type cells with the ribozyme transgenes.

Figure 6.3 displays the results of the expression analysis and knockdown verification at the mRNA level. All CRC cell lines displayed similar WAVE 1 expression in their wild type and pEF6 plasmid control cells (wild type cells transfected with a closed pEF6 plasmid alone to demonstrate that the plasmid by itself would have no effect on cellular functions being assayed). WAVE 3 expression was similar in CaCo2 WT and CaCo2 pEF6 cell lines.

Conventional PCR techniques demonstrated a reduction in WAVE 1 mRNA expression levels in the RKO W1R1 cell line and a reduction in the mRNA expression levels of WAVE 3 in the CaCo2 W3R1 cell line when compared to their respective wild types and pEF6 controls.

The expression analysis was performed along with that of the housekeeping gene, GAPDH (glyceraldehyde 3-phosphate dehydrogenase). This worked as a control to ensure that a uniform level of cDNA was present within the samples and, as a consequence, that any reduction in band intensity was due to a reduction in expression levels. GAPDH expression analysis illustrated a uniform expression level.

A negative control was provided, with water substituted for cDNA, so that contaminants would be highlighted. There was no evidence of DNA contamination in the primers or PCR water used as demonstrated by the lack of PCR product band.

Figure 6.4 demonstrates the quantitative analysis of WAVE 1 and WAVE 3 knockdown at the mRNA level using Q-PCR techniques. This focused on those cell lines already implicated in having reduced expression levels as described above. Therefore, expression analysis of RKO W1R1 and CaCo2 W3R1 was undertaken using this method, along with GAPDH to normalise the values obtained for WAVE 1 and 3. In addition, standards of known transcript level were simultaneously amplified enabling a calculation of the expression level in the WAVE 1 and 3 samples. These Q-PCR experiments were repeated at least three times.

Statistical analysis using a 2 sample, 2 tailed t-test shows a significant reduction in the expression of WAVE 1 in RKO W1R1 cell line ($p = 0.0042$) and WAVE 3 in the CaCo2 W3R1 cell line ($p = 0.0067$) compared to their respective pEF6 control cell lines. These cell lines were subsequently used in experiments to test the biological impact of WAVE 1 and 3 knockdown. No significant difference was seen in the expression of WAVE 1 and WAVE 3 when the wild type and pEF6 control cells of either cell line were compared.

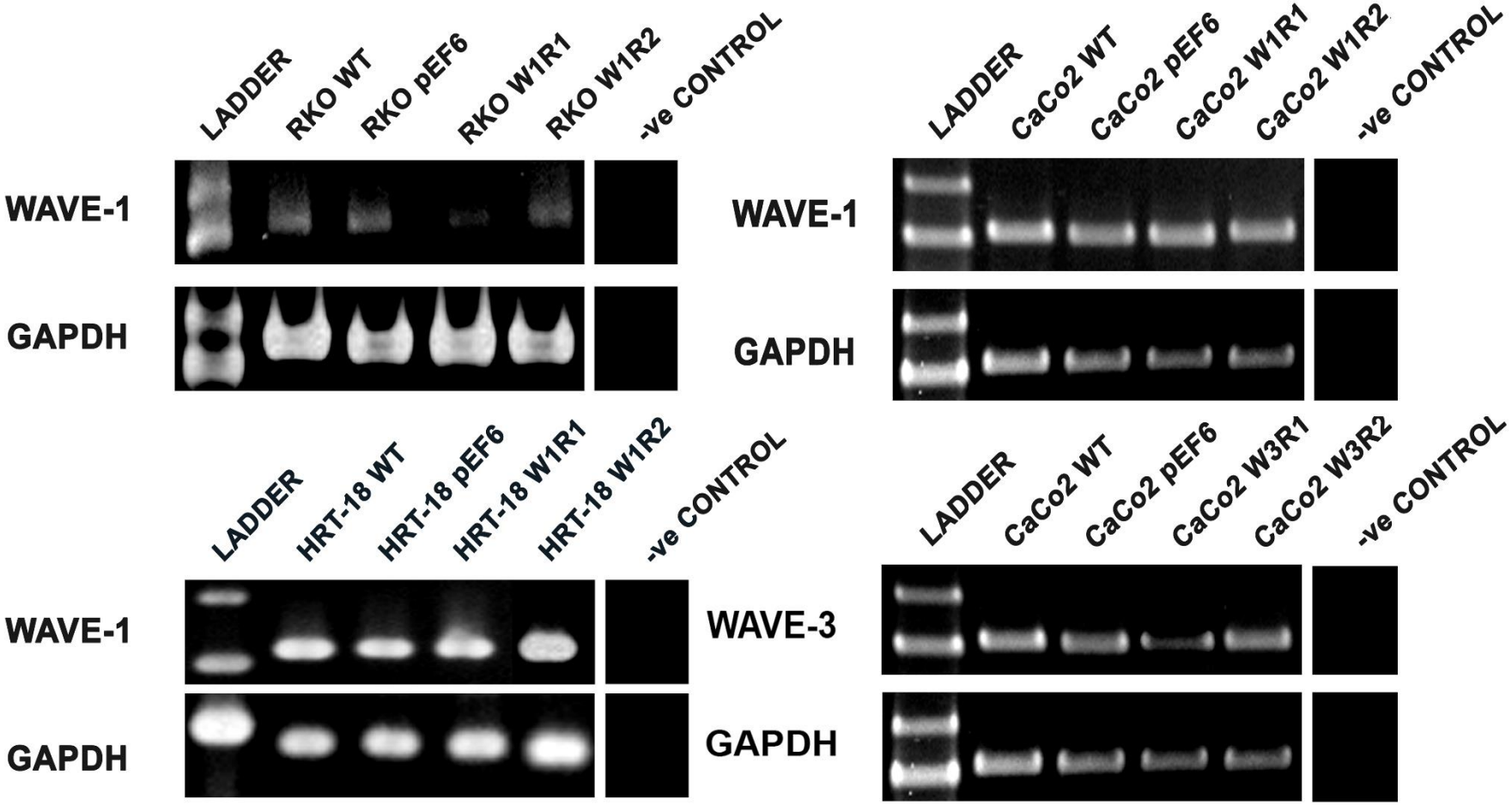
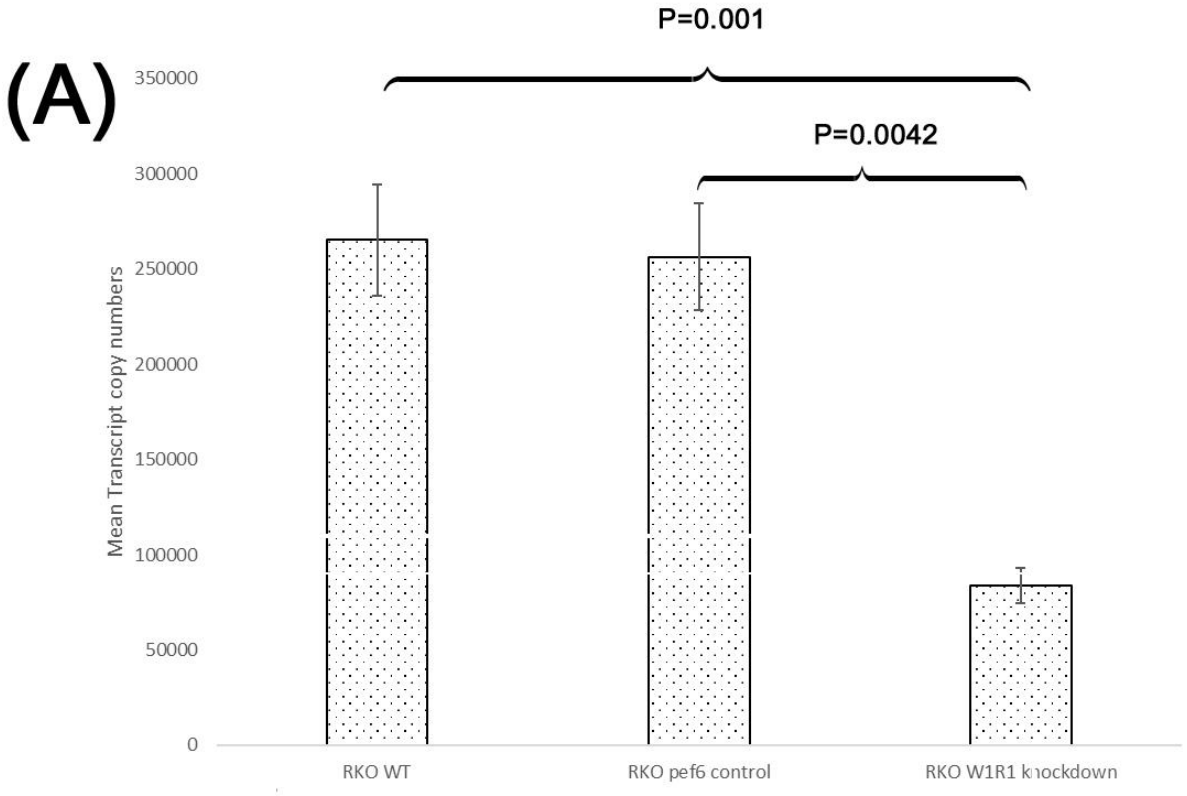


