ROLE OF MAGI PROTEINS IN INVASIVENESS OF HUMAN COLORECTAL CANCER CELLS

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Doctor of Medicine (MD) thesis

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Declaration

I hereby declare that this thesis has been composed by myself. I confirm that the work submitted is my own. The work has not been submitted for any other degree or professional qualification. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

Zubair Khanzada

Acknowledgement

My sincere thanks to Dr Martin, Miss Hargest, Prof Jiang and every single person at the Cardiff China Medical Research Collaborative (CCMRC), Cardiff University who has helped me to make it possible. My special gratitude to my parents, my wife and my two daughters, my brothers and my friends, for their constant support and encouragement.

Abstract

Introduction: MAGI -1 plays an essential role in cancer metastasis. We aimed to study the expression of MAGI -1, -2 and -3 in colorectal cancer tissue with specific focus on the role of MAGI -1 in colorectal cancer metastasis, through its function in the maintenance of cell-to-cell tight junctions.

Materials and Methods: MAGI -1, -2 and -3, expression was determined in colorectal cancer tissue by using Q-PCR and RT-PCR. MAGI -1 knockdowns were created in cancer cell lines by electroporation method and were confirmed on RT- PCR, morphology and immunofluorescence. The impact of aberrantly expressed MAGI -1 on adhesion, invasion and migration of colorectal cancer tissue was studied by using trans-epithelial resistance (TER) and electrical cell impedance (ECIS) assays.

Results: Q-PCR was consistent with the reduced expression of MAGI-1, -2 and -3 in colorectal cancer tissue. Survival curves showed reduced survival in cohort with reduced MAGI expression. MAGI -2 lacked expression on RT-PCR. MAGI -1 KDs showed decrease trans-epithelial resistance in the KDs. Reduced electrical resistance across epithelial cell lines in MAGI -1 KDs and also reduced adhesion and increase in migratory function was observed in MAGI -1 KDs on Trans-epithelial resistance (TER) and electronic cell impedance sensing (ECIS) experiments respectively.

Conclusion: Our experimental work suggests that MAGI-1 plays a vital role in maintaining the barrier function of tight-junctions in colorectal cancer tissue, which when knocked down, has a significant implication in colorectal cancer metastasis.

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Abbreviations

AGS	Human gastric cancer cell line
CAR	Coxsacki adenovirus receptor
CEA	Carcinoembryonic antigen
COX	Cyclooxygenase
CREST	Stenting for obstructing colorectal cancer – CReST trial
CRC	Colorectal cancer
CT	Computerized tomography
CTX	Cholera toxin protein
DAPI	4',6-diamidino-2-phenylindole
DLG	Dorsophila large disc tumor suppressor protein
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ECIS	Electrical cell impedance sensing
ECACC	European collection of animal cell culture
EMT	Epithelial mesenchymal transition
ESAM	Endothelial cell adhesion molecule
ETOH	Ethyl alcohol
FAP	Familial adenamatosis polyposis
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FOLFOX	Folinic acid plus flourouracil plus oxiplatin
FOLFIRI	Folinic acid plus flourouracil plus irinotecan
GuK	Guanylate Kinase domain
HPV	Human piploma virus

HBV	Hepatitis B virus
HCV	Hepatitis C virus
HGC27	Human gastric cancer cell line
HRT18	Human colorectal cancer cell line
HTLV	Human T cell lymphoma virus
HT115	Human colorectal cancer cell line
IBD	Inflammatory Bowel Disease
JAMs	Junctional Adhesional Molecules
KDs	Knock downs
LPA	Lysophosphatidic acid
MAGI	Membrane associated guanylate kinase with inverted arrangement of protein domain
MDT	Multi-disciplinary meeting
MMPs	Matrix metalloproteinases
MMR	Mismatch repair gene
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
NHERF	Sodium hydrogen antiporter regulator
NHS	National Health Service
NICE	National institute of clinical excellence
PCR	Polymerase chain reaction
PDZ	Post synaptic density protein, *Drosophila* large disc tumour suppressor, Zona occludins
PEF	Ribozymes containing empty plasmids
PET	Positive emission tomography
PTEN	Phosphatase and tensin homolog
QPCR	Quantitative polymerase chain reaction

RNA	Ribonucleic acid
RKO	Human colorectal cancer cell line
RT-PCR	Reverse transcription polymerase chain reaction
SCPRT	Short course pre-operative radiotherapy
S-SCAM	Synaptic scaffolding molecule
TAMP	Tight junction associated marvel proteins
Taq	Thermus aquaticus
TBS	Tris-buffered saline
TER	Trans-epithelial resistance
TJs	Tight junctions
TRITC	Tetramethylrhodamine-isothiocyanate
VEGF	Vascular endothelial growth factor
WHO	World health organization
WT	Wild type
XELOX	Capecitabine plus oxiplatin
ZO	Zona occludins

ROLE OF MAGI PROTEINS IN INVASIVENESS OF HUMAN COLORECTAL CANCER CELLS.

CHAPTER 1: INTRODUCTION

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1.1. Introduction

Cancer is the leading cause of human morbidity and mortality worldwide. 14 million new cases were diagnosed in 2012, with more than 8 million cancer-related deaths reported in one year. The global cancer burden is on the increase. The World Health Organisation (WHO) publishes annual cancer mortality statistics for most countries. It is expected that the number of new cases will increase by 70% over next two decades. The majority of human cancers are carcinomas, which are malignant epithelial tumours, followed by lymphomas (malignant tumour of lymph tissue). Worldwide, the most prevalent cancer in men is lung, followed by prostate, colorectal, stomach and liver. In women, the most common cancers are breast followed by colorectal, lung, cervical and stomach. Lung cancer leads the mortality count by causing 1.59 million deaths, with liver cancer following by causing 745000 deaths, stomach cancer causing 723000 deaths, colorectal cancer-causing 694000 deaths, breast cancer-causing 521000 deaths and oesophageal cancer-causing 400000 deaths, as reported mortality in 2012 by WHO (WHO cancer statistics 2012).

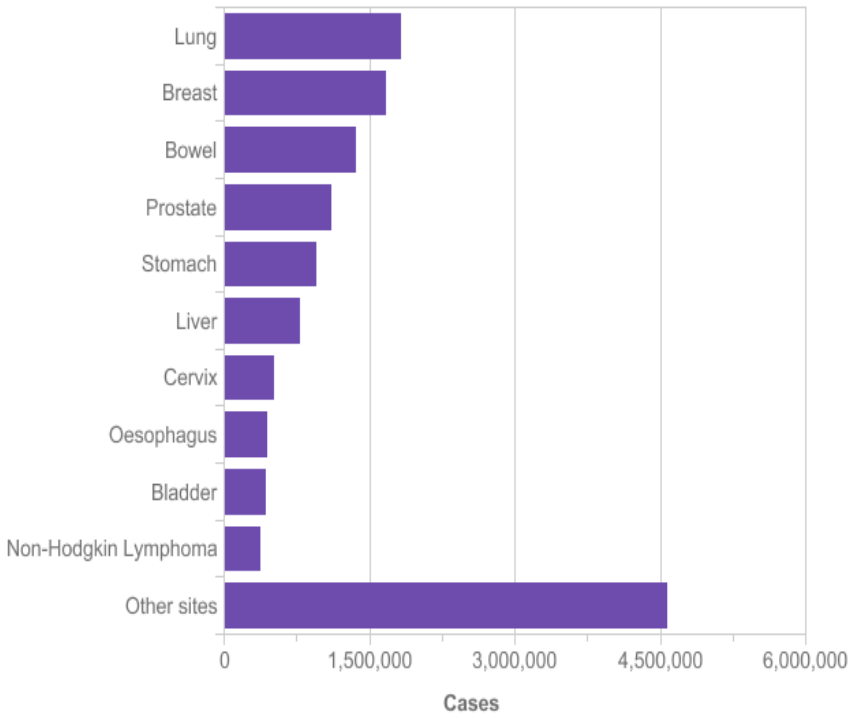


Figure 1.1.1. WHO cancer statistics 2012

1.1.1. Risk factors

Smoking single-handedly causes 20% of overall cancer deaths and 70% of lung cancer deaths. Cigarette smoking is responsible for at least a quarter of cancer deaths, especially from cancers of the lung, larynx, oral cavity and to a lesser extent the urinary tract. Most of the effects are due to direct contact with carcinogens in smoke, but these are also absorbed and excreted through the kidneys. Many carcinogens are present in smoke including benzopyrene and dimethylnitrosamine. There is a direct relationship between the number of cigarettes smoked and the risk of lung cancer, and, although stopping smoking reduces this risk, its effects are not reversible. One-third of the cancer morbidity and mortality risk is associated with dietary and behavioural factors - the top five of which include high BMI (obesity), low fruit and vegetable intake, lack of regular

exercise, alcohol, and smoking. The risk of developing cancer depends on many different factors, including age, sex, geographical location, race, occupational history, social habits and socio-economic class. Cancer mortality could be reduced more than 30% by altering the modifiable risk factors such as avoiding smoking, obesity, alcohol, radiations (ionising and non-ionizing), pollution, viral infections like hepatitis B virus (HBV) and human papillomavirus (HPV). A healthy lifestyle by maintaining regular exercise with a healthy diet also contributes towards avoidance of cancer. (WHO cancer statistics 2012)

1.1.2. Regional variations

More than 60% of the overall world's new cancers are diagnosed in Africa, Asia, Central and South America. The cancer mortality in these regions accounts for 70% of global cancer deaths. 20% of overall cancer deaths happen in low and middle socio-economic countries with viral infections such as HBV, HCV, and HPV. There are striking regional variations in cancer incidence throughout the world. Many of these variations appear to be due to environmental factors such as carcinogens rather than genetic factors. In south-east Asia and Africa, hepatocellular carcinoma is common due to the high prevalence of hepatitis B infection and environmental exposure to carcinogens, for example, aflatoxins present in mouldy ground nuts. Chewing betel-quid and areca-nut, a practice common in Asia is recognised to be carcinogenic. Malignant melanoma is mainly a disease of white-skinned people and is especially common in sunny climates such as Queensland, Australia, where many of the population are fair-skinned individuals of northern European extraction. Exposure to high levels of ultraviolet light can cause cancer. Gastric carcinoma is common in China, Japan, and Russia, whereas its incidence in Western countries has progressively fallen, perhaps due to altered dietary

habits and to the decline of infection with *Helicobacter pylori*. Breast and colorectal carcinoma are far commoner in Western countries. Careful epidemiological studies of large populations who have migrated around the world have demonstrated that the incidence of cancers in migrant populations rapidly moves towards that of the recipient country. For example, the incidence of gastric carcinoma in Japanese migrants to the west coast of America falls from the high level seen in Japan to the lower incidence in the USA by the second generation of immigrant families. The data strongly suggest that environment is more important than heredity in determining geographical variation in cancer risk. It is thought that environmental factors can account for over 80% of human tumours. (WHO cancer report 2014).

1.1.3. WHO action plan for cancer prevention and control

In 2013 WHO launched its global action plan for prevention and control of non-communicable and preventable diseases which aimed to reduce cancer mortality by 25% by 2025. The agenda included an increased political commitment for cancer prevention and control. There is a need for improved coordination and research on human cancer aetiology and mechanisms of cancer metastasis and need for the development of scientific strategies for cancer prevention and treatment. Emphasis was placed on the development and strengthening of cancer networks and health systems to improve cancer patient care. The generation and dissemination of new and existing knowledge were emphasised along with the development of protocols and tools for cancer prevention, early detection (screening and diagnosis), treatment and care of cancer patients.

1.1.4. Cancer incidence in the United Kingdom

There were 352000 new cases of cancer in the United Kingdom in the year 2013, which means 960 new cases diagnosed every single day, 40 new cases every single hour, literally someone diagnosed with cancer every other minute. 179000 new diagnoses of cancer were in men, and 173000 were in women. Only half of those with a cancer diagnosis are going to survive in the long term. Cancer killed 161823 individuals in the year 2013, and its vengeance is on the increase. In the Great Britain, cancer incidence has raised by more than a quarter over last 40 years, which is 15% in males and 35% in females. Bowel, lung, prostate and breast cancer make more than 50% of the new diagnoses of cancer patients and also makeup 46% of overall cancer deaths. Again, more than 50% diagnosed with cancer are 70 years and above. In particular an increase in cancers that are linked to lifestyles, e.g. the skin, has been observed. Many cancers are more common in white Caucasians than Asians by ethnicity, and almost all cancers are more common in socially deprived areas. It is said that, in the United Kingdom, people born after the 1960s, have one out of two chance of being diagnosed with some cancer during their lifetime. Individual risks for developing cancer depend on many factors, mainly age, genetics and exposure to external risk factors. The incidence can be reduced by addressing the preventable causes (42%) of cancer, of which smoking (19%) is the most significant single preventable cause. 4 out of ten are at risk due to their lifestyle, and modifying lifestyle may reduce this risk. It is estimated that 600,000 cancer cases were preventable in the UK. Lung cancer has highest evidence of linked cases to smoking. Currently, more than 10 million people continue to smoke in the UK that is one in five. It is estimated that up to 9% of cancers are related to the diet. Too little fruit, vegetable, fibre and high processed meat, salt are linked to cancer risk. Obesity is estimated to be linked to 5% of the new cancer cases each year, particularly oesophagal,

uterine and kidney cancer. Alcohol is linked to 4% of cancer cases in the UK, including oral cavity, larynx, pharynx and oesophagus. 6 out of 10 adults consume alcohol at least once a week in the UK, at least half of which drink more than the recommended amount. Alcohol consumption is more prevalent common in unemployed than employed and financially inactive class. (WHO cancer report 2014)

1.1.5. Bowel Cancer

It is estimated that 1.36 million new cases of bowel cancer were diagnosed in the year 2012. In Europe, 477000 new cases of bowel cancer were diagnosed 2012. In the UK, it accounts for 12% of all new cases and is overall the fourth most common cancer. It is the third most common in men (23000) after lung and prostate and also third most common in women (18200) after breast and lung. Approximately 41000 new cases of bowel cancer were diagnosed in the UK during 2013 equivalent to 110 people diagnosed with bowel cancer each day. Nearly 60% of patients diagnosed are over 70 years of age. Over the last forty years, the incidence of bowel cancer has increased by 14%. The most common site of occurrence is the rectum. 1 in 14 men and 1 in 19 women have a chance of developing bowel cancer in their lifetime.

It is estimated that bowel cancer killed around 694000 individuals worldwide, out of which 215000 were in Europe. White males have significantly higher incidence of bowel cancer compared to other ethnicities (55 per 100000). Significantly lower rates are found in the Asian population (19 – 28 per 100000 in men and 11 – 17 per 100000 in women). Bowel cancer is only second to lung cancer in killing individuals in the UK. 16200 died in the UK in 2012 due to bowel cancer, and this equals to 44 people each day. In England and Wales, 6 out of 10 (57%) diagnosed with bowel cancer are likely to survive the disease for ten years or more. Three quarters (76%) survive it for more than

a year. The 5-year survival curve drops, to 4 out of 10 from 6 out of 10, in patients aged 80 years and over. On a positive note, the mortality from this grave disease is declining. (WHO cancer report 2014)

Survival depends on the degree of spread of cancer at the time of diagnosis. When the cancer is diagnosed at its early stage, 9 out of 10 patients can survive their bowel cancer for five years or more, compared to metastatic disease, where five-year survival is poor (less than 1 in 10). Unfortunately, more than a quarter (26%) have the distant metastatic disease (stage 4) on diagnosis, and more people are diagnosed at a late stage (3 and 4) (55%) than early stage (1 and 2) disease (45%). It is estimated that bowel cancer can be prevented in up to 54% of cases linked to major lifestyle changes and other risk factors, which include processed meat consumption (21%), obesity (13%), alcohol consumption (12%), smoking (8%) and ionizing radiation (2%). Increased fibre consumption and physical activity reduce the risk of bowel cancer.

The majority of bowel cancer is located in the rectum, followed by sigmoid, caecum and ascending colon. A higher incidence of rectum (35%) and sigmoid (23%) cancer was recorded in men in comparison to the women (23% and 20% respectively), whereas higher incidence of caecum (17%) and ascending colon (9%) cancer was recorded in women in comparison to men (12% and 7% respectively). More than 90% of bowel cancers are adenocarcinoma which arises from adenomatous polyps. Benign adenomatous polyps can be as common as one-third of the western population. Only a tiny proportion (between 1 -10%) progress to malignant polyps, depending upon their size, type, and dysplasia. (WHO cancer report 2014).

1.2. Inherited bowel cancer

The lifetime risk for an individual to develop bowel cancer in the United Kingdom is 5%. Many people will have a chance of having an affected relative; as the number of relatives increases, the risk also increases. The risk due to genetic factors varies very widely from no risk to patients who will inevitably develop bowel cancer. 30% of the population is at a low to moderate risk of developing inherited colorectal cancer, while 5% of the population will be at high risk. The high-risk population has more than 50% chance of developing colorectal cancer. The high-risk groups include Lynch syndrome and familial adenomatous polyposis (FAP).

1.2.1. Lynch syndrome

Lynch syndrome is characterised by early onset of colorectal cancers, on average at 45 years of age. These tumours have certain distinguishable pathological features. There is a predilection for proximal colon and tumours are often multiple.

They tend to be mucinous, poorly differentiated and have a signet ring appearance with lymphocytes infiltration and lymphoid aggregation at margins. Other associated tumours of Lynch syndrome are endometrial, stomach, ovarian and urothelial in origin. The Amsterdam criterion I and II are used for diagnostic purposes.

<u>Amsterdam criteria I</u>

- At least three relatives with colorectal cancer, one of whom should be a first-degree relative
- At least two successive generations should be affected
- At least one colorectal cancer should be diagnosed before an age of 50 years

- FAP should be excluded
- Tumours should be verified by pathological examination.

Amsterdam criteria II

- At least three relatives with a Lynch syndrome-associated cancer (colorectal, endometrial, small bowel, ureter, renal pelvis), one of whom should be a first-degree relative
- At least two successive generations should be affected
- At least one colorectal cancer should be diagnosed before age 50 years
- FAP should be excluded
- Tumours should be verified by pathological examination.