Figure 6.3 - Expression analysis of WAVE 1 and WAVE 3 following targeted ribozyme transgene transfection of RKO, HRT-18 and CaCo2 wild type colorectal cancer cells.

The expression of WAVE 1 was shown to be knocked down at the mRNA level following transfection by ribozyme 1 in the RKO cell line. The expression of WAVE 3 was shown to be knocked down at the mRNA level following transfection by ribozyme 1 in the CaCo2 cell line.

Knockdown of WAVE 1 at the mRNA level was unsuccessful in the HRT-18 and CaCo2 cell lines.



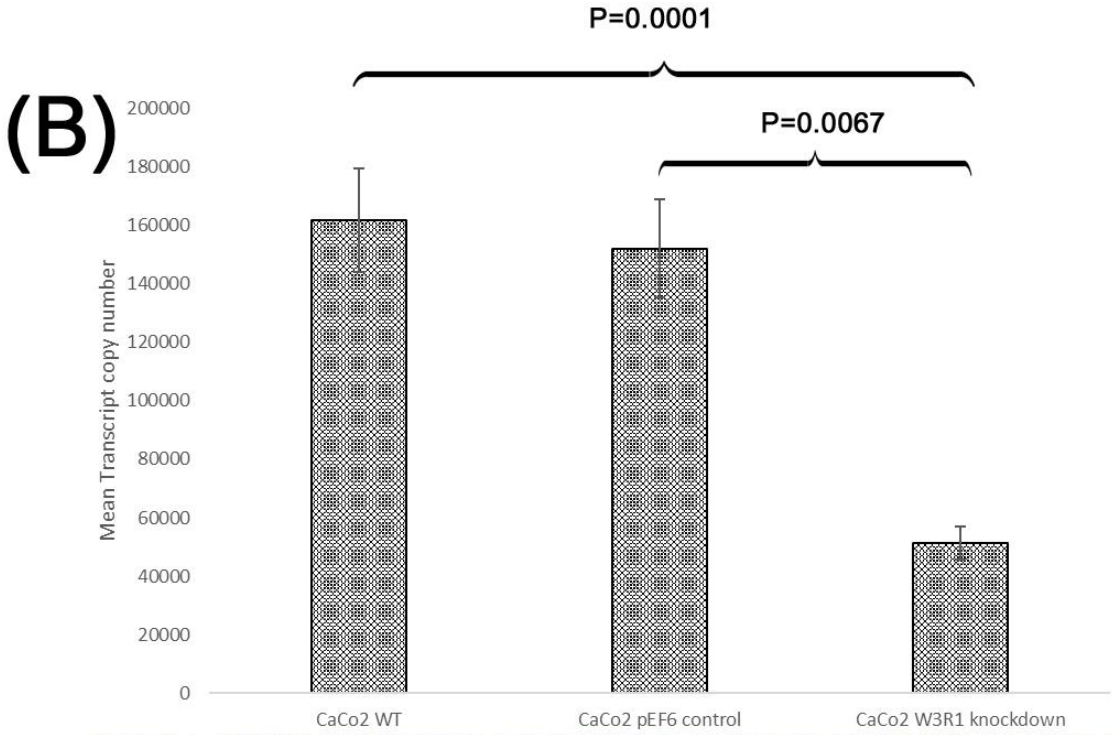


Figure 6.4 - WAVE 1 and 3 knockdown confirmed at the mRNA level using Q-PCR. (A) WAVE 1 expression knock-down in RKO cells transfected with WAVE 1 ribozyme 1 (W1R1) compared to both wild type (WT) and pEF6 plasmid control cells. (B) WAVE 3 expression knockdown in CaCo2 cells transfected with WAVE 3 ribozyme 1 (W3R1) compared to both wild type (WT) and pEF6 plasmid control cells. Expression levels of WAVE 1 and 3 were normalised against the housekeeping gene, GAPDH. Mean values +/- SEM are shown from a minimum of three independent repeats.

6.3.4 Impact of WAVE 1 and WAVE 3 knockdown on cell growth rate

The effects of suppressing WAVE 1 expression on the growth of RKO cell lines and suppressing WAVE 3 expression on the growth of CaCo2 cell lines was investigated following 3 and 5 day incubation periods using an *in vitro* cell growth assay as described in chapter 4. Figure 6.5 displays the results.

There was no significant difference in cell growth rates between the wild type, pEF6 control or WAVE 1 or WAVE 3 suppressed cells following a 3 day and a 5 day incubation period.

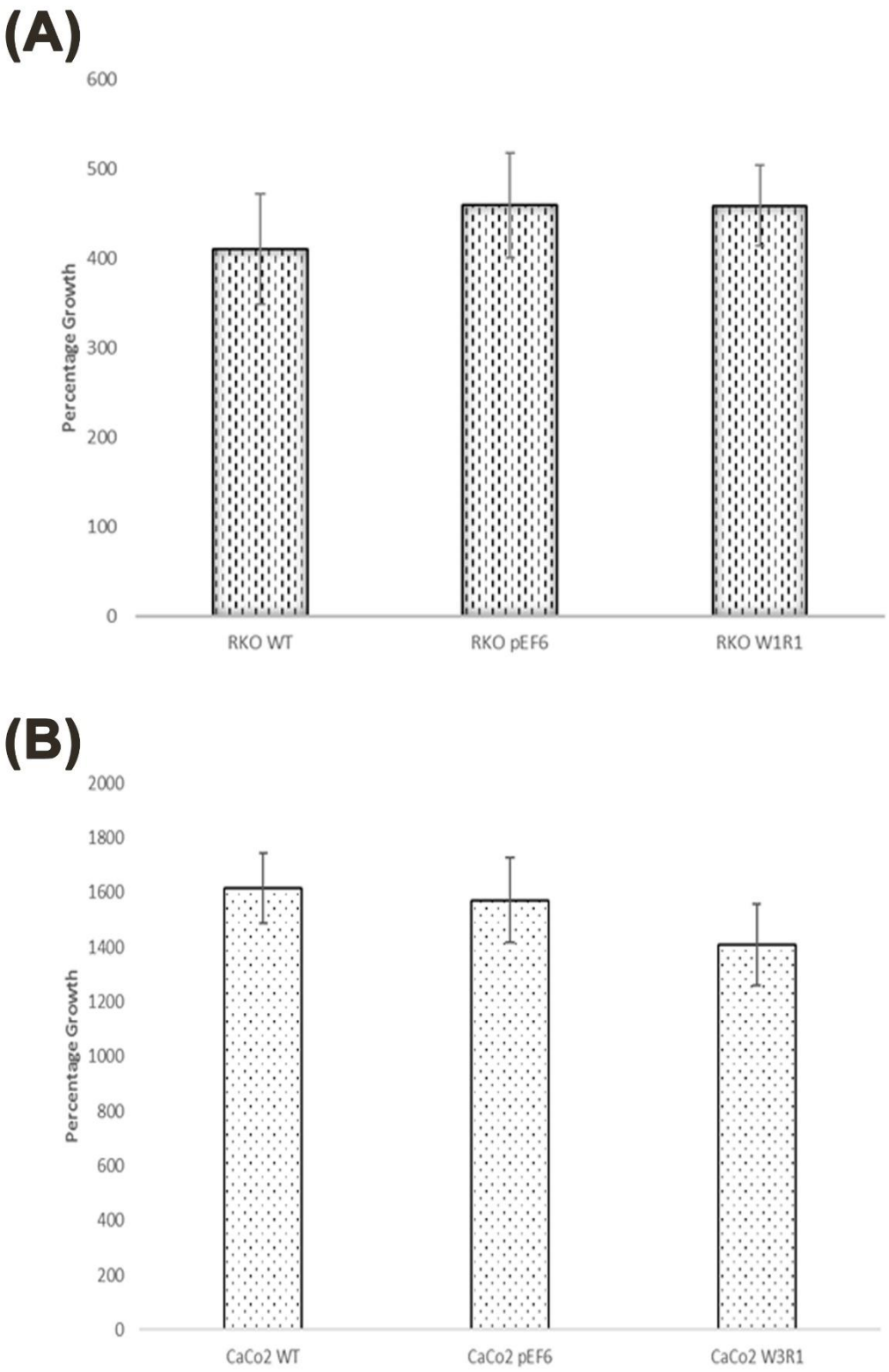


Figure 6.5 - The effects on cell growth over a 5 day incubation period of (A) WAVE 1 knockdown in RKO cell lines and (B) WAVE 3 knockdown in CaCo2 cell lines.

No significant difference in cell growth was seen in either cell lines when comparing knockdown cells (RKO W1R1 and CaCo2 W3R1) with their respective plasmid control cell lines (RKO pEF6 and CaCo2 pEF6) and wild types. Mean values +/- SEM are shown from a minimum of three independent repeats.

6.3.5 Impact of WAVE 1 and WAVE 3 knockdown on cell invasiveness

The invasive ability of the RKO and CaCo2 cell lines was examined using an *in vitro* Matrigel invasion assay (see section 4.5.2) comparing wild type cells and pEF6 control cells to RKO cells carrying the WAVE 1 ribozyme transgenes (Figure 6.6), and CaCo2 cells carrying the WAVE 3 ribozyme transgene (Figure 6.7).

Knockdown of expression of both WAVE 1 and WAVE 3 resulted in a significant decrease in the invasive ability of the CRC cells. Knockdown of WAVE 1 expression in RKO W1R1 cells produced a 50% reduction in invasive ability that was found to be significant ($p=0.02$) when compared to RKO pEF6 control cells.

Knockdown of WAVE 3 expression in CaCo2 W3R1 cells produced a 58% reduction in invasive ability that was also found to be significant ($p=0.04$) when compared to CaCo2 pEF6 control cells.

No significant difference was seen between the wild type and their respective pEF6 control cell lines.

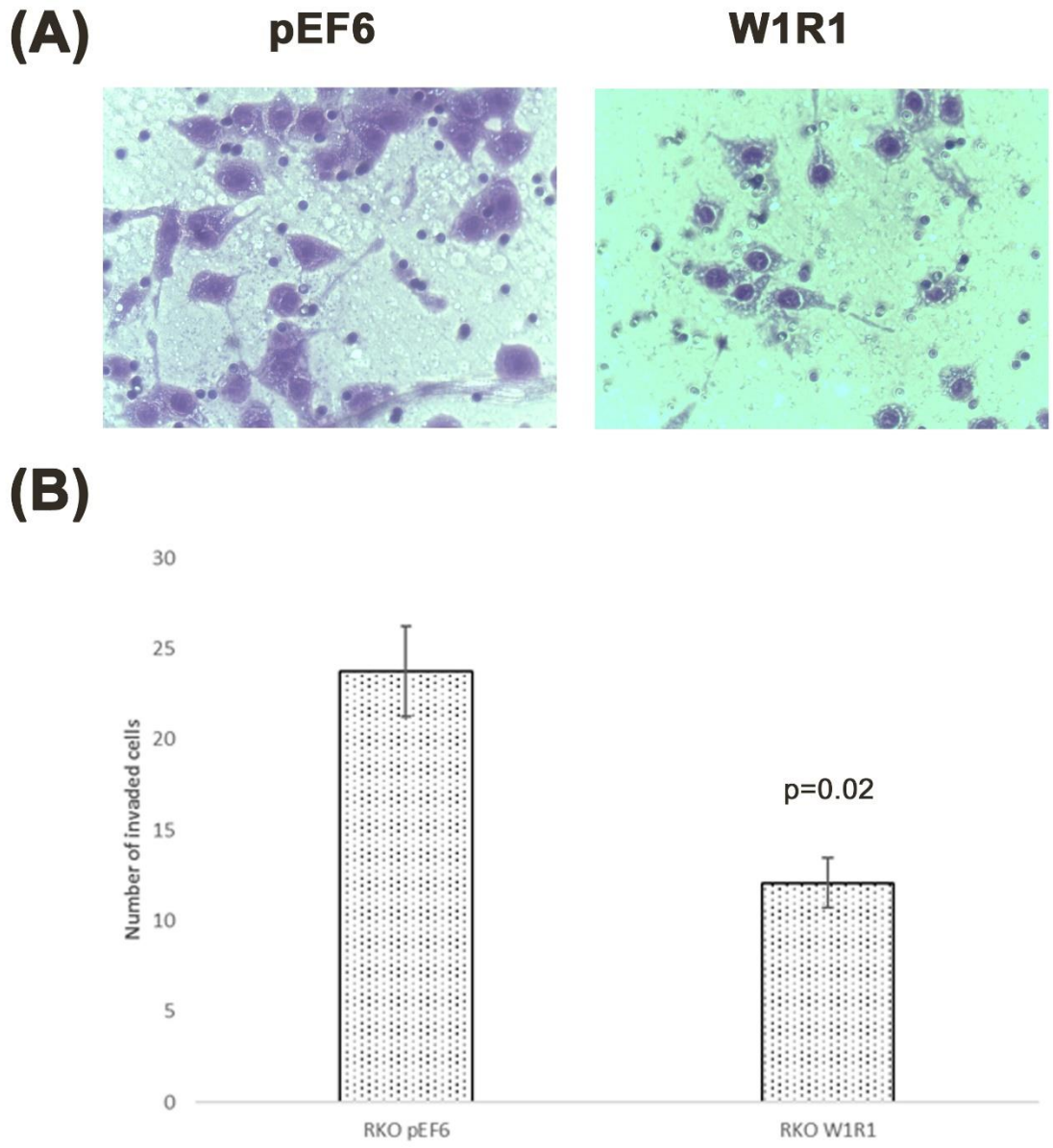


Figure 6.6 - The effect of WAVE 1 knockdown of expression on the invasiveness of RKO colorectal cancer cells.

(A) Displayed above the graph are representative images aquired under a microscope at X200 magnification of RKO pEF6 and RKO W1R1 cells during the cell invasion assay.
(B) WAVE 1 knockdown was shown to significantly decrease cell invasion when compared to a pEF6 control.

Mean values +/- SEM are shown from a minimum of three independent repeats.