Lynch syndrome is an autosomal dominant inherited disease which constitutes 2% of colorectal cancers. It results from a germline mutation in tumour suppressor mismatch repair (MMR) gene, whose role is to correct errors in base pair matching. A hallmark of tumours with defective MMR gene is microsatellite instability (MSI). MSI are regions where a short sequence of DNA (up to 5 nucleotides) is repeated, in which any errors during DNA replication are corrected by MMR gene. Hence in MMR deficiency, large segments of mutated DNA results. Around 15% of colorectal cancer patients show MSI. As genetic testing is expensive, Bethesda criteria are used for determining whether the tumour tissue from an individual with colorectal cancer should be tested for MSI. It is as follows,

- Colorectal cancer diagnosed at age <50 years
- Multiple colorectal or other Lynch syndrome-associated tumours, either at the same

time or occurring over a period

- Individuals diagnosed with colorectal cancer at <60 years, in whom a tumour has microscopic characteristics indicative of MSI
- Individuals with colorectal cancer who have one or more first-degree relatives diagnosed with a Lynch a syndrome-related tumour at age 50 years or younger
- Individuals with colorectal cancer who have two or more first or second-degree relative diagnosed with a Lynch a syndrome-related tumour

If a mutation is detected, treatment options include either 1-2 yearly surveillance colonoscopy from 25 years of age or 5 years earlier from youngest family member diagnosed; or a prophylactic colectomy. (Phillips, Robin K. S. Colorectal Surgery: A Companion to Specialist Surgical Practice, 4th Edition)

1.2.2. Familial adenomatous polyposis

FAP is less common than Lynch syndrome, but the risk of developing into colorectal cancer is 100%. The site of mutation is adenomatous polyposis coli (APC) gene and has autosomal dominant inheritance. It is characterised by the following.

- Hundreds of adenomatous polyps at a young age (second or third decade of life)
- Duodenal adenomatous polyps
- Extraintestinal manifestation

Diagnosis is made by either genetic testing or by the detection of FAP surveillance of at-risk family member. Treatment is prophylactic surgery before cancer develops. (Phillips, Robin K. S. Colorectal Surgery: A Companion to Specialist Surgical Practice, 4th Edition)

1.3. Tumour suppressor genes in colorectal cancer

The tumour suppressor gene maintains cell stability by regulating cell proliferation and differentiation. Therefore, uncontrollable cell proliferation and the formation of a tumour may be a result of inactivated tumour suppressor genes caused by somatic mutation or hereditary. The APC (Adenomatous polyposis coli), P53 and K-ras are involved in adenoma-cancer sequence (figure 1.2.1.) It is uncommon to have all the three mutations of APC, p53, and K-ras to coexist with same colorectal cancer, which suggests that mutations in these genes have separate pathways in colorectal tumorigenesis. (Smith *et al.* 2002).

1.3.1. APC (Adenomatous Polyposis Coli) Tumour Suppressor gene

APC gene controls many cellular functions of intestinal cells (e.g., proliferation, differentiation, migration and polarity). A good number of colorectal cancers have been reported to have loss or mutation of adenomatous polyposis coli. The APC gene is a negative regulator of beta-catenin concentrations and interacts with E-cadherin, which results in loss of cell adhesions. Thus, mutations in the APC gene result in the development of colorectal cancer (Markowitz *et al.* 2009).

1.3.2. p53 tumour Suppressor gene

p53 encodes the transcription factor that is involved in the regulation of cell cycle, apoptosis, cellular senescence (McBride *et al.* 1985, Benchinol *et al.* 1985, Rowley *et al.* 1986). Abnormalities of the p53 gene, transcript, and protein expression have been widely reported in human cancers, including colorectal cancer. Allelic loss of

chromosome 17p is observed in three of four colorectal carcinomas but fewer than 10% of adenomatous polyps. Mutations in p53 appear during the transition from adenoma to carcinoma. (Messerini *et al.* 1995).

1.3.3. K-ras tumour suppressor gene

Patients who have a K-ras mutation in their colorectal tumour have a significantly poorer prognosis than those without a K-ras mutation. Mutated K-ras cause increased and unregulated signalling down of pathways that normally are involved in growth and differentiation, such as the mitogen-activated kinase pathway. K-ras mutation is a marker of aggressive tumour phenotype. (Smith *et al.* 2002).

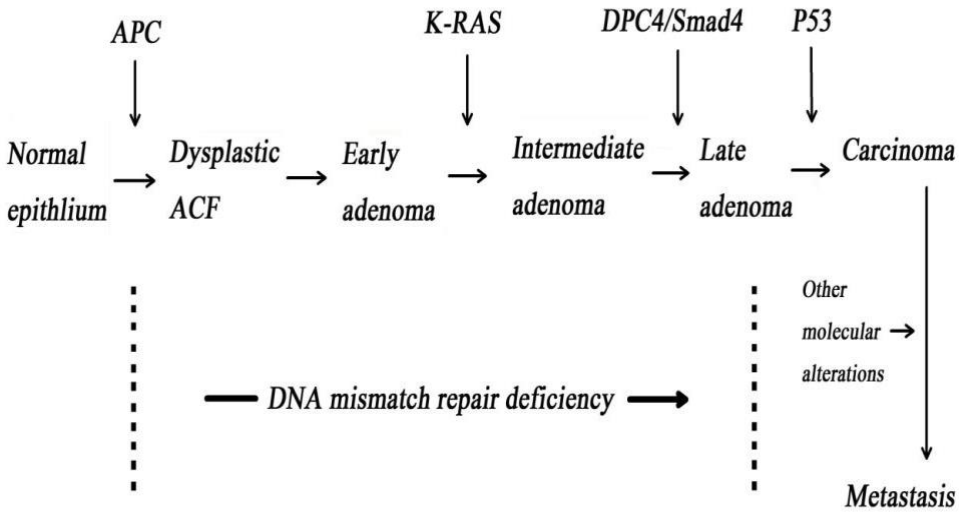


Figure 1.2.2. Flowchart demonstrates these gene mutations are consistent with the order of cancer progression (from adenoma to carcinoma). (1988. Bert Vogelstein).

1.4. Anatomy and physiology of the colon and rectum

The bowel is a part of the human digestive system. It is divided into two parts; small and large bowel. The large intestine is a muscular tube that extends from the ileocecal junction (where small bowel enters into the colon) to the anus. The colon is anatomically divided into the following parts (figure 1.4.1.).

- Caecum
- Ascending colon
- Transverse colon
- Descending colon
- Rectum

The caecum is the first part where the terminal ileum (the last part of small bowel) joins the large bowel through the ileo-caecal valve. The appendix is also attached to the caecum posterior-medially, lateral to the ileo-caecal valve. The taeniae coli of caecum converge on the base of the appendix. Taeniae coli three straps of muscle fibres, which run along the length of the colon from caecum to the rectum, where they fan out and become diffuse with the rectal wall muscle. The caecum is located in the right iliac fossa. It is completely covered in the peritoneum and relatively fixed in its position due to lack of any length its mesentery. From the caecum, large bowel travels upwards and becomes ascending or right colon as it is on the right side of the abdominal cavity. The ascending colon is covered with peritoneum on its front and sides only. It leads to hepatic flexure, where it lies in front of the right kidney and the second part of the duodenum. At the hepatic flexure, the colon curves to the left to become transverse colon. The transverse colon is very mobile, completely wrapped with the peritoneum and greater omentum hangs down from the anterior surface of the transverse colon.

The transverse colon leads to the splenic flexure, where it lies in front of the left kidney and is closely related to the spleen, which lies superior-laterally. From splenic flexure, the colon curves downwards to become descending or left colon as it lies to the left of the abdominal cavity, relatively fixed in position. The descending colon continues downwards and makes an "S" shape twist. Hence this part called the sigmoid. The sigmoid colon is mobile, and the length of mesentery varies.

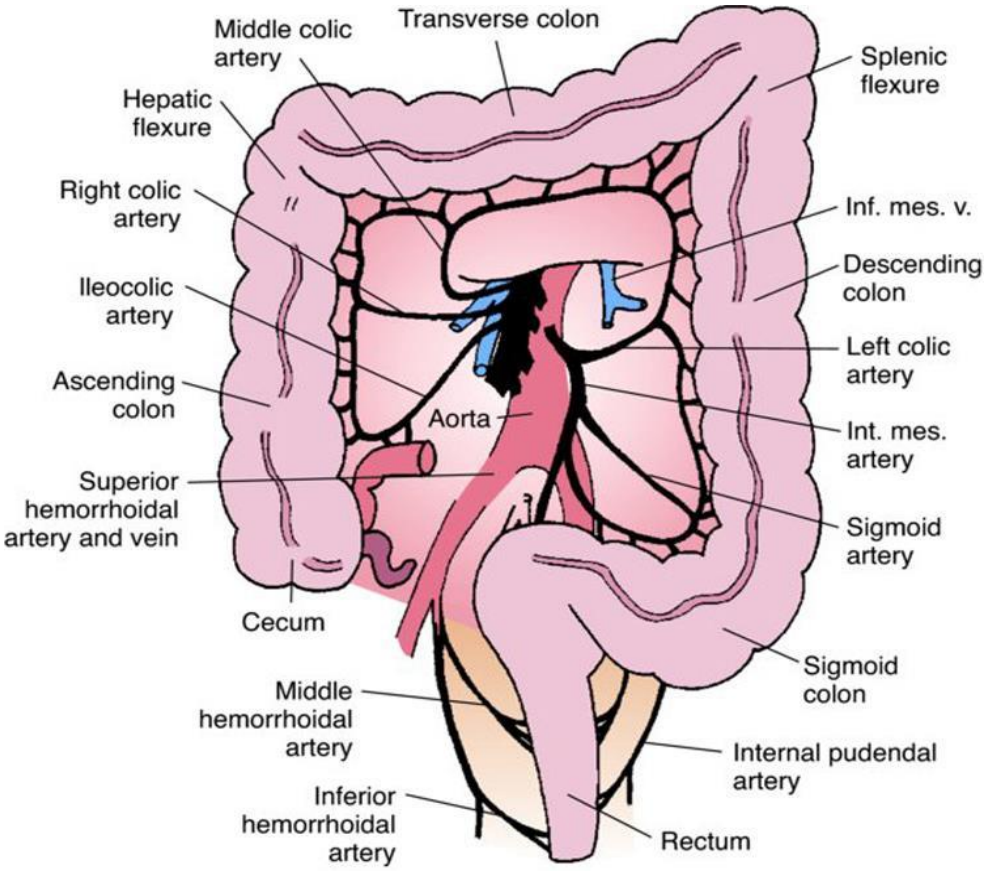


Figure 1.4.1. The anatomy of the colon with the vascular supply (Source: DeVita VT, Lawrence TS, Rosenberg SA: DeVita, Hellman, and Rosenberg's Cancer: Principles & Practice of Oncology, 9th Edition, 2011. By Lippincott Williams & Wilkins).

The sigmoid distally fans out to become the rectum (figure 1.4.2.). The recto-sigmoid junction usually lies against the third sacral vertebra. The rectum lies in the concavity of the sacrum, and it connects to the anus. It is covered by the peritoneum in its upper third, the middle third is covered anteriorly only, and the lower third is devoid of peritoneum. In men, in the lower third rectum is anteriorly separated from prostate, seminal vesicles, and base of the bladder by Denonvillier's fascia, which represents embryological fused peritoneal layers. In women, the rectum is related to the vagina anteriorly. The blood vessels, nerves, lymphatics and lymph nodes to the bowel are contained in a wrapped double layer of peritoneum called mesentery. Large bowel mesentery is called mesocolon and mesentery attached to the rectum is called mesorectum, the bulk of which lies posteriorly in the concavity of the sacrum. There is areolar plane between mesorectum and structures of the pelvis, which is essential for total mesorectal excision in cancer surgery of the rectum.

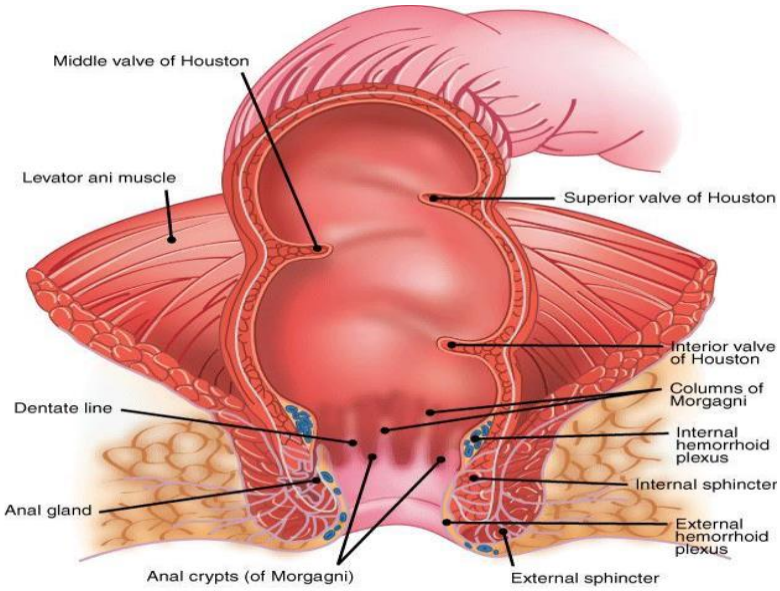


Figure 1.4.2. Rectal anatomy. (Source: Shrieve DC, Loeffler JS: Human Radiation Injury. 2011. By Lippincott Williams & Wilkins)

To understand and have an orientation of anatomy of the colon, it is crucial that one has some basic understanding of the embryological development of the gut as it is complicated by intrauterine folding of the gastrointestinal tract. In early intrauterine life, the entire gastrointestinal tract is one straight tube, which is hanging from midline dorsal mesentery. The blood supply is from three midline visceral vessels, supplying foregut, midgut, and hindgut. These blood vessels persist as the celiac axis, superior mesenteric artery, and inferior mesenteric artery, all arising in the midline from the front of the aorta. The small bowel persists on a mesentery arising approximately from the midline. On the other hand, the large bowel rotates in an anti-clockwise fashion attaining its adult life shape of ascending, transverse and descending colon. The mesentery of ascending and descending colon fuses with retroperitoneal tissue, which can be used as dissecting plane behind the colon to get to the root of mesentery for radical large bowel resections for its cancers. The fusion of the peritoneum in depth of pelvis creates rectovaginal or recto-vesical pouch forms the Denonvillier's fascia which is essential in radical resections of the rectum for cancer.

The wall of the colon is made up of the following layers (figure 1.4.3.):

- Mucosa
- Submucosa
- Muscularis propria
- Serosa

Mucosa is the innermost layer of the colon and is made of epithelium, lamina propria, and muscularis mucosa. Sub-mucosa is the layer of connective tissue that surrounds the

mucosa. The blood vessels, lymphatics, nerves and mucous glands lie in the sub-mucosa. The next layer is the muscularis propria, which is a layer of circular and longitudinal muscle fibres in the wall of the colon. The serosa is the outermost layer comprised of the peritoneum.

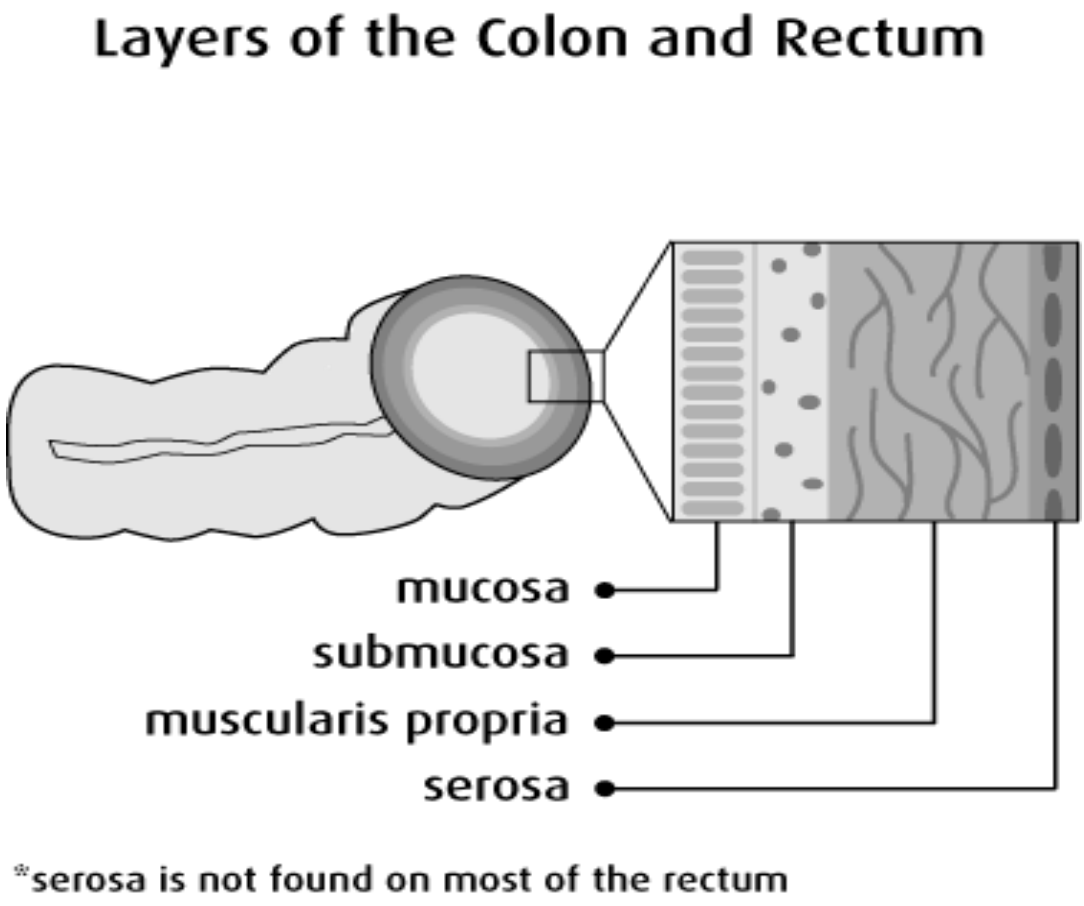


Figure 1.4.3. Layers of colon and rectum

The main function of the colon is to absorb important nutrients, electrolytes and water, and to give faeces their final form, store it and expel it when appropriate.

1.5. Natural history of the disease

It is said that in approximately 50% cases, colorectal cancer arises from the left side of the colon, whereas about 25% cases arise from the right colon. Moreover, 4-5% of cases have synchronous cancers, which is why a colonoscopy to look at the full length of the colon is indicated if cancer is found in the rectum or sigmoid. It is widely accepted that the majority of colorectal cancers arise from pre-existing adenomas. The distribution of adenomas is similar to that of cancer in the colon. Also in one-third of the cancer specimens, adenomas are found. The pathway of oncogenesis from adenoma to carcinoma in sporadic disease is broadly the same as for Familial Adenomatous Polyposis (FAP) coli, a pre-cancerous condition where the colon develops multiple polyps, which inevitably lead to cancer. (Leslie *et al.* 2002) Underlying genetic events were described above, not only for the common adenocarcinoma sequence, but also for the other most common alternative tumourigenic pathway, which is reflected by the Lynch syndrome.