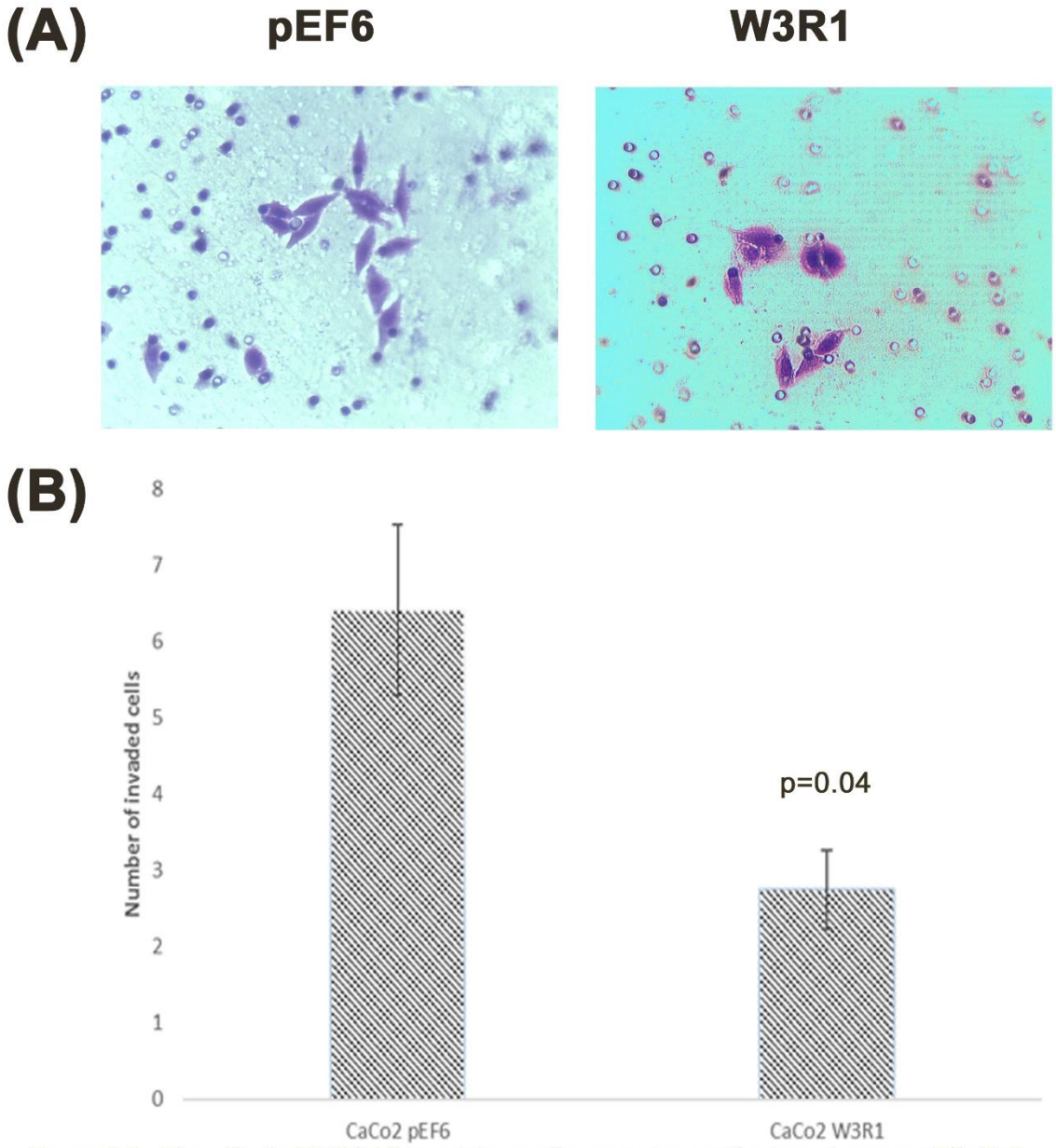


Figure 6.7 - The effect of WAVE 3 knockdown of expression on the invasiveness of CaCo2 colorectal cancer cells.

(A) Displayed above the graft are representative images acquired under a microscope at X200 magnification of CaCo2 pEF6 and CaCo2 W3R1 cells during the cell invasion assay.
(B) WAVE 3 knockdown was shown to significantly decrease cell invasion when compared to pEF6 plasmid control cells.

Mean values +/- SEM are shown from a minimum of three independent repeats.

6.3.6 Impact of WAVE 1 and WAVE 3 knockdown on cell adhesiveness

The ability of the RKO and CaCo2 cell lines to adhere was examined using an *in vitro* Matrigel adhesion assay (see section 4.5.3) comparing wild type cells and pEF6 control cells to RKO cells carrying the WAVE 1 ribozyme transgenes (Figure 6.8), and CaCo2 cells carrying the WAVE 3 ribozyme transgene (Figure 6.9).

No changes in the cells adhesive abilities were seen for WAVE 1 or WAVE 3 knockdown cells ($p=0.797$; $p=0.906$).

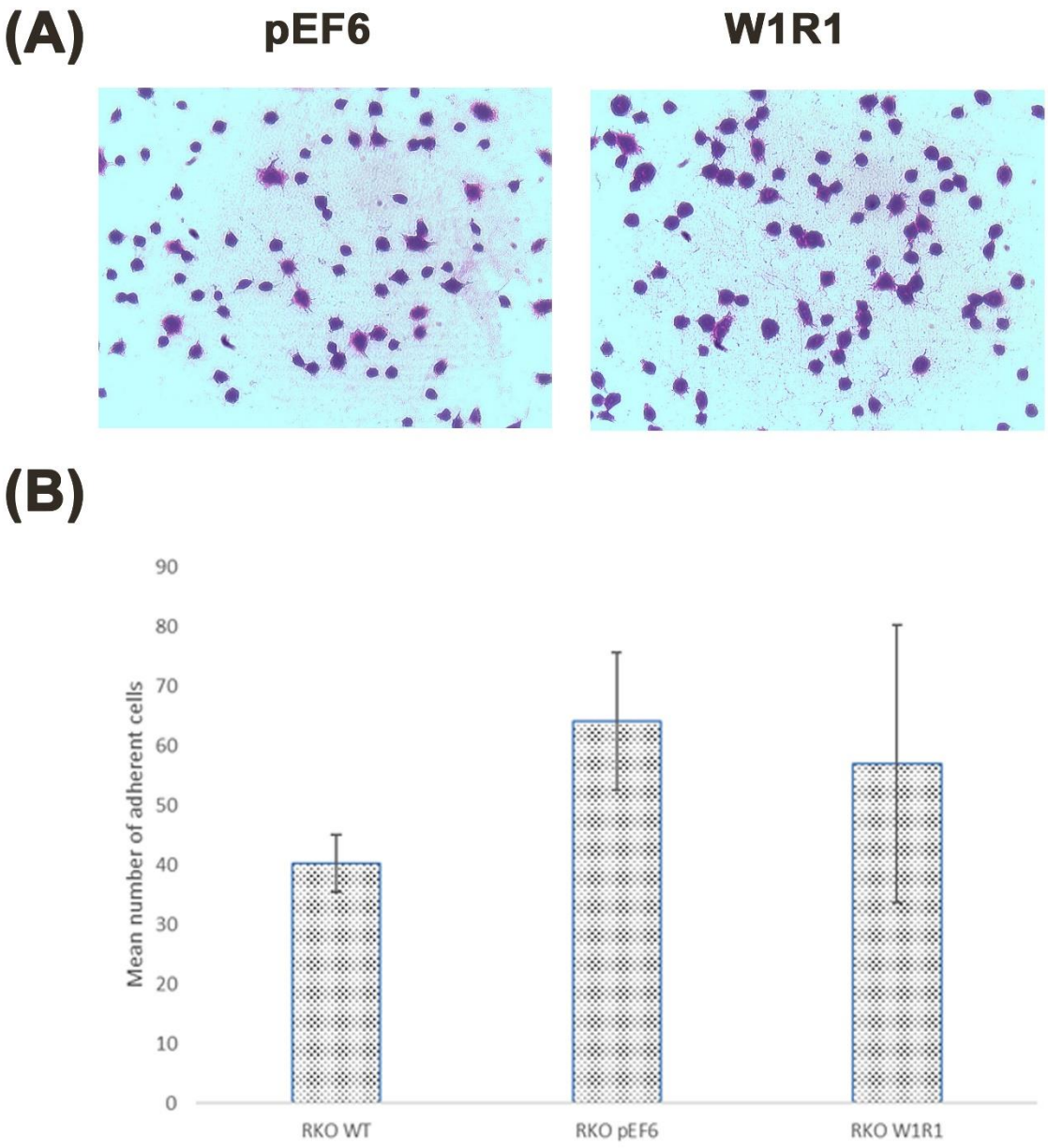


Figure 6.8 - The effect of WAVE 1 knockdown of expression on the adhesion of RKO colorectal cancer cells.

(A) Displayed above the graph are representative images aquired under a microscope at X200 magnification of RKO pEF6 and RKO W1R1 cells during the cell adhesion assay.
(B) No significant difference in cell adhesion was seen in RKO cells when comparing WAVE 1 knockdown cells (RKO W1R1) with plasmid control cells (RKO pEF6) and wild types.

Mean values +/- SEM are shown from a minimum of three independent repeats.