1.6. Presentation

Unfortunately, many symptoms of colonic cancer are common and nonspecific, so a high index of suspicion is warranted. The presentation may be acute or chronic depending on the location, stage, and grade of the disease. Anaemia, change in bowel habit more than six weeks, episodes of per rectal bleeding, family history of colorectal cancer, smoking and alcohol intake are not infrequently described symptoms by the patients at the colorectal cancer outpatient clinics. Patients can present as large bowel obstruction or

perforation as an emergency. Occasionally patient presents with abdominal mass or pneumaturia due to the encroachment of a tumour into the bladder and resulting into a colo-vesical fistula.

1.7. National Institute for Health Care and Excellence (NICE) – suspected cancer referral pathway

A patient suspected of colorectal malignancy should be referred for further investigation by using suspected cancer pathway referral in the UK to be seen by a colorectal surgeon within two weeks. The following criteria are used:

- 40 years and over with unexplained weight loss and abdominal pain
- 50 years and over with unexplained rectal bleeding
- 60 years and over with iron deficiency anaemia or change in bowel habit
- Rectal or abdominal mass
- In adults under the age of 50 years with rectal bleeding, consider a suspected cancer pathway in case of the following unexplained symptoms or findings:
- Abdominal pain
- Change in bowel habit
- Weight loss
- Iron deficiency anaemia
- Faecal occult blood – offered to patients without rectal bleeding

50 years and above with unexplained abdominal pain or weight loss or 60 years and under with change in bowel habit or iron deficiency anaemia or 60 years and above with anaemia in the absence of iron deficiency. (Colorectal cancer NICE guidelines [CG131] Dec 2014 update)

1.8. UK department of health criteria for high risk and low-risk colorectal cancer

Higher risk:

- Rectal bleeding with a change in bowel habit to looser stools or increased frequency of defecation persisting for six weeks (all ages). Change in bowel habit persisting for six weeks (>60 years).
- Persistent rectal bleeding without anal symptoms* (>60 years).
- Palpable right-sided abdominal mass (all ages).
- Palpable rectal mass (all ages). Unexplained iron deficiency anaemia (all ages).

Low risk:

- Rectal bleeding with anal symptoms with no persistent change in bowel habit (all ages).
- Patients with no iron deficiency anaemia, no palpable rectal or abdominal mass
- Rectal bleeding with an obvious external cause, e.g. anal fissure (all ages)
- Change in bowel habit without rectal bleeding (<60 years).
- Transient changes in bowel habit, particularly to harder or decreased frequency of defecation (all ages).
- Abdominal pain as a single symptom without signs and symptoms of intestinal obstruction (all ages). (Thompson *et al.* 2003).

*Soreness, discomfort, itching, lumps, prolapse or pain.

1.9. Diagnostic investigations

Usually, more than one investigation is necessary for confirmation of colorectal cancer.

Patients with suspected colorectal cancers, who have not got any major co-morbidity, should be offered colonoscopy as a first line investigation.

If a suspicious lesion is seen, a biopsy should be taken at the time for histological diagnosis, unless contraindicated. Computerized Tomography (CT) colonography can be an alternative to colonoscopy or flexible sigmoidoscopy. A subsequent biopsy to confirm tissue diagnosis will be needed if a lesion is detected on CT. If the CT is indeterminate, a colonoscopy should also be considered. Since the Siggar Trial, (SIGGAR), Barium Enema is now rarely used, and many hospitals in the UK no longer provide this service. Once the diagnosis of colonic or rectal cancer has established, the next step would be to determine the grade and stage of cancer.

1.9.1. Screening

Colon cancer is a suitable disease for screening. Prognosis after treatment is much better in the early-stage disease, and the polyp carcinoma sequence offers an opportunity to prevent cancer by treating premalignant disease. The ideal screening test should detect the majority of tumours without a large number of false positives, i.e., it should have high sensitivity and specificity. Also, it must be safe and acceptable to the population offered to screen. In colorectal cancer, the most widely studied test is faecal occult blood testing, a guaiac-based test which detects the peroxidase-like activity of haematin in faeces. But unfortunately, the sensitivity of faecal occult blood testing is only about 50–70%. Another approach is to use endoscopy as a primary screening test. As 70% of cancers and large adenomas are found in the distal 60cm of the large bowel, which is more sensitive than FOB testing. Currently, all men and women between 60 to 74 years of age are invited to participate in faecal occult blood testing once every two

years. A screening colonoscopy is offered at the age of 55.

1.9.2. Surveillance

Surveillance strategies for patients with adenomatous polyps are divided into low, moderate and high-risk groups. The low-risk category includes patients with one or two adenomatous polyps that are less than 1cm in diameter. No follow-up or a repeat colonoscopy at five years is recommended. Intermediate risk group is defined as patients with three to four adenomatous polyps, or at least one adenoma greater than 1cm in diameter. Colonoscopy at three years is recommended. The high-risk group includes patients with five or more small adenomatous polyps or three or more, where at least one is greater than 1cm in diameter. Colonoscopy at one year is recommended.

1.10. Staging of colorectal cancer

A contrast-enhanced CT of the chest, abdomen and pelvis is done to establish the extent of the spread of cancer. Magnetic resonance imaging (MRI) is done to assess the localised spread of rectal cancer in the pelvis. An endo-rectal ultrasound scan is of value in rectal cancer if the MRI shows disease amenable to local resection.

1.10.1. Duke's classification

In 1932 Cuthbert E. Dukes developed a classification for colorectal cancer metastasis, predicting the prognosis of colorectal cancer patients. In 1936 Dukes had modified the C stage by the different levels of para-rectal lymphatic spread from the superior rectal

artery to inferior mesenteric artery. Astler and Coller further divided stages B and C in 1954 (table 1.10.1).

Table 1.10.1. Modified Dukes classification of colorectal cancer.

A	Invasive carcinoma not breaching the muscularis propria
B	Invasive carcinoma breaching muscularis propria, but not involving regional lymph nodes
C_1	Invasive carcinoma involving the regional lymph nodes (apical lymph node negative)
C_2	Invasive carcinoma involving the regional lymph nodes (apical lymph node positive)
D	Distant metastasis

Astler VB, Coller FA: The prognostic significance of direct extension of carcinoma of the colon and rectum. Ann Surg 139:846, 1954

1.10.2. TNM Classification

In 1954, Pierre Denoix proposed the TNM (Tumour, Node and Metastasis) Classification based on local invasion and lymphatic metastasis (table 1.10.2). TNM classification for colorectal cancer is widely accepted.

Table 1.10.2. TNM classification of colorectal cancer

T	Primary tumour
T_x	A primary tumour cannot be assessed
T_0	No evidence of a primary tumour
T_{is}	Carcinoma in situ
T_1	Tumour invades submucosa
T_2	Tumour invades muscularis propria
T_3	Tumour invades through muscularis propria into subserosa or non-peritonealised peri-colic or peri-rectal tissue
T_4	Tumour perforates the visceral peritoneum or directly invades other organs or structures
N	Regional lymph nodes
N_x	Regional lymph nodes cannot be assessed
N_0	No regional lymph nodes metastasis
N_1	Metastasis in 1-3 peri-colic or peri-rectal lymph nodes
N_2	Metastasis in 4 or more peri-colic or peri-rectal lymph nodes
N_3	Metastasis in any lymph nodes along a named vascular trunk
M	Distant metastasis
M_0	No distant metastasis
M_1	Distant metastasis

Source: www.cancerstaging.org/staging/index.html

1.11. Management of colorectal cancer

Management depends on whether the patient has the localised or metastatic disease. The multidisciplinary team should determine at the outset, whether curative treatment can be achieved, or whether the situation is palliative. NICE Guidelines (Colorectal cancer `NICE guidelines [CG131] Dec 2014 Update) can be useful in guiding management plans. There are different aspects of the management of rectal and colonic cancers which will be considered in the sections below. Patients with non-metastatic rectal cancer can also be classified into three different groups of patients according to the risk of local recurrence (table 1.11.1).

Table 1.11.1: Risk of local recurrence for rectal tumours as predicted by MRI

Risk of local recurrence	Characteristics of rectal tumours predicted by MRI
High	A threatened (<1mm) or breached resectional margin or Low tumours are encroaching on to the inter-sphincteric plane with levator involvement.
Moderate	Any T3b or greater, in which potential surgical margin is not threatened or Any suspicious lymph node not threatening the surgical resection margin or The presence of extra mural vascular invasion (Associated with high risk of systemic recurrence)
Low	T1 or T2 or T3a and No lymph node involvement

1.11.1. Patients with resectable primary rectal cancer

For patients who have low risk operable rectal cancer, surgery is the mainstay of treatment if the patient is fit for anaesthetic. Patients who have moderate risk operable rectal cancer can be considered for SCPRT in the immediate preoperative period (Colorectal cancer `NICE guidelines [CG131] Dec 2014 Update), although the evidence for and against this approach is controversial. (Dutch Colorectal Cancer Group, COIN Trial Investigators, Sailor Trial).

1.11.2. Patients with high-risk rectal cancer

A patient who have locally advanced rectal cancer, preoperative long course chemo-radiotherapy should be offered with an interval before surgery to allow for tumour

shrinkage with high risk. Habr-Gama *et al.* have reported excellent outcomes on a wait and watch approach following complete shrinkage of a tumour following the long course of chemo-radiotherapy. (Habr-Gama *et al.*)

1.11.3. Role of colonic stents in acute large bowel obstruction

Patients presenting with acute large bowel obstruction should have a CT scan of abdomen and pelvis to confirm the diagnosis of the mechanical cause of their obstruction, but also to rule out perforation. If cancer is suspected as the cause of obstruction, further CT chest should be done to look for chest metastasis. Contrast enemas studies as the only imaging modality are not best suited for diagnosis and staging. Patients who present as an emergency with left-sided obstruction secondary to colorectal cancer, which is deemed to be resectable, could be managed with emergency surgical resection. Following the recent publication of CREST trial results, the use of stenting as a "bridge to surgery" in otherwise operable patients should be reconsidered. However, for patients who present as an emergency with left-sided obstruction secondary to colorectal cancer, which is deemed to be unresectable and metastatic, a self-expanding metallic stent should be inserted with the help of an endoscopist and interventional radiologist with suitable experience. Self-expanding stents should not be inserted in patients with low rectal cancers, nor in cases where there is evidence of perforation or peritonitis (CReST trial).

1.11.4. Patients with stage 1 colorectal cancer

Patients with stage 1 colorectal cancer should have MDT discussion as to whether a tumour is amenable to local excision. If this is undertaken, further MDT discussion to confirm histological grade whether the excised cancer had clear margins and lymph node status. Further resection should be offered to patients who had less than 1 mm of

a clear resection margin. (Colorectal cancer NICE guidelines [CG131] Dec 2014 update) There are several technical methods for removal of early rectal cancers including Endoscopic Mucosal Resection (EMR), Endoscopic Submucosal Dissection (ESD), Trans-Anal Microscopic Surgery (TEMS) and Trans-Anal Minimally Invasive Surgery (TAMIS), a detailed discussion of which is beyond the scope of this thesis.

1.11.5. Surgical Resection of colorectal cancer

If the cancer is deemed to be resectable and the patient is fit for surgical intervention, it is the preferred and first line of treatment. Cancers of colon often involve the lymph nodes at presentation, even in the absence of distant metastasis. Therefore, to offer potentially curable resection of cancerous part of the colon, it is vital to be removed en-bloc with its lymphatic drainage. As the lymphatic drainage follows the arterial supply, radical colonic resection for cancers with its lymphatic drainage is carried out along the lines of arterial supply of the colon. The length of colon needed to be excised determines the reconstruction options. Once the cancerous part has been removed during the operation, the healthy ends are aimed to be joined together if possible. The healthy ends must have a good blood supply and should be able to reach each other without any tension for the anastomosis to successful. The radical resection of lymph nodes inevitably ends up compromising the blood supply. Therefore, a longer segment of the bowel has to be resected. Hence the extent of lymph node removal determines the length of the colon that has to be excised. The standard operations for colonic cancer resection include:

- Right hemicolectomy
- Extended right hemicolectomy
- Transverse colectomy

- Left hemicolectomy
- High anterior resection
- Low anterior resection
- Abdominoperineal resection
- Hartmann's procedure

Every operation has to be tailored to the individual patient depending upon their vascular anatomy, length and mobility of the colon, the exact position and extent of cancer, all of which might dictate adaptabilities to the standard resections listed above. A bypass procedure can be considered with palliative intent where resection not possible.

The decision between laparoscopic and open resection is made after informed discussion with the patient, considering the suitability of the lesion for laparoscopic resection, evaluating risks and benefits of the two procedures and experience and technical skill of the surgeon. The surgeon's skills should meet the criteria and standards set by the trust and colorectal cancer network. (Farquharson's Text Book of Operative General Surgery)

1.11.6. Adjuvant chemotherapy in patients with colorectal cancer

After the colorectal cancer MDT discussion, a detailed discussion should be carried out with patients and their family involving risks and benefits. For patients with high-risk stage 2 and stage 3 rectal cancer, adjuvant chemotherapy should be considered to reduce the risk of local and systemic recurrence. For patients with high-risk stage 2 colonic cancer, adjuvant chemotherapy should be considered. For patients with high-risk stage 3 colonic cancer, adjuvant chemotherapy (capecitabine and oxaliplatin) should be

offered. (Colorectal cancer `NICE guidelines [CG131] Dec 2014 Update).

1.11.7. Management of metastatic disease

If both a primary and metastatic tumour is considered to be resectable, anatomical site-specific MDT discussions are carried out with consideration of initial systemic chemotherapy followed by surgery. FOLFOX (folinic acid plus fluorouracil plus oxiplatin) or XELOX (capecitabine plus oxiplatin) is used as first-line systemic chemotherapy for advanced metastatic disease. Second line chemotherapeutic agents would include FOLFIRI (folinic acid fluorouracil plus irinotecan) or single-agent irinotecan. Raltitrexed should be only considered in patients who are intolerant to 5 fluorouracil. A full discussion should take place between the oncologist and the patient taking individual patient's preferences into account.

(Colorectal cancer `NICE guidelines [CG131] Dec 2014 Update).

1.11.8. Imaging hepatic and extrahepatic metastasis

A contrast-enhanced CT of chest abdomen and pelvis should be offered to all patients diagnosed with colorectal cancer not only to stage but to pick up hepatic and extrahepatic metastasis. Each case, depending on the anatomical site of metastasis, is discussed by the corresponding MDT. A positron emission tomography CT (PET CT) of the whole body may be appropriate if metastasectomy is being considered. (Colorectal cancer `NICE guidelines [CG131] Dec 2014 Update).

1.11.9. Ongoing care

Patients following major curative resection are offered outpatients' clinic appointment in 4-6 weeks after their surgery. Six monthly blood tests with measured serum carcinoembryonic antigen (CEA) and a minimum of 2 CT scans of chest abdomen and

pelvis are offered as surveillance during the first three years. Surveillance colonoscopy is offered at the one-year interval, and after that at five years interval provided no concerns raised on any of the other investigations. (Colorectal cancer `NICE guidelines [CG131] Dec 2014 Update).

1.12. Mechanism of spread

Cancer metastasis is a complex multi-step process, involving cell and cell-matrix interactions, in which cell adhesion molecules of various types are involved. Cancer cells can spread by one or more of the following mechanisms.

1.12.1. Reduction in cell to cell adhesion

Normal epithelial cells bind tightly to one another by adhesion molecules. Malignant epithelial cells are less firmly attached to one another, due at least in part to reduced expression of binding proteins. The cancer cells invade the basement membrane by the interaction between cell surface integrins and matrix proteins. The cancer cells produce proteolytic enzymes such as matrix metalloproteinases (MMPs), which break up the matrix proteins laminin, fibronectin and collagen. These proteolytic enzymes are under complex control mechanisms. (Patel *et al.* 2011)

1.12.2. Tumour cell motility

Movement is generated by the cytoskeleton of cross-linked actin molecules and is stimulated by a wide variety of growth factors, stromal components and cytokines, for example, auto toxins.

1.12.3. Angiogenesis

Angiogenesis is the process by which new blood vessels are formed. Normally angiogenesis is seen in wound healing and embryogenesis. The importance of

angiogenesis in tumour development has been established. The development of blood supply to a tumour is critical in progression. The mechanisms involved are complex. New blood vessels are formed by outgrowth of endothelial cells into the tumour mass. The stimulus for this is the increased production of angiogenic factors by the tumour cells, especially vascular endothelial growth factor (VEGF), fibroblast growth factors and angiogenin. The steps involved in angiogenesis include:

- Proteolytic digestion of basement membrane
- Migration of endothelial cells
- Proliferation of endothelial cells
- Organization of endothelial cells into new blood vessels with lumens.