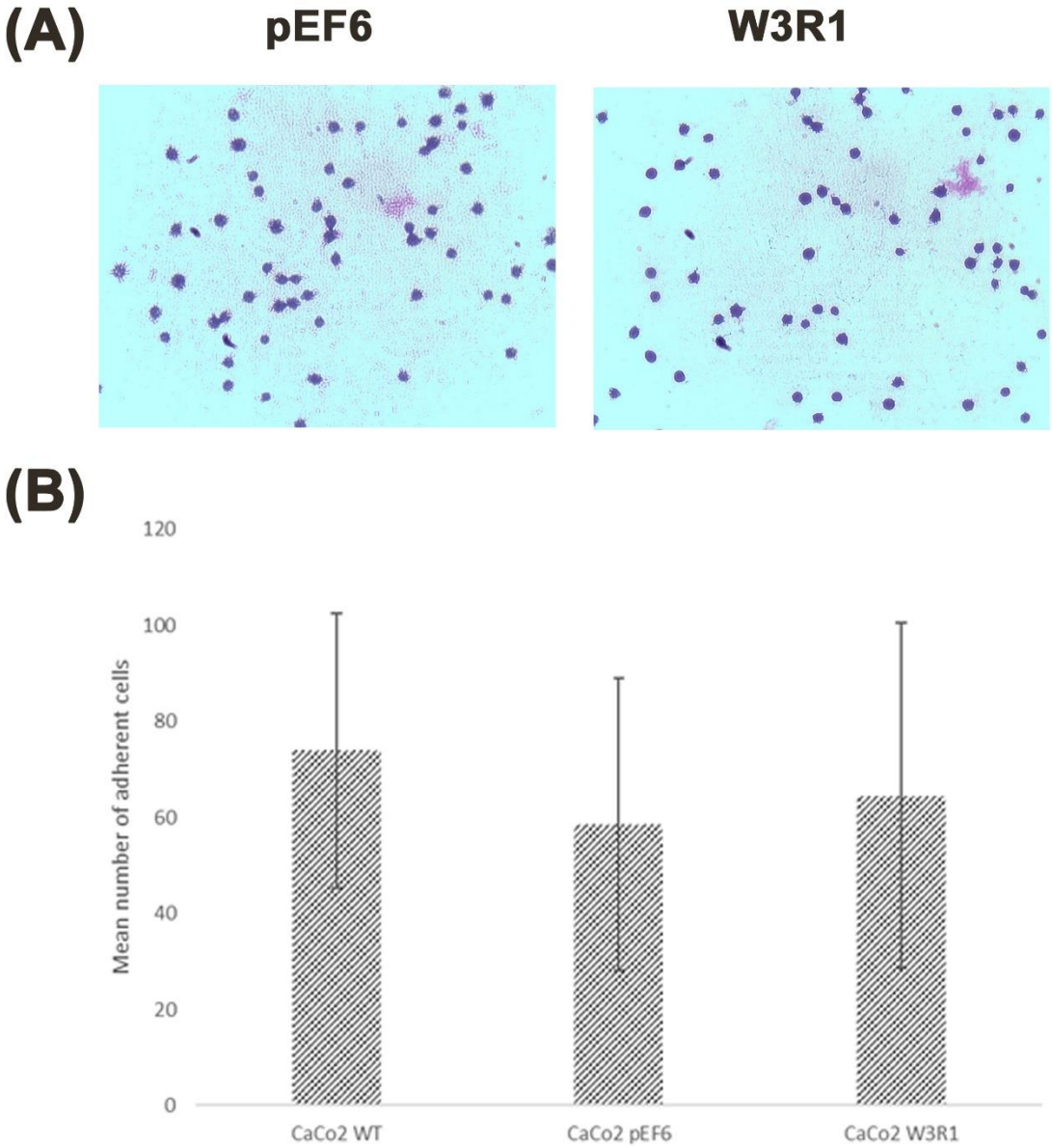


Figure 6.9 - The effect of WAVE 3 knockdown of expression on the adhesion of CaCo2 colorectal cancer cells.

(A) Displayed above the graph are representative images aquired under a microscope at X200 magnification of CaCo2 pEF6 and CaCo2 W3R1 cells during the cell adhesion assay.
(B) No significant difference in cell adhesion was seen in CaCo2 cells when comparing WAVE 3 knockdown cells (CaCo2 W3R1) with plasmid control cells (CaCo2 pEF6) and wild types.

Mean values +/- SEM are shown from a minimum of three independent repeats.

6.3.7 Results of WAVE 1 and WAVE 3 knockdown on cell motility

A migration assay (see section 4.5.4) was used to assess the migratory capacity of the RKO and CaCo2 cell lines (Figure 6.10). WAVE 3 suppression was found to significantly decrease cell motility in CaCo2 W3R1 cells ($p=0.02$) relative to both wild type and pEF6 cells. The motility of RKO cells carrying the WAVE 1 ribozyme transgene (RKO W1R1) was reduced but not significantly so ($p=0.058$) when compared to wild type and pEF6 control cells.

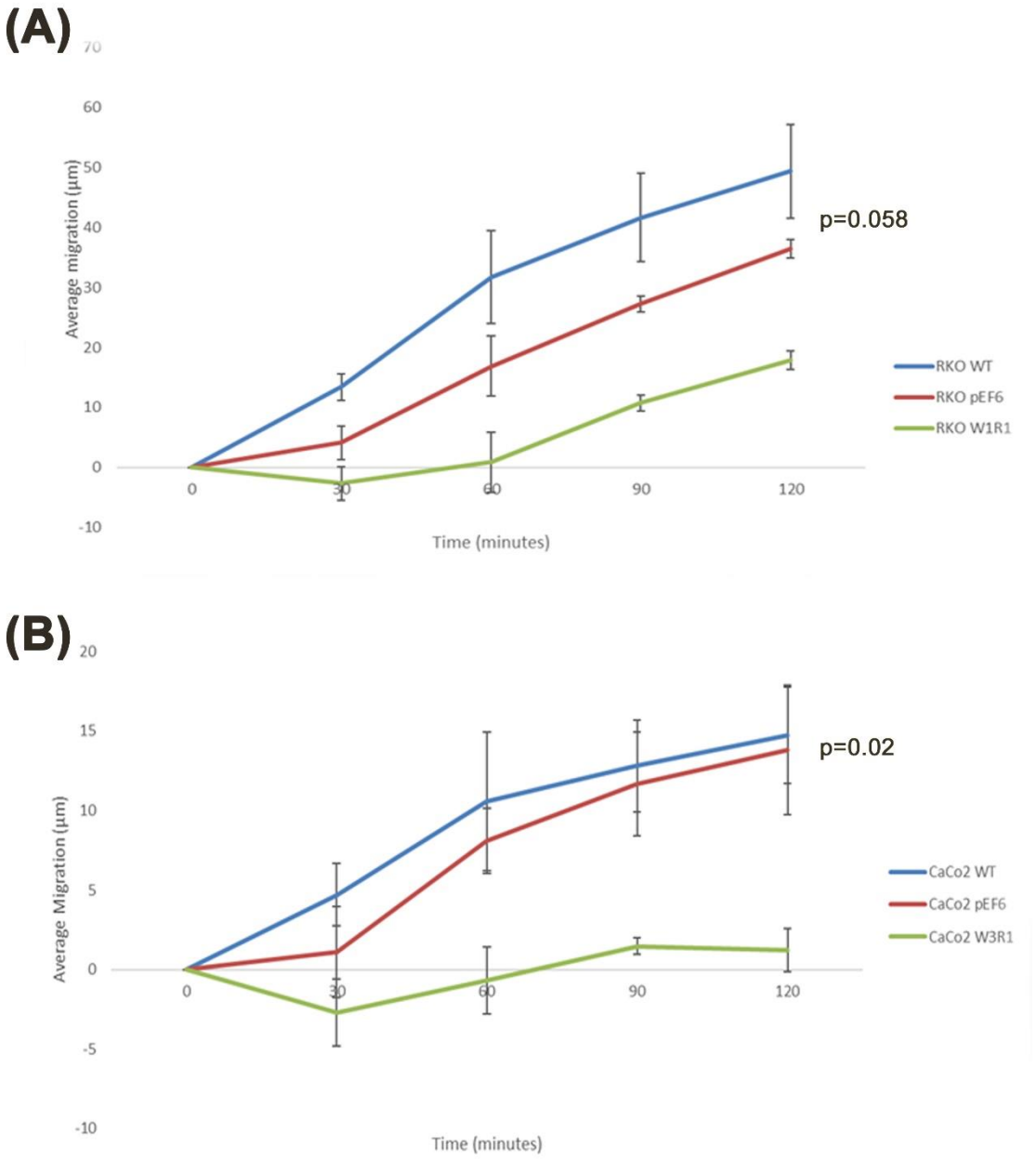


Figure 6.10 - The effects on cellular migration of (A) WAVE 1 knockdown in RKO cell lines and (B) WAVE 3 knockdown in CaCo2 cell lines.