(Patel *et al.* 2011)

1.12.4. Vascular invasion

Tumour cells must breach the basement membrane and penetrate between endothelial cells; the thin walls and poorly formed basement membranes of newly formed blood vessels are easy to penetrate. Once tumour cells are free within the lumen of the blood vessel, they are carried into the circulation and lodge in a capillary bed. At this site, further complex interactions are required. The tumour cells bind to endothelial cells. The cells then penetrate the capillary basement membrane by mechanisms similar to those described above. (Patel *et al.* 2011)

1.12.5. Establishment of a new colony

This involves cell proliferation and the development of a tumour blood supply by stimulation of angiogenesis as previously described. It will be apparent that the process of metastasis is not simply determined by simple mechanical factors. Stephen Paget in

1889 proposed that cancer metastasis depends on the cancer cells (the seed) and the microenvironment of the host organ (the soil), which still holds true. The potential of cancer cells to metastasise depends on their interaction with the homeostatic factors that promote cancer cell growth, survival, angiogenesis invasion and metastasis. The phenomena are known as ‘seed and soil'. Also, not all cells in a tumour are equally likely to metastasise: the acquisition of the ability to metastasise is determined by the expression of a series of genes whose normal function appears to be the control of cell migration during embryogenesis, normal tissue homeostasis and inflammation and repair. (Patel *et al.* 2011)

1.13. Pathophysiology of metastasis

Cancer cells can invade the surrounding tissues. In epithelial tumours, the cells breach the basement membrane, *i.e*. proceed from the stage of intraepithelial neoplasia to invasive cancer. At this point tumour, cells are classified as malignant. Usually, the cancer cells take the paths of least resistance. Cancer cells continue to spread through lymph, blood or can spread across the peritoneal cavity. Metastatic spread is not uncommon and directly impacts the patient's survival. Metastasis is a complex multi-step process involving cell and cell-matrix interactions in which various types of cell adhesion proteins are involved. Patients who are diagnosed at an early stage have a much better prognosis than those who present with more extensive disease. Over 93% of patients diagnosed with Dukes stage A (the earliest stage of the disease) survived five years compared with less than 7% of patients with advanced disease (Dukes stage D). (Cancer Research UK). At the time of diagnosis of cancer, at least half of the patients have metastatic disease. The metastatic spread of cancer is most commonly responsible for death in most of the patients. (Devita *et al.* 1975) Another significant number of patients will have micro-metastases which might not get detected with the conventional

investigation modalities. The spread of the cancer is the key event, which leads to mortality in most of the cancer patients. The spread of cancer is known to be a process which involves some sequential events, termed the metastatic cascade. The metastatic cascade can be broadly divided into three main processes: Invasion, intravasation and extravasation (figure 1.14). (Martin *et al.* 2011)

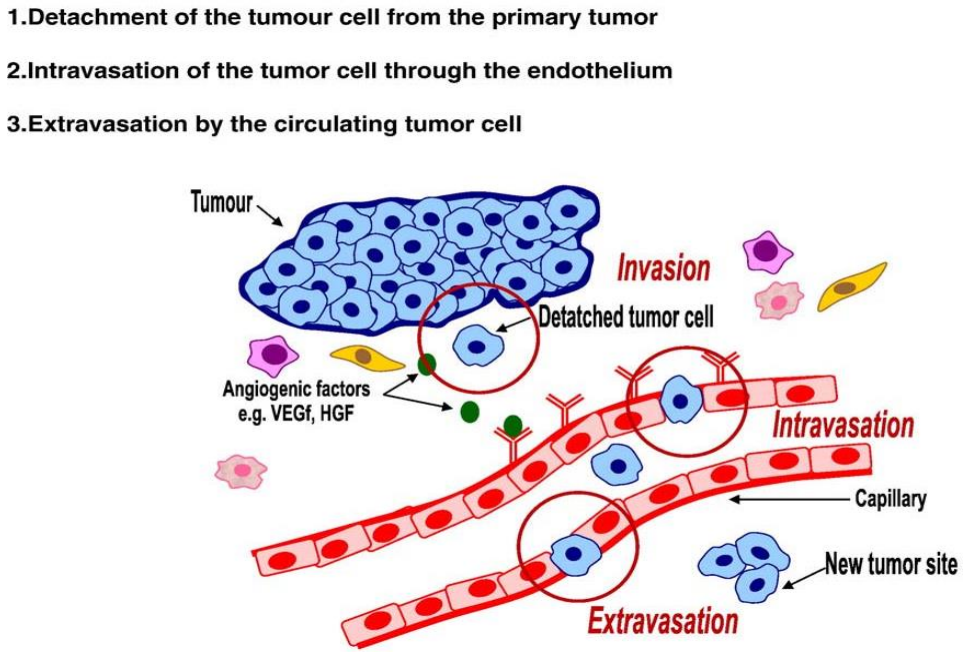


Figure 1.14. Schematic illustrating events in the metastatic cascade (Martin et al. 2009)

This initial dissociation of malignant cells from a primary tumour happens by loss of cell adhesion between cells, which subsequently leads to invasion of the cells to the surrounding stroma, hence process of angiogenesis begins involving specific proteins expression and suppression, degrading basement membrane and extracellular matrix and also affecting cell motility and migration (Brooks *et al.* 1996). Once a tumour cell has detached, it makes entry to the nearest blood or lymph vessel (by process of intravasation) and metastasises to distant sites. (Folkman *et al.* 1992, Folkman *et al.* 1996). Once the tumour cell arrives at the distant site, it develops adhesions to the

blood vessel endothelial cells by undergoing chemical interactions and penetrates through the endothelium and basement membrane (by process of extravasation), leading to proliferation at the distant metastatic site.

1.14. Tight junctions

Tight junctions (TJ) are a fundamental structural adhesive structure which is sited firstly between adjacent epithelial cells (which can prevent detachment of cancer cells) and secondly between the epithelial/endothelial cells and their basement membrane (which can prevent intravasation and extravasation of cancer cells). Tight junctions have been of much focus of research recently in cancer metastasis, as for cancer cells to metastasise successfully, TJs must be disrupted. (Martin *et al.* 2011)

Farquhar and Palade first described tight junctions (TJ) in 1963. They provided evidence that, in epithelial cell linings, the apically located belt-like structures form an impermeable barrier that restricts diffusion process through the luminal spaces (Farquhar *et al.* 1963). Body cavities, lumens and surfaces are lined by epithelial cells. These epithelial cells are exposed to repeated insults by the environmental agents and carcinogens, which cause repeated damage. To cope with the repeated, ongoing damage, the body constantly renews these epithelial cell linings, which have a very high regeneration and proliferation rate. This leads to a high probability of acquiring genomic mutation, which is probably why nearly ninety percent of cancers arise from epithelial cells. There is good evidence in the literature on the loss of TJs and spread of cancer. (Martin *et al.* 2010, Kominsky *et al.* 2003, Sauer *et al.* 2005, Seok *et al.* 2007, Usami *et al.* 2006, Krämer *et al.* 2000, Osanai *et al.* 2006).

As discussed earlier, one of the important early steps in cancer metastases is interaction and penetration of the vascular endothelium by the cancer cells. The TJs in the vascular

endothelium act as a barrier to molecules and cells. TJs in the epithelium acts as an adhesive, which prevents cells from detachment. (Hollande *et al.* 2001).

There has been enough evidence to prove that TJs are the first line of defense, which cancer cell must overcome to metastasise. (Martin *et al.* 2002, Hoevel *et al.* 2002, Ren *et al.*1990, Satoh *et al.*1996). TJs have been reported to control cell polarity which effects epithelial-mesenchymal-transition (EMT), as the acquisition of tumour of epithelial origin, which is regarded as a pathological version of EMT. EMT is a cellular mechanism long recognised as a feature of the normal development process. In EMT cells lose their epithelial characteristics and gain mesenchymal characteristics. This results in the cell to cell cohesiveness, which leads to the increased migratory capacity of the cell. Multiple genes and proteins are involved in EMT by up and down regulation. The most critical molecule which down regulates in EMT is E-cadherin. E-cadherin acts as a tumour suppressor by inhibiting invasion and metastasis. (Ikenouchi *et al.* 2003, Martin *et al.* 2005, Ohkubo *et al.* 2004, Gopalakrishnan *et al.* 1998)

1.14.1. Structure of TJs:

TJs are the most topically located structures, and they govern the endothelial and epithelial permeability. The TJs appear to be completely occluding the extracellular space by creating an intracellular barrier, which acts as an inter-membrane diffusion fence. They exist in the plasma membranes of adjacent cells by forming a series of contacts (Figure 1.14.1.1). (Jiang *et al.* 1999)

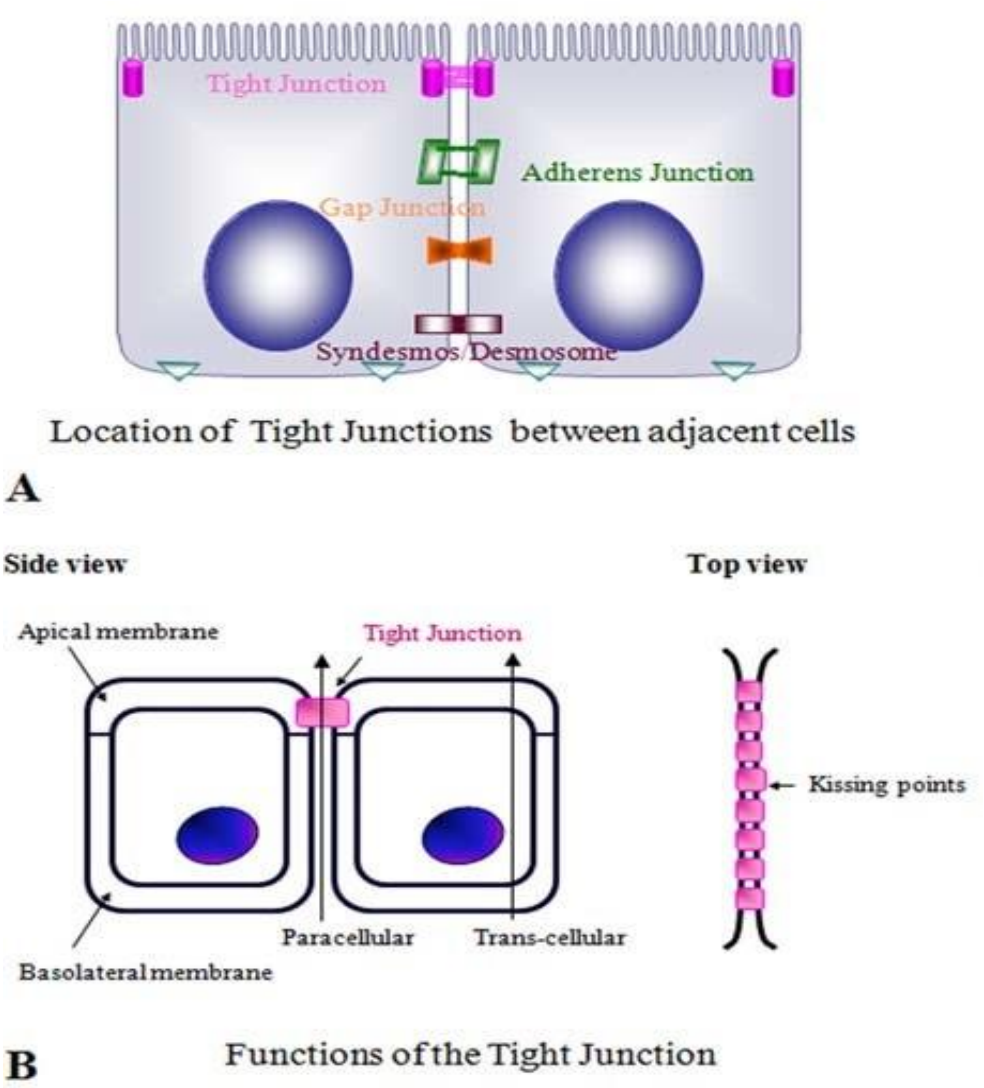


Figure 1.14.1.1. Schematic illustration of TJs structure and location between adjacent cells. (Martin et al. 2011).

The TJs are characteristic in structure and appear as discrete fusion sites between the outer plasma membrane of adjacent cells. On freeze-fracture and electron microscopy, they appear as continuous intramembrane particle strands on the protoplasmic face with complementary grooves in the extracellular face in adjacent cells. (Furuse *et al.* 1998). The appearance of these networks of complete circumscribed apices of intramembrane fibrils has also been described as of kissing points. (Wong *et al.*1997)

The structure of TJs can be represented by a conglomerate of proteins (figure 1.14.1.2), which has remained an area of interest for scientists and has been extensively investigated. TJs consists of 3 regions (table 1.14.1.1):

1. Integral trans-membrane proteins: These include occludin and other

   TJ associated Marvel proteins (TAMP), claudins and junctional adhesion molecules (JAM), together with other cholera toxin proteins (CTX). The integral membrane proteins are essentially the adhesions proteins which have the responsibility of for maintaining correct assembly of the TJs structure. The integral membrane proteins are also involved in the control of the TJ functions.
2. Peripheral or plaque anchoring proteins: These include PDZ motifs like Zonula Occludens (ZO) -1, -2, -3 and Membrane Associated Guanalytes Inverted Orientation (MAGI) -1, -2, -3 etc. The PDZ domain is a common structural domain of 80 to 90 amino acids found in signalling proteins. PDZ domain plays a key role in anchoring receptor proteins in the cytoskeleton. PDZ are initials of the first three proteins discovered. The P is from postsynaptic density protein (PSD), the D is from Drosophila large disc protein (Dlg), and the Z is from zona occludins (ZO). The anchoring proteins play their key role in the assembly and maintenance of the TJs by the anchorage of transmembrane proteins. They also play their role in the signalling mechanism of the cell and binding of the

cytoskeletal.

3. The peripheral TJs-associated regulatory proteins: These include alpha-catenin and cingulin *etc*. The TJ associated regulatory proteins work in conjunction with the anchoring proteins.

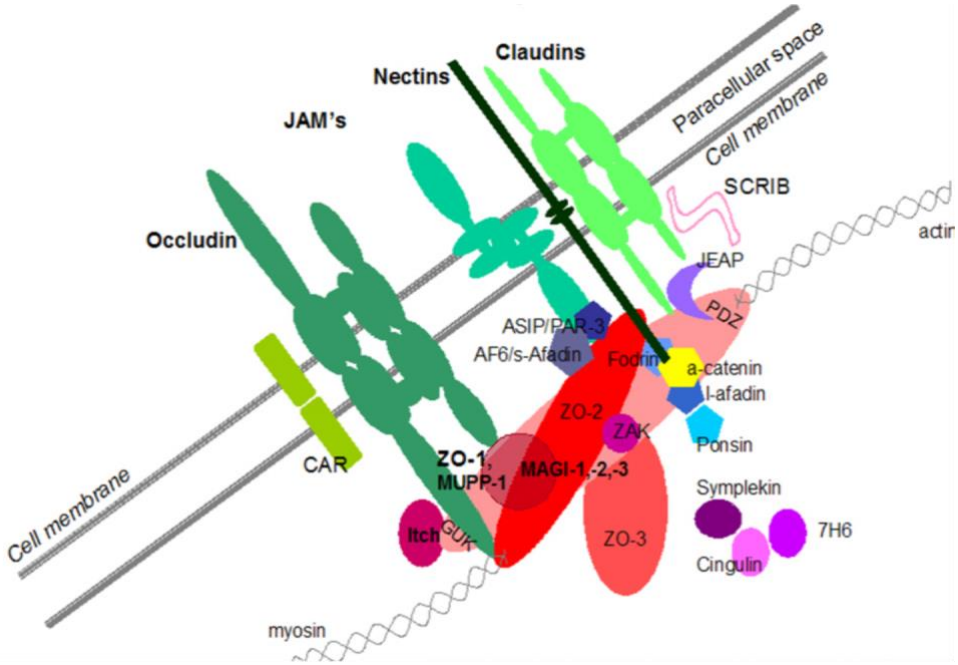


Figure 1.14.1.2. Schematic presentation of TJs and its associated proteins. (Martin et al. 2011)

Table 1.14.1.1: List of proteins involved in TJ structure, function and regulation. (Martin et al. 2011)

Location	Protein
Integral transmembrane proteins	Occludin, Tricellulin, MARVELD3
	Claudins 1-24
	JAM
	CAR, ESAM, Vmp1
Peripheral plaque proteins	ZO-1, ZO-2, ZO-3
	MAGI-1, -2, -3
	MUPP-1
	PAR-3/ASIP
	PAR-6
	AF-6/s-afadin
	CASK
	CAROM
Associated proteins	Cingulin, 7H6, Symplekin, ZONAB, Rab-13, 19B1, Ponsin,
	Rab 3B, PKC, l-afadin, c-src, Gi-2, Gi-12, alpha-catenin,
	Pals, PATJ, PKA, JEAP, Pilt, PTEN, ZAK, SCRIB, ITCH,
	Rho-GTPases, WNK4, Vinculin

1.14.2. Functions of TJs

The cell adhesions to adjacent cells and the extracellular matrix play a vital role in the regulation of cellular processes like gene expression, differentiation, growth and motility. These regulatory functions are mediated by TJ molecules, cytoskeletal proteins and transmembrane receptors, all of which constitute multi-molecular complexes, involved in signaling pathways. TJs separate the apical and basolateral fluid epithelial and endothelial compartments and seal the intracellular space. TJs work as a diffusion barrier to plasma membrane lipids and proteins, hence define apical and basolateral membrane domain of the polarised epithelial and endothelial cell lining. This is why TJs are crucial for the generation of chemical and electrical gradients across mono-layered epithelial cell linings, which is needed for vectorial transport processes. TJ molecules are involved in cell signaling as intermediates and transducers, playing a role in processes of maintaining cell growth, cell polarity, cell differentiation and proliferation. TJs function as barriers to cell migration. These regulatory functions are mediated by TJ molecules, cytoskeletal proteins and transmembrane receptors, all of which constitute multi-molecular complexes, involved in signaling pathways.

In addition to the above roles, evidence suggests that TJs play an important role in tumorigenesis. (Chlenski *et al.* 1999). Furthermore, there is increasing evidence that the spread of human cancer is frequently associated with failure of TJs in epithelial cells. (Latorre *et al.* 2005). The loss of cell-to-cell adhesion has been widely accepted as the starting point for metastasis in the epithelial cell linings of cancer tissues. (Kominsky *et al.* 2003). Tobioka *et al.* showed that optimising the function of TJs can decrease in the penetration of cancer cells through the mesothelial cell linings. (Tobioka *et al.* 1996).

1.14.3. Tight junctions in colorectal cancer cells

Increased leakiness in TJs during the metastatic process has also been related to the oncogene mutations in cancer cell lines. It was also reported by Soler *et al.* that the Trans-Epithelial Resistance (TER) and para-cellular influx rate of TJs were effected in colonic cancer cells when compared to normal colonic cells, suggestive of increased permeability and decreased barrier function, hence leakier colon cancer epithelial cells than that of normal colonic epithelial cells. (Soler *et al.* 1999, Mullin *et al.* 2005). Stromberg *et al.* reported that invasiveness of colorectal cancer cells had a co-relation with cell adhesion proteins and polymorphism in their genes. (Stromberg *et al.* 2008). Landy *et al.* suggested that the dysregulation of TJs proteins precede the development of inflammatory bowel disease (IBD) and also lead to the development of IBD associated colorectal cancer (CRC). IBD results from the compromised integrity of epithelial barrier. Intestinal epithelium acts as a static barrier and has complex mechanisms to control and regulate bacterial interactions with the mucosal surface, during which apical tight junction proteins are critical. Alterations in tight junction proteins compromise epithelial barrier function in inflammatory bowel diseases. (Landy *et al.* 2016)

1.15. MAGI proteins

MAGI (Membrane Associated Guanylate Kinase with the Inverted arrangement of protein interaction domains) is one of the peripherally associated cytoplasmic proteins, active in TJs (figure 1.15.1). MAGI proteins were first reported by Dobrosotskaya *et al.* in 1997, recognised as prototypical members of membrane-associated guanylate kinase (MAGUK) family, which is a part of DLG (*Drosophila* disc large tumour suppressor protein). The MAGUK family of proteins are mainly found in the assembly of multiple protein complexes on the inner surface of the plasma membrane at regions of the cell to cell contact points (TJs) in epithelial cells and synaptic junctions in neurons. MAGUK

proteins are characterised by their specific three type of protein-to-protein interactions:

1) Guanylate kinase (GuK) domain
2) PDZ domain (one or three in number)
3) Src homology 3 (SH3) domain

Guanylate kinase (GuK) domain helps to catalyse reactions. PDZ domain plays a role in the anchoring of proteins. Src homology 3 (SH3) domain is active in signalling pathways. The pattern of the above domains was conserved in previously known MAGUK proteins, which would be, either one or three PDZ domains in the NH2 – terminal half would be followed by SH3 domain, which would then be followed by a COOH-terminal GuK domain. However, in MAGI, the GuK domain is at the NH2 terminus, the SH3 domain is substituted by two WW (in full) domains, not found in other MAGUK proteins. Also, MAGI contains five PDZ domains. MAGI is the only protein that contains a GuK domain, which is combined with five PDZ domains and two WW domains. This combination of PDZ and WW domains in MAGI proteins can be used to predict their interactions with other proteins. (Dobrosotskaya *et al.* 1997).

There are three types: MAGI -1, MAGI -2, and MAGI -3, based on their highest degree of to the central PDZ domains with the inverted orientation of the GuK domain and SH3 domain and their significant resemblance in protein to protein interactions. MAGI -1 is located on chromosome 3p14.1. MAGI -2 is located on chromosome 7q21 and MAGI -3 is located on chromosome 1p12-p11.2. MAGI is reported to be widely expressed in tissues and tumours. MAGI -1 reported being expressed widely expressed in different tissues and tumours, whereas MAGI -2 was reported to be predominantly expressed in the brain and renal tissue. MAGI -3 is reported to be expressed in various tissues, including fetal and adult, and also in tumours. (Wu *et al.* 2000).

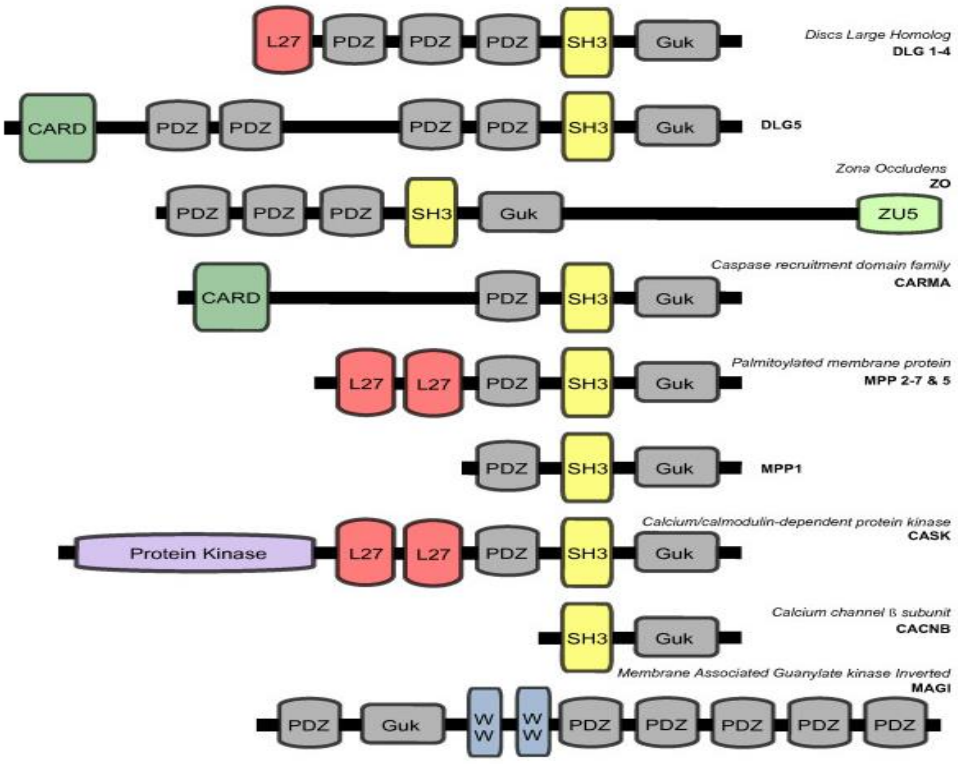


Figure 1.15.1. Unique Arrangement of PDZ Domains in MAGI.

1.15.1. MAGI -1

MAGI -1 is located on chromosome 3p14.1. MAGI -1 occurs in three splice variants (MAGI -1a, MAGI -1b and MAGI -1c), which is primarily due to the divergence in the COOH termini of fifth PDZ domain. MAGI -1b also has a small insert near second PDZ domain. The COOH termini of MAGI -1a and MAGI -1b contain 16 and 48 unique sequence amino acids respectively, with a 3.5 kDa molecular weight difference. The COOH terminus of MAGI -1c extends 251 amino acids beyond the splice site and is highly charged. The short COOH-terminal sequence is unique to MAGI -1a and MAGI -1b and does not constitute any features that would suggest the functional difference between the two. (Dobrosotskaya *et al.* 1997).

MAGI -1 is widely expressed at and have been found to be localised to the epithelial and endothelial cell to cell TJs, and acts as a scaffolding molecule to stabilise cadherin-

mediated adhesions and to recruit molecules in TJ. (Laura *et al.* 2002).

MAGI-1 cleavage has proven to be an important step in the disassembly of cell-to-cell adhesion during apoptosis. (Gregorc *et al.* 2007). Roles attributed to MAGI include organisation of protein complexes at the cell and synaptic junctions, regulation of cell polarity, connecting trans-cellular proteins with the cytoskeleton in TJs. (Martin *et al.* 2011), and regulation of synapse formation and plasticity. (Emtage *et al.*2009). Kimura *et al.* suggested that Endothelial cell-selective adhesion molecule (ESAM) regulates MAGI-1 recruitment to the cell contacts and subsequently promotes actin polymerisation and mature cell-to-cell adhesion (Kimura *et al.* 2010).

Stetak *et al.* reported that the knockdown of MAGI protein causes a loss of junctional compartmentalisation along the lateral membrane, and reduces the overall robustness of cell-to-cell adhesion. Stetak *et al.* also concluded from their experiments that MAGI-1 proteins functioned in organisation and segregation of different cell adhesion complexes into distinct domains along the lateral plasma membrane. (Stetak *et al.* 2010). MAGI have also been reported to be involved in cancer cell signalling as a tumour suppressor regulator protein (Matsuda *et al.* 2013). It is advocated in the literature that MAGI -1 has not only an important role to play in providing a connection with the cytoskeleton and components of the electromechanical transduction cell complexes (XU *et al.* 2008), but it also forms an important component of the glomerular slit diaphragm, which is closely associated with nephrin (a protein necessary for the proper functioning of the renal filtration barrier) (Hirabayashi *et al.* 2005).

Furthermore, MAGI-1 has also been proposed as the recruiter of PTEN (a tumour suppressor gene in mammals) at adherens junctions, as an effector system at the site of cell-to-cell contact junctions, which are focal points for restraining of not only the disruption of junctional complexes, but also commencement of tumour cell invasion

(Kotelevets *et al.* 2005). Kranjec *et al.* highlighted the fact that MAGI-1 expression can induce cell growth arrest and apoptosis in Human Papilloma Virus (HPV) tumour cells. (Kranjec *et al.* 2014).

1.15.2. MAGI -2

MAGI -2 is located on chromosome 7q21. There are three protein isoforms of MAGI -2, previously known as AIP1, S-SCAM and ARIP1, derived from alternative splicing and different initiation of ATG methionine. MAGI -2 was initially identified by Hirao *et al.* as novel proteins with the inverse structure of membrane-associated domains that bind with SAPAP through the GK-like domain and NMDA receptors and neuroligins through the PDZ domains. MAGI -2 were initially named as S-SCAM due to their assembly in the cell adhesion proteins at synaptic junctions. Initial high expressions of MAGI -2 were noted at synaptic junctions in the brain tissue of mice. (Hirao *et al.* 2000, Shoji *et al.* 2000, Hirao *et al.* 1998, Wood *et al.* 1998). MAGI-2, in association with PTEN, has been shown to play a critical role in inhibition of cell migration and proliferation function in hepatic cancer cells (Hu *et al.* 2007).

Wu *et al.* published their work on MAGI -2 and identified it for its possible tumour suppressor activity by its interaction, which results in stabilising PTEN expression. They suggested that MAGI-2 enhances PTEN function, which raises the possibility of MAGI-2 in itself to act as a tumour suppressor gene. MAGI-2 was found to be localised on chromosome 7q21, which was found deleted in tumours like a region that is deleted in uterine leiomyomas, prostate cancer, and glioblastoma. (Wu *et al.* 2000). MAGI -2 has also been identified as playing an important role in human prostate cancer. (Berger *et al.* 2011). Recent deep sequence studies have shown that genetic deletions and rearrangements in MAGI -2 are involved in human melanomas. (Pleasance *et al.* 2010).

MAGI -2 has been identifying cervical cancer. (Chen *et al.* 2014). MAGI -2 mRNA are potential target areas which are up-regulated in tumours. (Sachdeva *et al.* 2011, Kitamura *et al.* 2014). MAGI -2 has been identified in renal glomeruli in mice, where it is found to interact with the nephrin, which is one of the essential components in the cell to cell junctions and prevents urinary protein loss. (Lehtonen *et al.* 2005, Welsh *et al.* 2010, Patrakka *et al.* 2007). Moreover Balbas *et al.* published their evidence that in vivo that MAGI -2 is essential for the kidneys for their functions such as filtration and preservation of podocytes. (Balbas *et al.* 2014). Kitamura *et al.* identified MAGI -2 is a potential target in patients with advanced adenocarcinoma of the lungs, as suppression of MAGI -2 resulted in the loss of PTEN stability, leading to Epithelial-Mesenchymal Transition (EMT). (Kitamura *et al.* 2014).

1.15.3. MAGI -3

MAGI -3 is located on chromosome 1p12-p11.2. Wu *et al.* reported MAGI -3 as one of the MAGUK proteins with inverted orientation, based on its homology to the group in its domains and its protein to protein interactions. (Wu *et al.* 2000). Lee *et al.* found that over-expression of MAGI -3 inhibited LPA induced migration and invasion of colon cancer cells, whereas by knocking down MAGI -3 continues the repeated effect of Sodium hydrogen antiporter regulator (NHERF -2) over-expression, which led to the evidence that MAGI -3 has a negative regulatory effect on LPA_2 mediated cellular pathways and the PDZ domain-containing proteins has critical role in regulation of LPA_2 mediated effects in colorectal cancer cell lines. The signaling and functioning of LPA_2 are reciprocally modulated by the dynamic coordinated interaction of the two PDZ scaffolding proteins and NHERF -2 and MAGI -3. (Lee *et al.* 2011). Adamsky *et al.* suggested a possible role of MAGI-3 as a scaffolding molecule that acts on receptor tyrosine phosphatase and its substrates at the plasma membrane. (Adamsky *et al.*

2003). Ohashi *et al.* suggested that HTLV-1 viruses, the causative agent of Human T Cell Lymphoma, interact with MAGI -3 in rat cells, which possibly play a role in transformation. This may relate to the pathogenesis of HTLV-1-associated diseases. (Ohashi *et al.* 2004). Thomas *et al.* suggested MAGI -3 as a possible target site for the Human Papilloma Virus 6 (HPV-6), which is responsible for some human cancers. (Thomas *et al.* 2002)

1.15.4. MAGI proteins in colorectal cancer

Zaric *et al.* reported that COX -2 was inhibited by up-regulation of MAGI -1. Colorectal cancer cells when treated in vitro with COX -1/2 inhibitors (COX is cyclooxygenase pathway involved in the synthesis of prostaglandins in humans), showed an increase in expression of MAGI -1 mRNA and MAGI -1 proteins. The increased expression of MAGI -1 was seen to induce stabilisation of E-cadherin and B-catenin between cell to cell junctions, increased paxillin-positive focal adhesions and increased actin stress fibres and cell adhesion to matrix protein. Increased expression of MAGI -1 was seen to suppress migration and invasion of the cancer cells. It was also seen that knocking down MAGI -1 decreased the localisation of E-cadherin at the cell to cell junction, increased disruption of stress fibres and focal adhesion points resulting in increased migration and invasion. Also, that MAGI -1 over-expression in colorectal cancer cells suppressed the cancer growth and lungs metastasis in vivo in an orthotopic transitional intestinal model. Zaric *et al.* also suggested that morphological and functional effects modulated by the over-expression of the MAGI -1 in colorectal cancer cell lines were independent of the epithelial-to-mesenchymal transition. In conclusion, over expression of MAGI -1 was identified as a suppressor of colorectal cancer and its metastatic activity. (Zaric *et al.* 2012) Lee *et al.* found that over-expression of MAGI -3 inhibited LPA induced migration and invasion of colon cancer cells, whereas by knocking down MAGI -3

continues the repeated effect of NHERF -2 over-expression, which led to the evidence that MAGI -3 has a negative regulatory effect on LPA_2 (Lipophosphaditic acid pathway – involved in platelet aggregation) mediated cellular pathways and the PDZ domain-containing proteins has critical role in regulation of LPA_2 mediated effects in colorectal cancer cell lines. The signaling and functioning of LPA_2 are reciprocally modulated by the dynamic coordinated interaction of the two PDZ scaffolding proteins and NHERF -2 and MAGI -3. (Lee *et al.* 2011).

In our experimental work on MAGI proteins in colorectal cancer cell lines, we had a constant lack of MAGI -2 RNA expression on PCR in colorectal cancer cell lines (RKO, HT115, HRT 18), which was consistent with the literature, as there is nothing reported to date in regard to MAGI -2 expressions in colorectal cancer cell lines. MAGI -1 and MAGI -3 were used as positive controls, which were expressed and gastric cell lines (AGS and HGC -27) were used as controls for MAGI -2 expressions. Our study had significant differences from Zaric *et al.* study, who looked at the tumour suppressor activity of MAGI -1 in colorectal cancer cells. The key difference being in the cell lines as Zaric *et al.* used SW40, SW680, HCT116, T84 and HT29. Also, that we used different methods than Zaric *et al.*, that is we used ECIS and TER to study cancer cell behaviour.

1.15.5. MAGI associated proteins in colorectal cancer

Martin et al. revealed that low expression levels of Claudin -1 and ZO -1 were directly related to high-grade colorectal cancer cells. ZO -1 levels were frequently found to be reduced in primary colorectal cancers with liver metastasis, possibly to their tyrosine phosphorylation, which down-regulates their function in TJs in colorectal cancer cell lines. Low levels of Claudin -1 were also deemed as independent predictors of recurrence and associated with poor survival. (Martin *et al.* 2011). Oshima *et al.* found

that Claudin -1, -3, and -4 have a relatively higher expression in colorectal cancer cell lines, whereas Claudin -7 had a reduced expression in venous and liver metastases of colorectal cancer. Thus, decreased Claudin -7 suggests invasiveness of colorectal cancer, and increasing the expression of Claudin -7 can have an important therapeutic role. (Oshima *et al.* 2008).

Korn *et al.* suggested a significant correlation between expression of CAR and the grade of gastrointestinal cancers. Moderate to poorly differentiated cancers showed reduced or loss of CAR expression. (Korn *et al.* 2006)

1.16. Hypothesis and aims of study

There is evidence in the literature that MAGI proteins along with the other TJ proteins play an important role in regulation and maintenance of TJs. Disruption of TJs is one of the crucial steps in the initiation of cancer metastasis. There is limited information on individual types of MAGI protein, especially on their types (MAGI -1, MAGI -2 and MAGI -3), their expression and their roles in human colorectal cancer metastasis. The focus of this project is on MAGI in cancer metastasis and the role of MAGI-1 protein in colorectal cancer cell lines.

It was hypothesised that MAGI protein plays a role in human colorectal cancer by way of aberrant expression and, by molecular and cellular mechanisms to be identified, that translates into disease progression of patients.

Thus, the aims of this study were as follows:

1. Investigation of the mRNA expression of MAGI -1, MAGI -2 and MAGI -3 in human colorectal cancer cell lines using Q-PCR and RT-PCR.

2. Generation of cell models (sublines) from human colorectal cancer cell lines with

differential expression of MAGI -1 (that is MAGI -1 knockdowns).

3. Investigation of the impact of aberrantly expressed MAGI -1 on adhesion, invasion and migration of colorectal cancer cells using trans-epithelial resistance (TER) and electrical cell impedance (ECIS) assays, and subsequently, to deduce the underlying mechanisms by the results of these tests.

4. Investigation of the expression of MAGI -1, MAGI -2, and MAGI -3 in human colorectal cancer and deduce the potential clinical and prognostic implications.

ROLE OF MAGI PROTEINS IN INVASIVENESS OF HUMAN COLORECTAL CANCER CELLS.

CHAPTER 2: MATERIALS AND METHODS

2.1.1. Materials

2.1.2. Culture mediums, buffers and standard solutions

2.1.3. 0.05M EDTA

EDTA solution at 0.05M was made as follows:

One gram of potassium chloride KCl (Fisons Scientific Equipment, Loughborough, UK), 5.72g of disodium phosphate Na2 HPO4, 1g of monopotassium phosphate KH2PO4, 40g of NaCl and 1.4g EDTA (Duchefa Biochemie, Haarlem, The Netherlands) was added to distilled water with a final volume of 5 litres. The pH of the solution was adjusted to 7.4 and sterilised by autoclaving before use.

2.1.4. Trypsin

Five hundred milligrams of Trypsin was dissolved in 20 ml 0.05 M EDTA. The solution was mixed and filtered through a 0.2-micron minecart filter (Sartorius Epson, UK), aliquoted in 250 micro-litre samples and stored at -20 Centigrade. When needed for use, one aliquot was diluted in 10 ml of EDTA solution.

2.1.5. Balanced salt solution (BSS)

The following were dissolved into distal water to make a final volume of 10 litres. Seventy-nine and a half gram of sodium chloride NaCl, 2.2gm of potassium KCl, 2.1gm of potassium monophosphate KH2PO4, 1.1gm of sodium bi-phosphate Na2HPO4. Solution PH was adjusted to 7.2 and was ready to use.