The data shows a significant decrease in cellular migration over a 120 minute period in the WAVE 3 knockdown CaCo2 cell line (CaCo2 W3R1) when compared to the pEF6 plasmid control cell lines (CaCo2 pEF6) and the wild type cells (CaCo2 WT).

No significant difference in cellular migration over a 120 minute period is seen in the WAVE 1 knockdown RKO cell line (RKO W1R1) when compared to the pEF6 plasmid control cell lines (RKO pEF6) and wild type cells (RKO WT).

Mean values +/- SEM are shown from a minimum of three independent repeats.

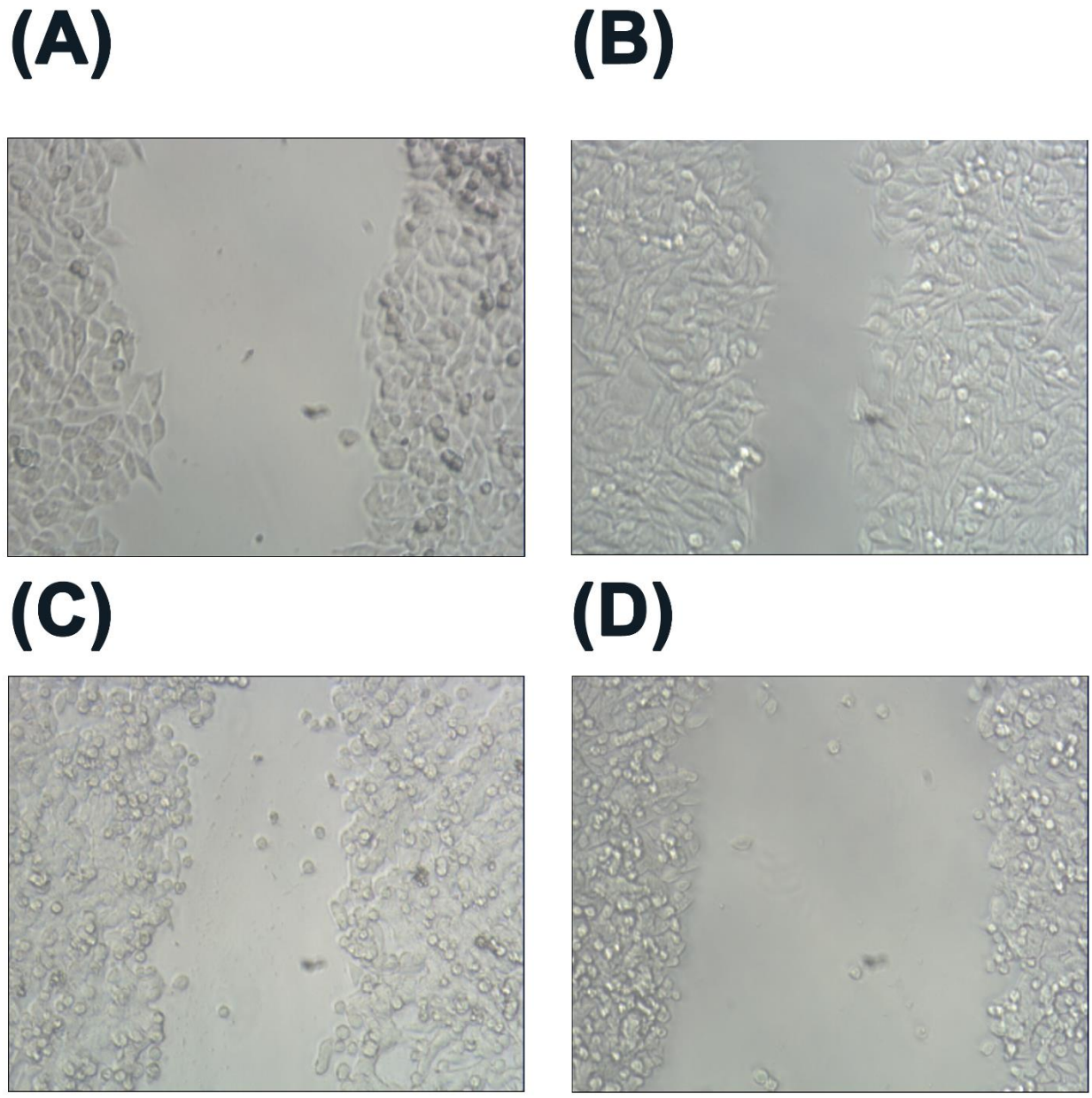


Figure 6.11 – Representative images of the RKO and CaCo2 cell lines during the migration assay at Time = 120 minutes. (A) WAVE 1 knockdown in RKO cell line. (B) RKO WT cell line. (C) CaCo2 WT cell line. (D) WAVE 3 knockdown in CaCo2 cell line.

6.4 Discussion

Due to the role of WAVE proteins in cell migration it is a logical progression to hypothesise a role for them within the cancer metastasis pathway, a route which itself is associated with uncontrolled levels of cell migration. Indeed, elevated expression levels of WAVE 2 have been linked with aggressive and metastatic colorectal cancer and co-localised with Arp2/3 (Iwaya et al., 2007) when compared to normal colorectal tissues.

Unfortunately, the WAVE 2 primers that had been designed using the Beacon designer programme and synthesised by Invitrogen (Paisley, UK) were not functioning when the expression analyses were being carried out. This prevented a WAVE 2 expression profile being compiled and therefore a cell line to target WAVE 2 expression in could not be identified. A decision to proceed with WAVE 1 and WAVE 3 expression knockdown was made at this point with the hope that should the WAVE 2 primers be successfully redesigned and synthesised during the time course of this study then the gamut of experiments performed on WAVE 1 and 3 would be repeated for WAVE 2. Unfortunately, this was not to be the case and has meant that WAVE 2 has not been as thoroughly investigated as WAVE 1 and 3 have been in this study.

Transfection of wild type RKO, CaCo2, and HRT-18 with either of the two hammerhead ribozyme transgenes designed to target WAVE 1 (WAVE 1 ribozyme

1, WAVE 1 ribozyme 2) mRNA transcripts, resulted in the successful knockdown of the target gene at the mRNA level in the RKO cell line with WAVE 1 ribozyme 1 (W1R1). This was successfully demonstrated using conventional PCR and Q-PCR techniques. Transfection of wild type CaCo2 cells with either of the two hammerhead ribozyme transgenes available for targeting WAVE 3 (WAVE 3 ribozyme 1, WAVE 3 ribozyme 2) mRNA transcripts, resulted in the successful knockdown of the target gene at the mRNA level with WAVE 3 ribozyme 1 (W3R1). Again this was demonstrated using conventional PCR and Q-PCR techniques. Therefore, the RKO W1R1 and CaCo2 W3R1 cell lines were used in the subsequent functional assay experiments.

In addition, wild type RKO and wild type CaCo2 cells were transfected with the empty vector, pEF6, to generate RKO pEF6 and CaCo2 pEF6 plasmid control cell lines. Conventional PCR techniques assessed mRNA expression levels of WAVE 1 and WAVE 3 in the wild type and pEF6 control cell lines. These confirmed similar expression levels to each other and, therefore, that the reduction in WAVE 1 and 3 expression levels in the RKO W1R1 and CaCo2 W3R1 transfected cells was caused by specific gene targeting as oppose to being an artefact caused by the process of gene manipulation. With this established, the pEF6 cell lines were used as controls for the functional assay experiments.