2.1.6. Antibiotic solution

To prevent colonisation of cell cultures by microbes and fungi, an antibiotic solution was used in all cell media and standard solution.

Dissolving the following in 500ml of BSS made the stock solution:

3.3gm Penicillin

12.5gm Amphotericin B

5gm Streptomycin

Due to lipophilic nature of Amphotericin B, it was first dissolved into dimethyl sulphoxide (DMSO) solution to yield a 30mg/ml solution. Once fully dissolved, the solution was passed through a 0.4 filtration unit and was stored at -20C in 5ml universal tubes, ready to be added to the 500ml bottle of the culture medium (Dulbecco's Modified Eagle's Medium *DMEM),* resulting in the following final concentrations.

100 U / ml Penicillin

0.25 microgram/ml Amphotericin B

0.1 mg / ml Streptomycin

2.1.7. 2.1.1.5. Normal culture medium

The normal culture medium used for wild-type cell growth was Dulbecco's Modified Eagle's Medium (DMEM) with 10% (50ml) of FCS (Fetal Calf serum or Fetal Bovine Serum) and 5ml antibiotics. Cell cultures were grown and maintained in 25 cm^2 and 75 cm^2 tissue culture flasks (Grenier Bio-One limited, Gloucestershire UK).

2.1.8. Selection culture medium

Selection culture medium used for the selection of transfected cells contained Dulbecco's Modified Eagle's Medium (DMEM) with 10% of FCS (Fetal Calf serum or Fetal Bovine Serum) plus antibiotics and also 250 micro-litres of Blasticidin.

2.1.9. Maintenance culture medium

Maintenance culture medium used for the transfected cells contained Dulbecco's Modified Eagle's Medium (DMEM) with 10% of FCS (Fetal Calf serum or Fetal Bovine Serum) plus antibiotics and also 27.5 micro-litres of Blasticidin.

2.1.10. DPC water

Two hundred and fifty micro-litres of Diethyl Pyro Carbonate (DPC) was added to 4750 micro-litres of distilled water. The solution was then autoclaved for use.

2.1.11. PCR water

Distilled water was filtered and irradiated with ultraviolet light to ensure the breakdown of contaminating nucleic acids.

2.1.12. T5X Tris, Boric acid, EDTA (TBE)

A 5 times stock solution was made by using the following:

- 545gram of Tris-Cl (Melford Laboratories Ltd., Suffolk, UK),
- 275gram of Boric acid (Melford Laboratories Ltd., Suffolk, UK)
- 46.5gram of disodium EDTA (Duxhefa Biochemie, Haarlem, The Netherlands)

The above was dissolved in distilled water to make a final volume of 10 litres. The solution was stored at room temperature and was diluted as required for use.

2.1.13. RNA-ase free water

Sigma-Aldrich RNAse water was used.

2.1.14. Master mix

Go Taq Green PCR master mix from Promega UK was used.

2.1.15. RT mix

PCR master mix was used from Invitrogen Life Science Technologies.

2.1.16. DNA ladder

DNA ladder was used to confirm the size of the product on Agarose Gel.

2.1.17. Electrophoresis gel

0.8% agarose gel was used by the product size.

2.1.18. Syber safe

Syber safe was used for safe DNA staining from Invitrogen Life Science Technologies.

2.1.19. TBS X 10

The following were dissolved to make a final volume of 5 litres, adjusted to pH 7.4:

TRIS 121 grams, NaCl 400.3grams

2.1.20. Cell lines

We have used human colorectal cancer cells, namely HT-115, HRT-18 and RKO with epithelial morphology established from highly invasive human colorectal carcinoma, which expresses high amounts of the tumour marker carcinoembryonic antigen. Human gastric cancer cells, namely AGS and HGC-27, were chosen as controls due to consistent

lack of MAGI -2 expression on agarose gel electrophoresis following RT PCR. The cells were obtained from the European Collection of Animal Cell Culture (ECACC), HECV, human endothelial cells were purchased from Interlab, Milan, Italy (Table 2.1.20.1. and Table 2.1.20.2.). Different types of cells for both gastric and colonic cancer were chosen due to, firstly, their varying invasiveness properties and differential expression patterns of types of MAGI proteins *i-e*, MAGI -1, -2 and -3.

Table 2.1.20.1. Specification of colorectal cancer cell lines

Cell line	HHT -115	HRT -18	RKO
Species	Human	Human	Human
Tissue	Colon	Rectum	Rectum
Age	63	67	82
Gender	Male	Male	Female
Morphology	Epithelial	Epithelial-like	Epithelial
Growth mode	Adherent	Adherent	Adherent
Karyotype	2n=46	2n=46	2n=46
Tumorigenic	Specified	Yes	Yes
Country	UK	UK	UK

Table 2.1.20.2. Specification of gastric cancer cell lines

Cell line	AGS	HGC - 27
Organism	Human	Human
Tissue	Stomach	Lymph node
Age	54	Unspecified
Gender	Female	Unspecified
Morphology	Epithelial	Epithelial
Growth mode	Adherent	Adherent

2.1.21. MAGI primers used:

MAGI-1, MAGI-2 and MAGI-3 primers (table 2.1.21.1) were designed in-house by using Beacon Designer Programme, synthesised by Sigma-Aldrich UK, and were stored in -20^{0}C freezers. The required primers (MAGI -1, -2, -3) were taken out when needed and defrosted on room temperature, following which they were diluted to 1:10 concentration using PCR water. Subsequently, the diluted primers were stored in a designated primer fridge for future use, and the originals were returned to the freezer.

Table 2.1.21.1. MAGI primer sequences

Primers	Forward	Reverse
MAGI-1 (F11 R11)	CTCC AAGAAA CAGCA TTACC	CTGAA GTGG AACTT CTCTGG
MAGI-2 (F11 R11)	CAGTG ACTAC GCAA CCT ACA	GGATG TCT GGTTT CACA TCT
MAGI-2 (F12 R12)	TCAAGG ATGCA GGTCTT AGT	CTTATT GCTGG TCCA TCTTC
MAGI-3 (F12 R12)	GGGAAA TCA CAC AAACAA GT	TGAG ACAG TTCAA CAAT GGA

2.2. Methods

2.2.1. Preparation of growth culture medium

50 ml (10%) of FCS was added to 500ml of DMEM medium inside the hood. Antibiotics were taken out of the freezer, defrosted and added to DMEM inside the hood with non-touch technique.

2.2.2. Reviving cells

Stocks of frozen cells were available at the host department, which was securely stored away in liquid nitrogen tanks in a room, originally obtained from the European Collection of Animal Cell Culture (ECACC), HECV, human endothelial cells, Interlab, Milan, Italy. Protective gloves were worn. The safe distance was observed while standing close to the tank, while opening it, because of the risk of hypothermic burn. The shelf was taken out, and the rod was removed. The desired cell cones were taken out; the box

was closed and was put back on the shelf, with the rod back in place. The shelf was delivered back into the tank carefully, and the tank was closed. The cells taken out were marked in the folder for recording purposes. The room was locked. The cones were gently warmed up to the room temperature. Five universal containers (UC) were taken out, and the tops were cleaned. Four were labelled with the cell types names and one labelled and used for medium. The 5th UC was a transfer means for the culture medium. Four T25 flasks were labelled with the four cell type names and were dated. 5 ml of culture medium was poured into each flask. The cells were transferred into labelled UCs and mixed with 5 ml of normal medium and then were centrifuged for 8 minutes at 1780 rpm. The cell sediment formed was re-suspended into 1ml of the normal medium, which was carefully transferred to the designated labelled T25 flask. T25 Flasks were put into an incubator for cells to grow.

2.2.3. Maintenance of cells

All cell culture work was carried out inside a tissue culture facility which had class two laminar flow cabinets. We observed strict sterile and aseptic techniques and precautions. Gastric and colorectal cell lines were maintained in T25 and T75 flasks (Grenier Bio-One limited, Gloucestershire UK) as required using DMEM/Ham's F12 medium prepared as described earlier.

The flasks were maintained in the incubator (Sanyo MDA 15 AC), loosely capped at 37 C in 5% carbon dioxide and 95% humidification. Cells were grown to confluence before the start of any experimental work.

2.2.4. Trypsinization of cells

Cell lines were grown to reach significant confluence levels which were assessed visually first and then under a microscope. The medium was removed from the flask, and the

cells were rinsed with BSS and maintained at room temperature. The BSS was then aspirated, and 1 to 2 ml of trypsin added to the flask and flask were shaken to spread the trypsin equally over the surface of the flask. Flasks were then transferred to the incubator for 10 to 20 minutes. The flask was given a gentle tap to detach cells from the flask surface. The supernatant was transferred into a 20 ml universal container (Greiner Bio-One Ltd., Gloucestershire, UK) and the normal medium was added to make the volume to 5ml. The mixture was then centrifuged at 1700rpm for 8 minutes to pellet the cells. The supernatant was aspirated, and the pellet was resuspended in 5 ml of normal medium. The cells were then counted for immediate experimental work, or were put into new flasks to be re-cultured, or were put into cryo-tubes to be frozen down and stored in the liquid nitrogen.

2.2.5. Cell counting

Cells were counted using Neubauer haemocytometer (Figure 2.2.5.1 – figure 1&2) counting chamber (Mod-FuchsRosenthal, Hawksley, UK) in the haemocytometer counting chamber by using an inverted microscope (Reichert, Austria) at 10 x 10 magnification for *in vitro* functional assays.

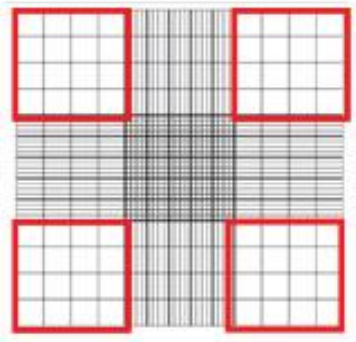


Figure 1: Hemacytometer grid

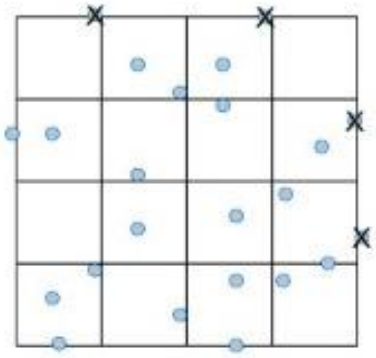


Figure 2: Cell counting guidelines

Figure 2.2.5.1. Haemocytometer grid and cell counting (Stem cell technologies)

Using a pipette, 100 µL of cell suspension was removed and applied to the haemocytometer. Both the chambers were filled underneath the coverslip, allowing the cell suspension to be drawn out by capillary action. The microscope was focused on the grid lines of the haemocytometer with a 10X objective. Using a hand tally counter, the cells were counted in one set of 16 squares as shown within a red square line in figure 2.2.5.1. The cells were only counted when they were set the right-hand or bottom boundary line. The full dimension of each 16 square areas, containing cells were counted. The dimensions were 1mm x 1mm x 0.2mm

Following equation was used:

Cell number / ml = (number of cells counted in 16 small squares / 2) x (1×10^4)

2.2.6. Cryopreservation of cells

Cells were regularly preserved at different stages during the experiments to have enough cells available, not only for future work but also in case of loss of cells. An extra set of T25 flasks were run beside the one used in the experiment, which was then used to cryopreserve cells. Once these flasks would become confluent, they were trypsinised and centrifuged for 8 minutes at 17000rpm. The cells were then re-suspended into 0.9 ml of normal medium. They were transferred to 1 ml cryo-tubes (Nunc, Fisher Scientific, Leicestershire, UK) and 0.1 ml of 10% dimethylsulphoxide (DMSO) (Sigma-Aldrich, Gillingham, Dorset, UK) was added on. Once DMSO was added, the cryo-tubes were transferred to the -80C freezer without any delay for overnight storage and subsequently to liquid nitrogen for long-term storage.

2.2.7. Generation of mutant RKO, HT-115 and HRT-18 cell lines with the MAGI-1 knockdown.

2.2.8. Discovery and the catalytic mechanism of ribozymes

To achieve human SOCS-4 mRNA (Figure 2.2.8.1) knockdown, hammerhead ribozyme transgenes that specifically target GUC or AUC sites of SOCS-4 mRNA (Figure 2.2.8.2) and cleave SOCS-4 transcripts were designed according to the predicted secondary structure of SOCS-4 mRNA by Zuker's RNA mFold software (Zuker, 2003). The hammerhead ribozyme was first discovered by Forster and Symons in 1987 as a self-cleaving domain in the RNA genome in a variety of plant viroids and virusoids (Forster and Symons, 1987). Such hammerhead motifs can form short synthetic oligonucleotides, acting as multiple turnover catalysts which are capable of cleaving RNA targets (Uhlenbeck, 1987, Haseloff and Gerlach, 1988). A general universal secondary structure which is integrated by several invariant nucleotides and consists of three helical stems (I, II and III) could be found in all hammerhead motifs. Stem II is incorporated intramolecular in such secondary structure, whereas stem I and III are formed by hybridisation based on the complementary sequences of the targeted substrate. Moreover, the best cleavage sites were discovered to be AUC, GUC and UUC.

Figure 2.2.8.1. Diagram of the predicted secondary structure of human SOCS-4 mRNA

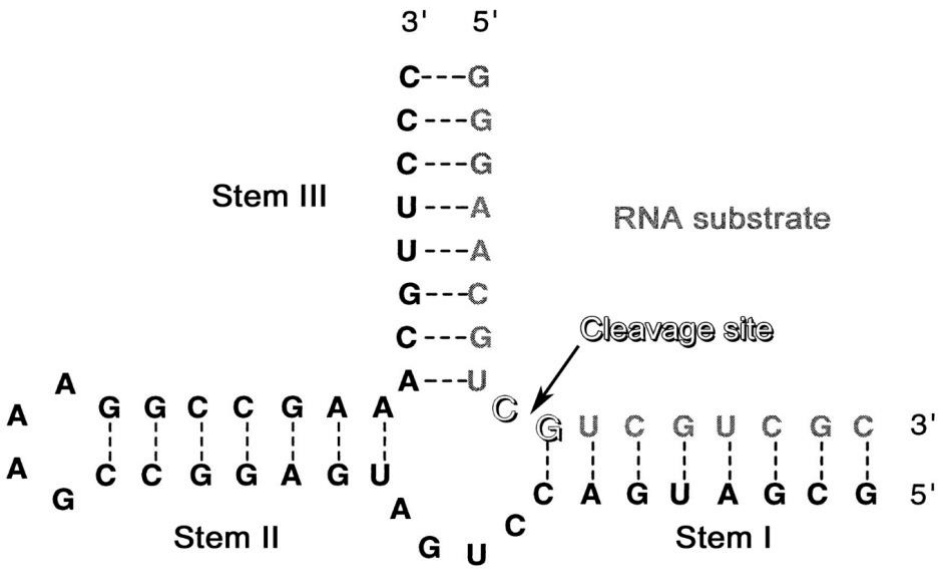


Figure 2.2.8.2. Diagram of hammerhead ribozyme secondary structure

Hammerhead ribozymes consist of three helices. Stem II, containing highly conserved sequences, is flanked by stem I and III that are adjacent to the cleavage site and are base-paired to the RNA substrate. Dash line indicates the Watson-Crick base pairs.

2.2.9. TOPO TA gene cloning and generation of stable transfectants

The pEF6/V5-His TOPO® TA expression system (Invitrogen, Paisley, UK) provides a highly efficient one-step cloning technique which does not require ligase, post-PCR procedures, or PCR primers containing specific sequences. Such an approach allows the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for constitutive expression in mammalian cells after transfection. The line raised pEF6/V5-His TOPO® vector in the kit consists of single, overhanging 3' deoxythymidine (T) residues which allow *Taq* polymerase-amplified PCR products, containing a single deoxyadenosine (A) at the 3’ ends, to ligate efficiently. The EF-1α promoter in pEF6/V5-His TOPO® then enables the constitutive expression of the PCR product after the transfection of the plasmid (figure 2.2.9.1).

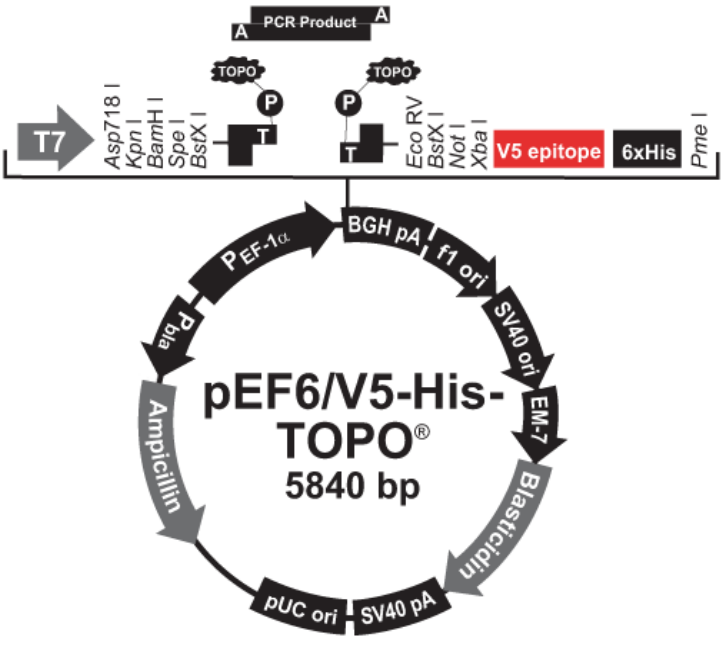


Figure 2.2.9.1. Diagram of the pEF6/V5-His TOPO® vector feature (From pEF6-His TOPO® TA Expression Kit manual)

The PCR products were cloned into a pEF6/V5-His TOPO® plasmid, and the total 6µl cloning reaction was mixed and stored on ice in advance to the One Shot Chemical Transformation (Invitrogen, Paisley, UK). The condition used for TOPO cloning reaction was as follows:

- PCR product - 4µl
- Salt solution - 1µl
- TOPO vector - 1µl

5µl of TOPO cloning reaction was added into each vial of One Shot TOP10 Chemically Competent *E. coli.* (Invitrogen, Paisley, UK) and mixed gently by stirring using the pipette tip. The vial was stored on ice for 5-30 minutes (based on the product size) before heat-shocking at 42°C for 30 seconds followed by immediately transferring into ice. To each vial, 250µl of room temperature S.O.C medium was added, and the tubes were shaken at a 45° angle at 225rpm on a horizontal orbital shaker (Bibby Stuart Scientific, UK) at 37°C for 1 hour. After the incubation period, the transformed products from each vial were spread in different seeding density on a pre-warmed agar petri dish containing 100µg/ml ampicillin followed by incubating overnight at 37°C. Several colonies from each petri dish were randomly picked, and the PCR product insertion and orientation of the incorporated PCR product were checked by PCR using primer combination of T7F vs BGHR and T7F vs specific forward or reverse primer respectively. The reaction mix is outlined as follows:

PCR product insertion primer pair mix (used for over-expression sequences)

- GoTaq Green master mix - 8µl
- T7F plasmid specific forward primer - 1µl

- BGHR plasmid specific reverse primer - 1µl
- PCR water -6µl

For ribozyme transgene insertion and orientation checking, the molecule specific forward and reverse primer was replaced in the above mixtures with forwarding (RbToPF) and reverse (RbBMR) sequences to the common region of the ribozyme transgene. Each colony was inoculated into both reaction mixes using a sterile pipette tip, and the reaction mixes were placed in a 2720 Thermal Cycler (Applied Biosystems, Paisley, UK). The PCR reaction conditions were used as follows: 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 40 seconds and 72°C for 1 minute with a final extension step of 72°C for 10 minutes. Gel electrophoresis on a 2% agarose gel stained with SYBR safe DNA gel stain was utilised to visualise the conventional PCR reaction products. Colonies with correctly orientated PCR product inserts were inoculated into universal tubes containing 5ml of selective LB broth (containing 100µg/ml ampicillin) followed by being incubated overnight at 37°C while being shaken with a 45° angle at 225rpm.

2.2.10. Plasmid amplification and extraction

Plasmid extraction was undertaken using the Sigma GenElute Plasmid MiniPrep Kit (Sigma, Dorset, UK) based on the protocol provided. The overnight incubated universal tubes containing the chosen colonies were centrifuged at 5,000RCF to acquire a bacterial pellet. The supernatant in each tube was discarded, and the bacterial pellet was re-suspended thoroughly using 200µl Resuspension Solution containing RNase A and transferred into a 1.5ml Microfuge tube. To the Microfuge tube was added 200µl of Lysis Solution followed by eight gentle inversions. This lysis stage was conducted within

5 minutes to avoid permanent plasmid denaturation. Then, 350µl of Neutralisation Solution was added, and the Microfuge tube was gently inverted six times followed by centrifugation at 12,000g for 10 minutes to pellet the cell debris. The clear lysate in the Microfuge tube was transferred into a Mini-prep Binding Column which was placed into a micro-centrifuge Tube. Such Mini-prep Binding Column was washed with Column Preparation Solution in advance to maximise plasmid binding capacity. The clear lysate added micro-centrifuge Tube was then centrifuged for 1 minute at 12,000g to enable the plasmid binding to Mini-prep Binding Column. The flow-through liquid was discarded, and 700µl of Wash Solution (containing ethanol) was added to the Mini-prep Binding Column followed by centrifugation at 12,000g for 1 minute before discarding the flow-through liquid. The Micro-centrifuge Tube had then centrifuged again at 12,000g for 1 minute to remove the remaining Wash solution before the Mini-prep Binding Column was transferred into a fresh Collection Tube. The plasmid was eluted by adding 50µl of Elution Solution followed by spinning the fresh Collection Tube at 12,000g for 1 minute and was stored at -20°C for further use. Knockdowns (KDs) were created by using ribozymes containing purified plasmids for MAGI -1 gene KD. 2 different ribozymes were used for each WT cell line. Electroporation was used to introduce the plasmids into the cells.

2.2.11. Transfection by electroporation

The WT cancer cells were grown in T25 flasks to reach full confluence so that the TJs had formed and well established. The culture medium was removed, and the flasks were washed with BSS solution twice. 1 ml of TRI Reagent (Sigma-Aldrich) was added to the flask, and the flask was kept in the incubator for 5 minutes. After 5 minutes the detached cells floated in the reagent. The leftover attached cells were detached by gently from the surface by gently tapping on the flask. The cell lysate was transferred

into the pre-labelled universal tube, and 4ml of the normal medium was added to make up the total volume to 5ml. The mixture was centrifuged at 1700rpm for 8 minutes to pellet the cells. The supernatant was aspirated, and the pellet was re-suspended in 2 ml of normal medium.

500 micro-litres of the stock was transferred into pre-labelled four electroporation sterile cuvettes for each cell type. These included WT, two different ribozymes containing purified plasmids for MAGI -1 gene (KD1 and KD2), and ribozymes containing empty plasmids (PEF). The WT and PEFs were used as controls. Once the vector was added, 30 seconds were given for dispersion. The cuvettes were sealed by placing the lid back on and were put into the electroporation chamber. Standard settings were used for electroporation. Cells were subjected to electrical pulse 1500 farads and 300 volts. The injured cells were transferred to T25 flasks with 5ml of normal growth medium and were incubated at 37C, 5% CO_2 and 95% humidity.

2.2.12. Stabilisation and maintenance of transfected cells

Following 48 hours of incubation of transfected cells in to normal medium, the medium was changed to the selection medium, containing Dulbecco's Modified Eagle's Medium (DMEM) with 10% of FCS (Fetal Calf serum or Fetal Bovine Serum) and antibiotics from the stock (see above) and in addition 250 micrograms of Blasticidin (0.5microgram /ml). The transfected cells were kept in the selection medium for 5 to 7 days. This resulted in the selection of transfected cells and apoptosis of non-transfected cells. The medium was then changed to maintenance medium. Successful KDs were confirmed by microscopy, cell imaging, qPCR, agarose gel PCR and immunofluorescence.

2.2.13. RNA isolation using TRI reagent (Sigma)

Confluent T25 flasks of the desired cell lines were selected, and the cell culture medium was aspirated. Flasks were then washed twice with the BSS. 1 ml of TRI reagent was added to the flasks. Cells were scrapped, and cell lysate was passed several times through the pipette to produce a homogenous lysate. The sample was transferred to a sterile Microfuge tube and allowed to stand at room temperature for 5 minutes. 0.2ml chloroform TRI reagent was added to each tube and shaken for 15 seconds and was left to stand at room temperature for 2-15 minutes, then centrifuged at 12000 for 15 minutes at 4C. Three phases appeared. The bottom red phase was the protein. In between white phase was DNA and the clear top phase was the RNA. The clear top phase was carefully transferred to a fresh Microfuge tube. 0.5 ml of isopropanol was added to the Microfuge tube, mixed by shaking and was left for 5-10 minutes at room temperature, then centrifuge 12000 for 10 minutes at 4C. A pellet appeared at the base (not always clearly visible). The supernatant was discarded. The pellet was washed with 75% EtOH which was made up of RNA-ase free water and then centrifuged at 7500 for 5 minutes. 75% EtOH was removed, and the sample was air dried for 5-10 minutes. The pellet was dissolved in 20 micro litres of RNA-ease free water. RNA concentration was measured on the nano-photometer. Lid factor used for most samples was 50. A lid factor of 10 was used for more concentrated samples, and 100 was used for more diluted samples. Dilution of concentrated samples was achieved by gradually adding ten micro litres of RNAase free water and measuring the new concentration. Few of the RNA samples were stored at -80C freezer and others were used for immediate reverse transcription (RT).

2.2.14. RNA quantification

The concentration of RNA extracted from the cells was measured using a UV101 Biotech Photometer (WPA, Cambridge, UK), which was set to detect single strand RNA in micrograms per micro-litres at 260 nanometers. Samples were measured by using Starna glass cuvette (Optiglass limited, Essex, UK).

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

Once RNA had been isolated and quantified, RT-PCR was carried out to convert RNA into complementary DNA (cDNA) using DuraScript^{TM} RT PCR kit (Sigma-Aldrich, Dorset, UK). A fixed amount of 0.5 micrograms of RNA was converted to cDNA as per protocol. Standardized RNA concentrations were achieved by adding RNA-ease free water. The dilution amount of RNA-ease free water was calculated by 0.5 divided by the RNA concentration measured by the nano-photometer from earlier. Once diluted to standardised concentration forms, the new RNA concentration was measured on the nano-photometer. The Abgene reverse transcription kit was used for RT PCR. The following volumes were used:

Volume reaction was 20 micro-litres:

PCR water was added to RNA to make volume 10 micro-litres

ten micro-litres of AB RT Mastermix 2X

All of above were added to a dome-capped Microfuge tube's and placed into a thermo-cycler (Applied GeneAmp PCR sys 2700). Each reaction was run as per the following protocol:

Stage 1: 25◦C for 5 minutes

Stage 2: 37◦C for 120 minutes

Stage 3: 85◦C for 5 minutes

Stage 4: held at 4◦C

The final product was diluted with PCR water in 1:3 resulting in a final volume of 80 micro-litres, which was then labelled and stored at -80C.

2.2.16. Real-time quantitative polymerase chain reaction (q-PCR)

q-PCR was undertaken to analyse the transcript expression level of target genes. Such a technique is more sensitive than conventional RT-PCR and capable of detecting small quantities of cDNA, providing a more reliable value of the template copy number in each sample. This method requires a sequence-specific DNA-based fluorescent reporter probe which only quantifies the target transcript containing such a probe sequence to greatly improve the specificity of the detection. Amplifluor™ Uniprimer™ Universal system (Intergen company®, New York, USA) was available at the home department and was used in this study. The Amplifluor probe consisted of a 3' region specific to the Z-sequence, which acted on the target-specific primers and a 5' hairpin structure labelled with a fluorescent tag (FAM). While in the hairpin structure, such a fluorescent tag was linked to an acceptor moiety (DABSYL) which effectively quenched the fluorescence and produced no fluorescence signal. Primers were designed to the specific target transcript using Beacon Designer Software. To each of the primer pairs specific to the target sequence, a Z-sequence was added to allow incorporation of the Amplifluor uni primer probe during PCR. Such Z-sequence incorporated DNA transcript that acted as a template for the Amplifluor uni primer probe, and the hairpin structure in the probe was subsequently degraded and unfolded by DNA polymerase disrupting the stable structure

between fluorophore and quencher to enable sufficient fluorescence emission to be detected. The principle of this process was demonstrated in figure 18. Pre-prepared cDNA samples were amplified and detected on a Step One Plus Real-Time PCR System (Applied Biosystems, Paisley, UK), using Precision FAST 2X q-PCR Master-Mix (Primerdesign Ltd, Chandler's Ford, UK), specific forward primer (10pM), reverse primer containing the Z sequence (1pM) and the FAM-tagged Uni-primer probe (10pM). Each sample was loaded into a 96-well plate and the reaction mixture. The q-PCR conditions used are shown below:

q-PCR Reaction mix:

- Precision FAST 2X q-PCR MasterMix - 5µl
- Specific forward primer - 0.3µl
- Specific reverse primer with Z-sequence - 0.3µl
- Amplifluor probe – 0.3µl
- PCR H_2O – 3.1µl
- Prepared cDNA sample - 1µl

q-PCR conditions:

- Step 1: Initial denaturation - 94°C for 10 minutes
- Step 2: Denaturation - 94°C for 10 seconds
- Step 3: Annealing - 55°C for 30 seconds
- Step 4: Extension - 72°C for 10 minutes

Step 2 - 4 were repeated for 100 cycles.

The fluorescent signal was detected at the annealing stage, and the fluorescent signal emitted during PCR correlated to the amount of DNA template that had been incorporated into Z- sequence. The geometric increase in fluorescent signal directly correlated to the exponential increase of DNA template, which was used to determine a threshold cycle for each reaction. The transcript copy number was determined by the time/cycle number when fluorescent signal reached a specific threshold. Expression of the target sequence was detected in conjunction with a range of standards of the known transcript, PDPL, that are used to generate a standard curve to enable the calculation of transcript copy number in each unknown sample (Figure 2.2.16.1 and 2.2.16.2). Finally, the transcript copy number was normalised by the detected copy number of GAPDH.

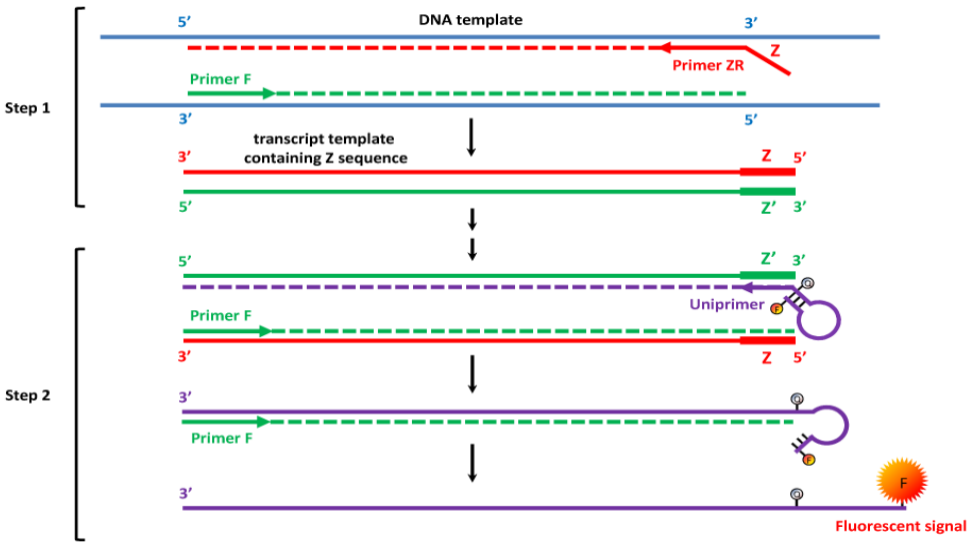


Figure 2.2.16.1. Diagram indicating the principle of the Amplifluor Uniprimer Universal system. (Intergen company®, New York, US)

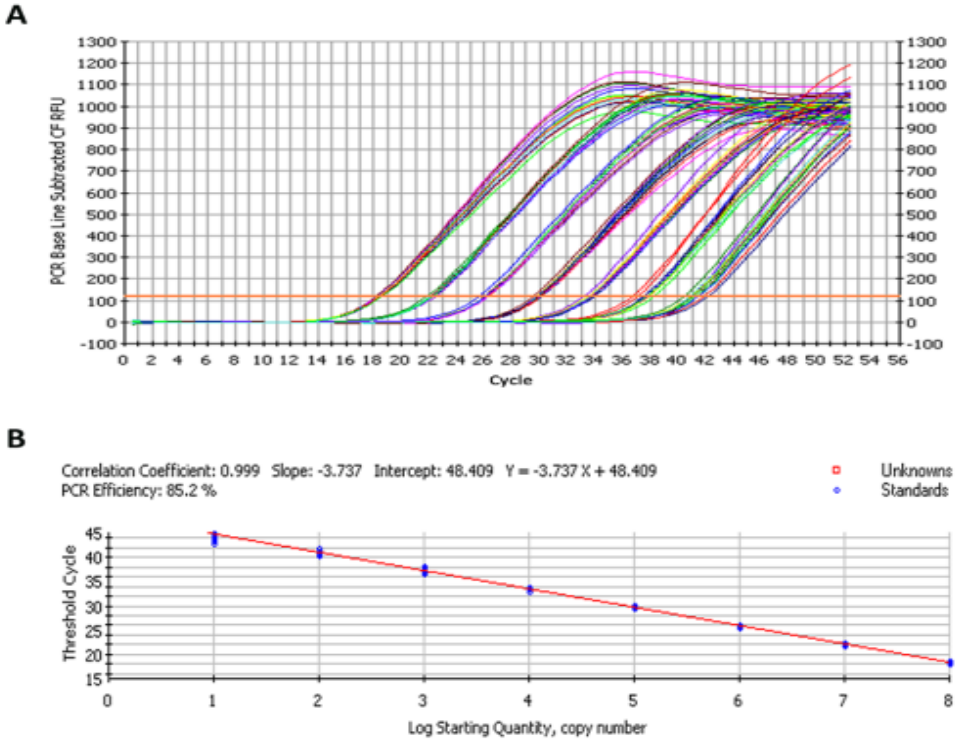


Figure 2.2.16.2. Figure 19: Diagram of standard curve generation (Intergen company®, New York, UK.

Reaction curves for serial diluted standard samples (10^8 to 10 copy number) using StepOne Plus Real-Time PCR System; The standard curve generated based on the threshold cycle and copy number of the serial diluted standard samples. Red line indicates the standard curve generated and blue dots stand for different standard samples with known copy number.

2.2.17. Agarose gel electrophoresis, DNA staining and visualisation

The amplified DNA products from the PCR were analysed using 2% agarose gel electrophoresis. The analysis was based on the separation of DNA products according to their size on electrophoresis. The procedure was as follows:

2.2.18. Formation of 0.8% agarose gel

3 grams of agarose (Melford chemicals, Suffolk, UK) was added to 150 ml of TBE solution in a flask. The mixture was heated in the microwave to boiling temperature, and the agarose was fully dissolved, and the mixture became transparent in colour. Then the gel was left to cool down on room temperature just for a few minutes, and ten micro-litres of the cyber safe was added into it, following which the liquid gel was poured down into the electrophoresis cassette. Plastic combs were already inserted into the electrophoresis cassettes, and the gel was allowed to settle down at room temperature, which took between 30-40 minutes. The plastic combs were then carefully removed, leaving indentation slots for loading of the PCR products for later on.

2.2.19. TBE Buffer

Once the gel was set, TBE buffer was carefully poured into the electrophoresis tank, directly on top of the gel, filling up to the maximum fill mark on the tank.

2.2.20. Loading gel with PCR products

Five micro-litre of 100 Kb DNA ladder (Invitrogen, Paisley, UK) was loaded into the first indentation slot which was used as a reference guide in measuring of the DNA products once the electrophoresis was completed. Ten micro-litres of the PCR products were carefully loaded into the rest of the indentation slots on the gel.

2.2.21. Electrophoresis

The electrophoresis cassette was connected with the power supply by its leads, making sure that the flow of the charge/current is in the right direction, that is from the positive to the negative electrode, which separated the DNA products out on the gel.

Consort EV 243 electrophoresis device was used. The following setting was applied.

Current: 110mA

Voltage: 90V

Time: 42 minutes.

2.2.22. Detection of spread of PCR products using UV light

The TBE was poured away from the gel, and the gel was placed inside the UV light container (UVTech. Cambridge, UK). The degree of spread of PCR products was captured by using UV camera in the UV Tech. The light settings were optimised.