The functional assays performed looked at the effects knockdown of WAVE 1 and 3 expression had on growth, adhesion, invasion and motility.

The growth assays performed to determine the effects of WAVE 1 knockdown on cell growth showed no significant difference between the RKO W1R1 knockdown cells and the corresponding RKO wild type and pEF6 control cell lines. The knockdown of WAVE 3 in the CaCo2 W3R1 cell line showed a very mild reduction in cell growth (11%) when compared to the CaCo2 wild type and pEF6 control cell lines, however, it was not statistically significant.

Similarly, assays carried out to determine the effects of WAVE 1 and 3 knockdown on cell adhesion revealed no significant difference between the knockdown cell lines (RKO W1R1 and CaCo2 W3R1) and their respective wild type and pEF6 control cell lines. The values obtained from this assay were widely distributed, yielding a very large standard error.

Cell invasiveness was found to be significantly reduced in both WAVE 1 and WAVE 3 knockdown cell lines (RKO W1R1, CaCo2 W3R1) compared to the pEF6 control cell line. WAVE 1 knockdown resulted in a 50% reduction in invasive ability and WAVE 3 knockdown resulted in a 58% reduction in invasive ability.

When analysing the consequences of WAVE knockdown on cell motility in RKO W1R1 cells, it was found that a decrease in WAVE 1 expression resulted in a 51% decrease in cell motility, which did not reach significance. In contrast, WAVE 3 knockdown in CaCo2 W3R1 cells showed that a decrease in WAVE 3 expression resulted in a significant reduction in motility. In fact a reduction of 91%.

These functional assays revealed similar roles for WAVE 1 and 3, particularly regarding cell invasion and cell motility where both traits were suppressed following WAVE 1 or 3 knockdown. The extent of trait suppression differed between the WAVE members as WAVE 3 knockdown was observed to suppress invasive and migratory ability to a greater and more significant extent than WAVE 1 knockdown when compared to wild type and pEF6 control cells. Although WAVE 1 knockdown was seen to inhibit cellular motility, these changes were not significant.

These effects on motility and invasion when WAVE 1 and 3 expression are knocked down have been seen in other studies. Fernando et al (2008; 2010) knocked down endogenous expression of WAVE 1 and WAVE 3 in prostate cancer cell lines. Both showed a significant reduction in invasiveness. Sossey-Alaoui et al (2007) investigated the effects of WAVE 3 expression knockdown in breast cancer cell lines. This resulted in a suppression of the in vitro invasive capabilities of the cells. The team took this further and injected the knockdown cells into a xenograft mouse model and found that there was a reduced rate of tumour growth at the primary site and an inhibition of the metastatic spread of the cells to distant sites, as would normally be seen in mice implanted with this cell line.

The growth assays performed in this study showed no effect on proliferation when WAVE 1 or 3 expression was knocked down. There are some conflicting findings in other studies performed whereby Fernando et al (2008; 2010) found a reduction in growth when WAVE 1 was knocked down in prostate cancer cell lines, but not when WAVE 3 was. However, Sossey-Alaoui et al (2007) did find a reduction in growth when WAVE 3 was knocked down in breast cancer cell lines.

WAVE1 and 3 display similar effects, to differing degrees, on cell function when their expression is knocked down in the colorectal cancer cell lines, RKO and CaCo2. Such findings suggest they are involved in common signalling pathways in facilitating cell motility and that they have an effect on invasion, a trait over which they have previously been attributed to having less influence. Structurally, WAVE1 and 3 share the same protein domains fundamental to their functional roles in the cell, however an alignment of their protein sequences reveal only 49.7% identity (Pearson *et al,* 1997). As these two molecules do not share identical protein sequences, it can be postulated that these differences could translate into cellular traits that vary in their overall impact due to differing abilities to regulate or be regulated by different protein partners and thus potentially influence signalling pathways to a greater or lesser degree. Indeed, Sossey-Alaoui et al, (2010) found that WAVE 3 promotes cell motility through the regulation of MMP-1, 3 and 9 expression. This could explain the significant reduction in cellular motility, 91%, when WAVE 3 expression was knocked down compared to the 51% (and not statistically significant) reduction when WAVE 1 was knocked down, as Sossey-Alaoui et al found that a reduction in WAVE 3 expression reduced the expression of these MMP's also. WAVE 1 expression has been associated with regulation of MMP-2 expression (Suetsugu et al., 2003).

An additional observation made during the functional assays was of the appearance of the cells. During the migration, adhesion and invasion assays the RKO cell line had a distinct elongated/mesenchymal shape. In contrast, the Caco2 cell line appeared more rounded and amoeboid, except during the invasion assay where they too became more elongated/mesenchymal in shape. These observations are in keeping with previously discussed methods of cancer cell migration and invasion of

the ECM in 3D environments (Kurisu et al., 2010; Hanahan et al., 2011; Sanz-Moreno et al., 2008; 2007). However, it does raise the question as the rounded/amoeboid shape has been associated with infiltration of the ECM without the aid of MMP's whereas the elongated/mesenchymal shape is associated with motility and invasion in the presence of MMP's.

The results presented here provide evidence that WAVE 3 and to a lesser extent WAVE 1 are indeed pro-tumorigenic in that their presence is closely linked to the invasion and motility of cancer cells.

The *in vitro* assays performed in this study mimic key stages of the metastasis cascade. In particular the invasion of cells through a basement membrane and the capacity to migrate and disseminate through surrounding extracellular matrix and tissues is of vital importance in allowing tumour cells to escape the main tumour body and spread to secondary sites. The reduced capacity of WAVE 1 and 3 suppressed cells to invade through an artificial basement membrane and migrate across a substrate implicates WAVE 1 and particularly WAVE 3 in the processes of cancer progression and metastasis.

CHAPTER 7
GENERAL DISCUSSION

7.1 Introduction

The incidence rates of colorectal cancer have increased steadily over the past 50 years and is the fourth most common malignancy in the United Kingdom. The risk of colorectal cancer increases with age and is a significant health issue in modern society. Colorectal cancer is the second most common cause of cancer related deaths in the United Kingdom. More than half of patients diagnosed are diagnosed at a late stage (Stage III and IV) with around a quarter (26%) found to already have metastases at diagnosis (26%). Approximately 50% of diagnosed patients will progress to Stage IV, metastatic disease, “Metastases rather than primary tumours are responsible for most cancer deaths” (Chambers et al., 2002)

As with many other forms of cancer, the main factor in determining patient survival is the metastatic potential of the tumour. The metastatic spread of a tumour is a multistage complex process involving the loss of adhesion between cells of the primary tumour (comprising of changes in cellular adhesion properties) and dissemination of these tumour cells from the main tumour body, degradation of the basement membrane (via the production and secretion of proteases), invasion of the surrounding stromal tissue (through mesenchymal and amoeboid migration), entry into the circulatory system (aided by the secretion of angiogenic factors), transport around the body, extravasation and invasion at the secondary site and development of a secondary tumour. A number of changes must occur to enable a tumour cell to gain these varying abilities to metastasise. These changes may occur due to genetic alterations in the cell or in the regulation of gene expression. Numerous abnormalities may develop and accumulate to facilitate a metastatic phenotype.

WAVE 1, 2 and 3 are known to play an important role in cellular actin systems which are closely involved with cell migration and have implications for cell invasion too. When such processes are uncontrolled they are a major contributor to cancer metastasis. As such, tumor invasion and metastasis are increasingly being associated with deregulation of the actin system (Lambrechts et al., 2004).

This point continues to be emphasised by findings that several human cancers are associated with increased expression and/or activity of particular WAVE proteins (Kurisu et al., 2005; Yang et al., 2006; Sossey-Alaoui et al., 2007; Fernando et al., 2007, 2008 & 2010). Indeed, a trend of higher WAVE expression was linked with metastatic colorectal carcinoma (Iwaya et al., 2007).

7.2 The role of WAVE 1

WAVE 1 expression immunohistochemically stained for on frozen sections showed only a subtle change between normal colorectal tissues and colorectal carcinoma tissues, mainly in the distribution of expression as opposed to a change in expression levels. This correlated with the Q-PCR results which actually showed that there was no statistically significant change in overall WAVE 1 expression between the normal colorectal tissues and the colorectal carcinoma tissues. On knockdown of WAVE 1 expression within the RKO colorectal carcinoma cell line (RKO W1R1) there was no effect on cell growth or the adhesive ability of the cells as shown by the in vitro tumour cell growth assay and the in vitro tumour cell matrigel adhesion assay. There was a 51% reduction in cell motility as shown by the in vitro tumour cell motility assay, but this was not statistically significant. However, there was a 50% reduction in the cells invasive ability, as shown by the in vitro tumour cell matrigel invasion assay, and this was considered significant. Overall, the implication is that WAVE 1 is involved in the invasive ability of colorectal cancer cells and, to a less significant extent, their migratory ability and that as tissues become malignant, that expression becomes more generalised throughout the tumour allowing invasion and migration to occur through the stromal tissues, beginning the pathway toward metastasis.

7.3 The role of WAVE 3

WAVE 3 expression stained for on frozen carcinoma sections demonstrated a more substantial difference when compared to normal colorectal tissue sections and compared to WAVE 1 staining. There was overall an increase in the intensity of staining suggestive of an increase in WAVE 3 expression levels compared to normal tissue staining. The distribution of the staining was also more widespread and distinct, with a significant increase in cell membrane distribution along the basal cell layers of the crypts and a larger amount of cytoplasmic and membrane staining within the stromal areas, particularly in the higher T-stage tissues. The Q-PCR results revealed a significantly higher WAVE 3 expression level in carcinoma tissues compared to normal colorectal tissues, in keeping with the increased intensity of staining identified. There was higher WAVE 3 expression in T2, 3 and 4 tumours compared to normal colorectal tissue. There was no difference in WAVE 3 expression in tissues from tumours that had nodal disease compared to those that did not have nodal disease and no difference in WAVE 3 expression from tumour tissues that had metastatic disease compared to those that did not have metastatic disease.

On knockdown of WAVE 3 expression within the Caco2 colorectal carcinoma cell line (CaCo2 W3R1) there was no effect on cell growth or the adhesive ability of the cells as shown by the in vitro tumour cell growth assay and the in vitro tumour cell matrigel adhesion assay. There was, however, a significant reduction in cell motility of 91%, as shown by the in vitro tumour cell motility assay, and a significant 58% reduction in the cells invasive abilities, as shown by the in vitro tumour cell matrigel invasion assay.

Again, the overall implication is that WAVE 3 is significantly involved in the invasive ability of colorectal cancer cells and their migratory ability. As tissues become malignant increased expression becomes more generalised throughout the tumour bulk, allowing invasion and migration to occur through the stromal tissues and progressing along the pathway toward metastasis.

7.4 The role of WAVE 2

WAVE 2 expression immunohistochemically stained for on frozen sections also demonstrated a more substantial difference when carcinoma tissue was compared to normal colorectal tissue sections and in comparison to WAVE 1 staining. There was overall an increase in the intensity of staining suggestive of an increase in WAVE 2 expression levels compared to normal tissue staining. The distribution of the staining was also more widespread and distinct, with a significant increase in cell cytoplasmic and cell membrane distribution within the basal cell layers of the crypts and a larger amount of cytoplasmic and membrane staining within the stromal areas, particularly in the higher T-stage tissues.

The Q-PCR results revealed a significantly higher WAVE 2 expression level within carcinoma tissues compared to normal colorectal tissues, in keeping with the increased intensity of staining identified. There was higher WAVE 2 expression in T3 and T4 tumours compared to normal colorectal tissues, along with a significant difference in expression between the T stages. In addition, there was a higher WAVE 2 expression in the less histologically differentiated tumours, in the tumours of patients with a higher overall disease stage (stage II, III and IV) and in the carcinoma tissues of those patients classified as having Dukes B and C stage. All of this Implies that a higher WAVE 2 expression level is associated with more aggressive and higher stage primary tumours.

This is supported by the analysis of data that shows there is a significant inverse correlation between WAVE 2 expression and patient's disease free survival and overall survival time. As mentioned above, the majority of patients who die from colorectal cancer die from metastatic disease. As such, the clinical implications of

this suggest that WAVE 2 plays an important role in the metastatic potential of colorectal carcinomas.

In contrast, there is no statistically significant increase in WAVE 2 expression in those patients who had or developed distant metastases compared to those who did not. Nor is it more highly expressed in those with nodal disease compared to those who do not. It is possible that low numbers in certain categories may have impacted the statistical analysis when comparing expression in those tumours with nodal and metastatic disease to those without. It is also possible that the WAVE 2 expression profile would be different in tissues from the actual nodal and metastatic deposits as it is more likely that the cancer cells forming these deposits at distant sites to the primary tumour are expressing an invasive phenotype that includes overexpression of the WAVE proteins. (Klymkowsky et al., 2009; Polyak et al., 2009; Thiery at al., 2009; Yilmaz et al, 2009; Barallo-Gimeno et al., 2005).

Finally, the results from this study and with evidence from other studies (Semba et al., 2006; Iwaya et al., 2007; Fernando et al., 2007 and Yang et al., 2006) show that WAVE 2 has an important role in the metastatic potential and survival of patients with lung adenocarcinoma, colorectal cancer, breast cancer and liver cancer. It is an important gene and protein that could be potentially used as a prognostic marker (Yang et al., 2006) and once the mechanisms surrounding its action are better understood, potentially as a therapeutic target.

7.5 Final Thoughts and Recommendations/Future work

This study has highlighted the clinical significance of the WAVE proteins in colorectal cancer tissues and also the functional role of WAVE 1 and particularly 3 in colorectal cancer cell lines. It is clear that these proteins govern cell invasion and motility through complex signalling networks which, unfortunately, are not well defined. Their contribution to spread of colorectal cancer tumour cells may hold potential as a future therapeutic target, however the precise roles played in the process of cellular metastasis is not yet fully clear.

Future work in a number of areas may clarify some of the questions raised by this thesis:

Following on from the significance of WAVE 2 expression within colorectal tissue samples, as described within this thesis, generation of colorectal cancer cell lines with knockdown of WAVE 2 expression would be beneficial. The functional assays described within this study could then be performed to allow comparison with the work already described within this thesis.

It would be beneficial to utilise a range of cell lines. Those that originate from primary colorectal tumour sites, those that originate from metastatic deposits and those that originate from nodal deposits. This would give an idea as to if and how WAVE 1, 2 and 3 expression differs depending on the site and aggressiveness of the tumour and allow comparisons to be made.

Assessment of protein expression levels within a cell gives a more accurate indication of actual translated levels of the respective protein following any post translational regulations, as such the use of Western Blot analysis would be of use to clarify the knockdown of WAVE 1, 2 and 3 beyond the mRNA stage and form a complete picture.

In addition, the use of immunocytochemistry would allow an assessment of the location of the expressed WAVE proteins within the cells and of how the cytoskeleton is working, particularly of interest would be to see the expression and localisation of WAVEs and actin in cells showing both elongated/mesenchymal phenotype and round/amoeboid phenotype.

Further collection of primary colorectal cancer tissue samples would increase the availability of stage specific sub-group samples of tissues available to be analysed. The collection of tissue samples from metastatic deposits, such as from the liver and lung, is feasible due to the increase of metastatic resections (Socola et al., 2015; Padman et al., 2013) and would allow greater comparison of the expression of WAVE 1, 2 and 3 between the different locations and give a better understanding of the significance of WAVEs in metastatic disease (Iwaya et al., 2007).

To generate individual cell lines with multiple knockdowns of WAVE 1, 2 and 3 expression to assess the redundancy of the molecules on cell function.

As the functional assays used in this study were *in vitro*, the use of *in vivo* models would provide a better indication of the therapeutic implications of the WAVE

proteins in primary colorectal cancer and metastases as was seen in previous *in vivo* models used when investigating the effects in breast cancer and melanoma (Sossey-Alaoui et al., 2007; Kurisu et al., 2005).

Finally, further work into the complex signalling networks that the WAVE proteins govern would enable greater understanding of the importance of the WAVE proteins within the system and potentially highlight possible targets for therapeutic intervention.

CHAPTER 8

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