2.2.23. Electrical Cell Impedance Sensing (ECIS)

Electrical cell impedance sensing (ECIS) is a technique for analysing cell behaviour based on work of Giaever and Keese, who found that it was possible to monitor growth, migration and adhesion of cells growing on a small electrode by monitoring changes in impedance of the cells, that is, resistance to an alternating current) (Giaever and Keese 1984). The ECIS apparatus was supplied by Applied Biophysics (New York, USA). A 96 well plate array specific to the ECIS machine was used (IBIDI, Germany). Each well inside the 96 well plate has a unique design. It is built around two small gold electrodes, in the bottom of the wells in which the seeded cells are allowed to grow within the incubator. These electrodes work as sensing the impedance or resistance between the cells and the

cell and the bottom of the array. Each Individual well is a part of plate array, which consists of a plastic housing encasing an integrated circuit incorporating all the electrodes included in the array. An array can contain various numbers of wells and types of plates, but a 96 well plate is the most widely used configuration. The plate slots into a dock, in the incubator, which connects it to the ECIS machine. The incubator in the apparatus does not provide carbon dioxide; therefore DMEM used has to contain HEPES to compensate.

ECIS: Procedure

The protocol for the procedure was available in the home department (Jiang *et al.*), which had been used extensively in the past for study of tumour cell behaviour (Keese, Wegener *et al.* 2004; Jiang 2012) (Figure 2.2.23.1). A series of these experiments were performed in 96 well ECIS plates. All the 96 plates used were new, straight out of the packet inside the tissue culture hood, maintaining as sterile conditions as possible. 200 micro-litres of the normal medium was added to each of the 96 wells on the plate. The top cover of the 96 well plate was placed and secured in position by manual pressure while was being transferred from tissue culture to the ECIS machine. ECIS machine which connected to a computer, which runs the ECIS software and displays the graph by the cell impedance changes.

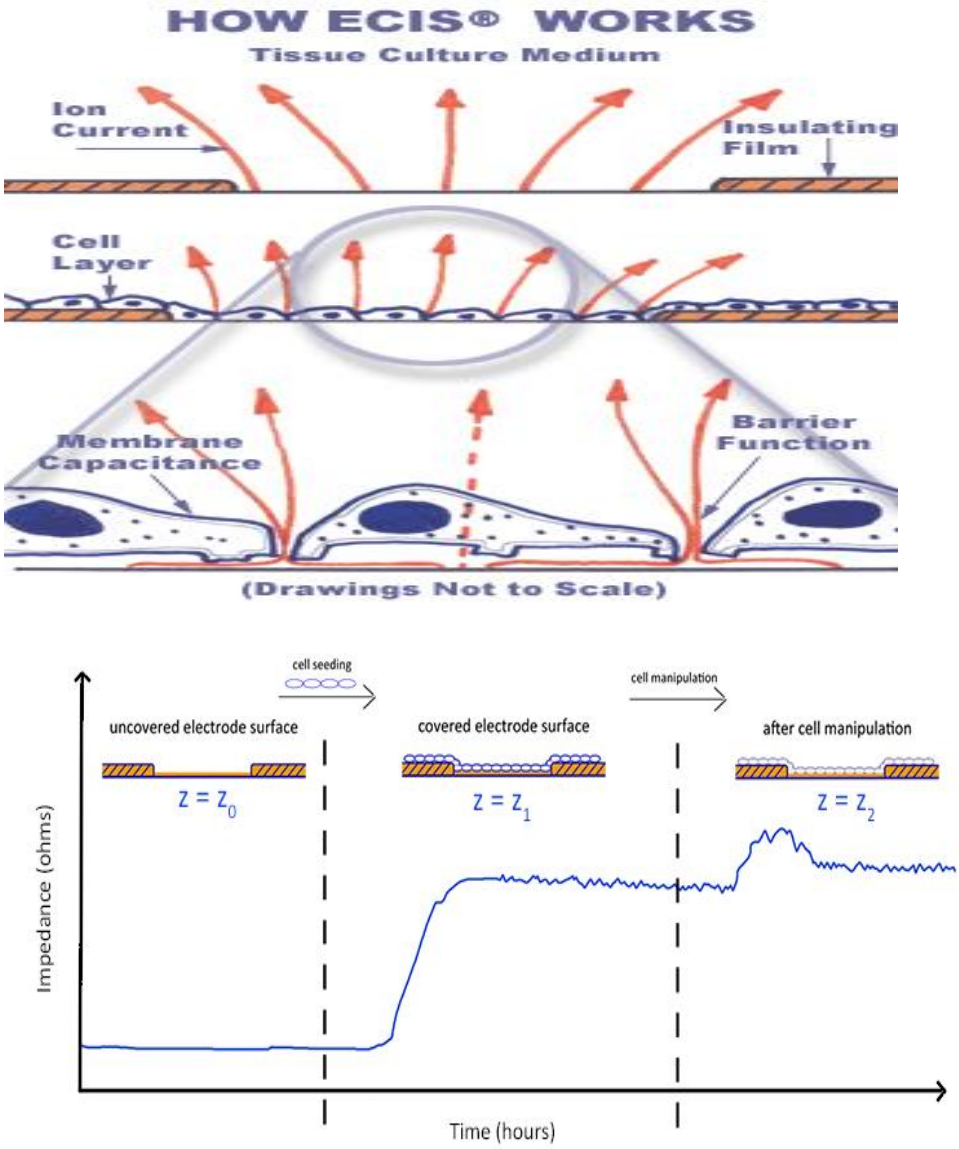


Figure 2.2.22.1. How ECIS works. The ECIS machine provides detailed readings on cell adhesion and growth, which is recorded in real time. Also, there is provision for inflicting the cells with a uniform wound by electroporation, thereby providing further detail of their migratory function. The home department not only has the largest array of ECIS machines in the country but also has published one of the most useful texts in regards to its uses (Jiang 2012). (http://www.biophysics.com/ecis-theory.php)

HRT-18 WT, HT-115 WT and RKO WT were the wild types used with their respective HRT-18 PEF, HT115-PEF and RKO PEF. Amongst the transfected types, two knockdowns were created and verified for each cell type, labelled as HT-115 KD1 and KD2, RKO KD1 and KD2 and HRT-18 KD1 and KD2. The cell flasks were trypsinised and centrifuged at 17000 revolutions per minute (RPM) for 5 minutes in universal containers (UCs), following which they were re-suspended in 1 ml of medium. Cell dilution was measured by using the following. http://www.celeromics.com/en/support/cell-dilutio-calculator.php

Each well was seeded with 100,000 cells in 200 micro-litres. Once final dilution volumes were measured, they were added to the ECIS plate, and the plate was taken back and put into the ECIS machine. Once the plate was fixed firmly into position inside the ECIS machine, the plate electrode connection with the machine electrodes was checked, which showed all the wells lit up in the green colour, meaning a good connection between electrodes had established. The incubator settings were set to 37°C and 95% humidity.

The experiment was then started and delayed wounding was enabled from options and the setting was set for eight hours for colorectal cancer cell lines and12 hours for gastric cancer cell lines. The current used for wounding was 3000 micro-amperes at a frequency of 4000 hertz with time set to 20 seconds. The cells impedance and resistance measurements were displayed on the screen off the monitor and were recorded for the all-new addition of the experiment and were also saved for a later reference. The average of the percentage of increase in impedance of the cells in the initial hours was calculated to study cell adhesion. Similarly, the average percentage of increase after a successful wounding to the cells was used to calculate the conventional migration essay. Once the experiment was completed, used plates were carefully washed with bleach,

and disposed of in the yellow clinical waste bins. In the end, the ECIS program was shut down, and the computer was switched off. The incubator was cleaned from inside with 70% ethanol. That experiment was repeated three times for each colorectal cancer and gastric cancer cell line.

The problems encountered during the ECIS experiments were to make sure that there were no quality control issues. It was realised that contamination could affect the quality of the readings generated inside the wells.

Great care was taken to prevent any infection or contamination of the cells inside the wells. We were also aware that sometimes air bubbles inside the wells could lead to defective functioning of the electrode sensors great care was taken to avoid it. Several repetitions of the ECIS experiment were undertaken to make sure that the readings were consistent and any errors were filtered out and were not included in the final analysis.

2.2.24. Trans-Epithelial Resistance (TER)

Trans-epithelial or trans-endothelial resistance (TER) is an excellent method for assessing barrier function of the reconstructed epithelial of the grown cell lines. It has a become method of assessing cell-to-cell tight junction function in vitro. The cells are cultured over a porous membrane and grown to become dense, forming cell-to-cell tight junctions and establishing cellular polarity. With the help of the chopstick type of electrodes in the apparatus, the electrical resistance across the barrier between the cell layers can be determined by applying a small AC voltage with varying frequency (Figure 2.2.24.1& 2.2.24.2).

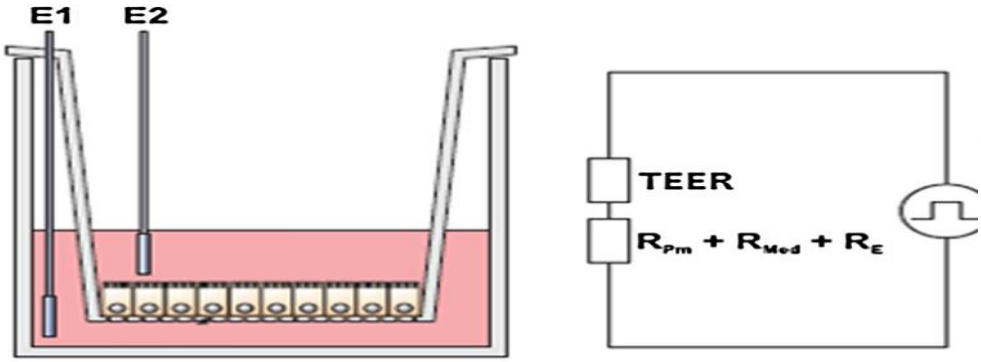


Figure 2.2.24.1: TER How it works

(http://sites.mc.ntu.edu.tw/sysdata/81/81/doc/efb6ccdbf3aa0193/attach/946.png)

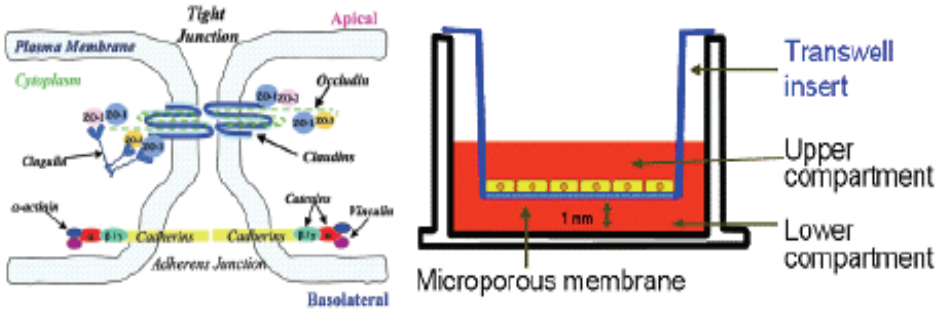


Figure 2.2.24.2: TER Mechanism of action

(http://sites.mc.ntu.edu.tw/sysdata/81/81/doc/efb6ccdbf3aa0193/attach/946.png)

2.2.25. TER: Procedure

The cells were trypsinised and re-suspended in DMEM, and cell count performed and cells diluted to 100,000 per 500 micro-litres. The cell mixture was added to the transparent 0.4 micrometre pores sized inserts (Greiner bio-one, Stonehouse, UK). The 0.4 micro-litre- sized pores membrane acts as a medium for selective diffusion. The inserts were placed inside a 24 wells plate. 400 micro-litres of the cell mix was added to the inserts and 1000 micro-litres to the wells, containing the inserts. The cells were grown to confluence. The medium was replaced with fresh DMEM containing 15Mm HEPES, L-gLutamine. (Lonza Laboratories, Verviers, Belgium). Medium alone was added to the base of the well (controls) (Martin *et al.* 2008). The classical way of measuring TER is by the use of hand held electrodes (chopstick-type electrodes). The electrode set up allows cell layer impedance measurements. We used the same method by using hand held static electrodes (WPI, FL, USA) and EVON volt-ohmmeter (EVON, World Precision Instruments, Aston, Herts, UK) (Martin *et al.* 2004a). Both electrodes were placed inside the well, one inside the insert and the other one outside the well. The resistance was measured across the colorectal cancer cell lines - HRT-18 WT, HT-115 WT and RKO WT were the wild types used with their respective KDs at intervals from 0 to 240 minutes. At the end of each experiment, the medium was removed, and the cells were stained with crystal violet and were examined under the microscope to ensure that the cell layers had remained attached throughout the experiment. TER experiments give us a relative estimation of cell functions such as migration, adhesion and resistance and comparison being made between the wild types and transfected cell lines, which are discussed in detail in the results section.

2.2.26. Immunofluorescence (IF)

2.2.27. Immunofluorescent Staining of Cells

Immunofluorescence technique is used for light microscopy with a fluorescent microscope. The technique uses specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets in a cell, which allows visualisation of the presence and distribution of the target molecule.

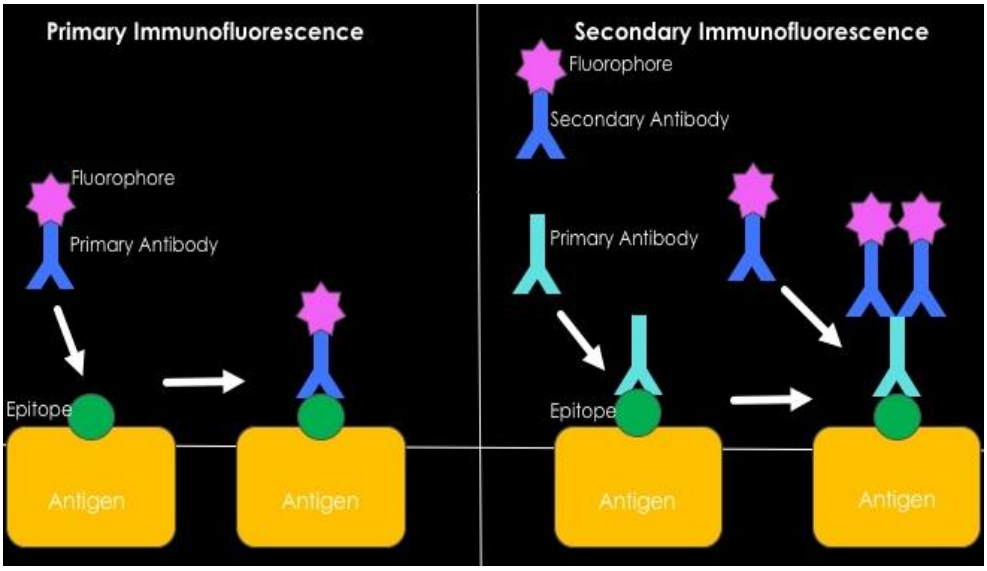


Figure 2.2.27.1. The basic mechanism of immunofluorescence. (By Alizee Pathology LLC. - http://alizeepathology.com)

Figure 2.2.27.1. demonstrates the basic mechanism of immunofluorescence. To the left, Primary immunofluorescence is depicted, which shows an antibody with a fluorophore group bound directly to the epitope of the specific antigen. Once the antibody binds to the epitope, the sample can be viewed under a fluorescent microscope to confirm the presence of the antigen in the sample. The right side of the figure shows secondary

immunofluorescence, which shows that first, an untagged primary antibody binds to an epitope of an antigen in a mechanism similar to the one described above. Once the primary antibody has bound to the target, a secondary antibody (which is specific for the primary antibody already bound to the antigen) binds to the primary antibody, thus increasing the fluorescent signal during microscopy.

30,000 cells were seeded into each chamber of 8 chamber slides x 8 (Millipore). The cells were incubated at 37C in 5% CO2 overnight to achieve confluence and development of tight junctions. The media was aspirated, and cells were fixed in 4% formalin for 10 minutes. The cells were rehydrated in BSS for 20minutes at room temperature. Cells were then permeabilised with 1% Triton x 100 made (made in BSS) for 5 minutes. 5% serum or TBS diluent was prepared while the cells were getting fixed and washed, which was used to dilute the primary and secondary FITC/TRITC reagents as well as blocking any non-specific staining. Each good chamber was covered with 150µl to cover the whole layer. The serum used in the diluent was from the animal in which the secondary was made.

Non-specific binding was blocked by covering the cells with the diluent and incubated at RT for 1 hour. The reagents were added very carefully on the side walls of the chamber, thus preventing any accidental detachment of cells. It was washed three times with TBS. Incubation was done with primary antibody at the concentration of 1:100 (made in dilute) for 1 hour. The following antibodies were used.

Primary antibodies used were as follows:

MAGI-1	SC – 25663	Rabbit (Santa Cruz Biotechnologies)

Secondary antibodies used were as follows:

Anti-rabbit IgG	Sigma Aldrich	Rabbit (for MAGI-1)

50µl of primary antibody (Rabbit) was added per 5 ml of solution, and secondary antibody concentrations were achieved by adding 5µl per 5ml solution for the anti-rabbit antibody. The negative control was diluted with no primary antibody added. Wash was repeated for another three times with TBS. Appropriate sections of were incubated with FITC / TRITC solution (stock = 4mg/ml, used 0.5µl per 100µl, gave final concentration of 2µg/100µl). TBS wash was repeated three times. Plastic frame from the slide was removed, and a coverslip was mounted over the cells using FluorSave™, avoiding any air bubbles. Slides were placed on a tray, wrapped with foil to prevent any fading, and store in the fridge until were viewed under the fluorescent microscope.

ROLE OF MAGI PROTEINS IN INVASIVENESS OF HUMAN COLORECTAL CANCER

CHAPTER 3:

EXPRESSION OF MAGI PROTEIN IN HUMAN COLORECTAL CANCER AND ITS CLINICAL CORRELATION

CHAPTER 3: EXPRESSION OF MAGI PROTEIN IN HUMAN COLORECTAL CANCER AND ITS CLINICAL CORRELATION

3.1. Introduction

Bowel cancer is the third most commonly diagnosed cancer in the UK. 41,000 new cases of bowel cancer were diagnosed in the UK during 2013. Overall survival depends on the degree of spread of cancer at the time of diagnosis since it is a metastasis of colorectal cancer which often leads to the death of the patient. Cancer spreads by cancer cells invading the surrounding tissues. Tight Junctions play an important role in the prevention of cancer spread. (Chlenski *et al.* 1999, Latorre *et al*. 2005). TJs have been widely accepted as the starting point for metastasis in the epithelial cell linings of cancer tissues. (Kominsky *et al*. 2003). The optimising function of TJs can decrease penetration of cancer cells through the mesothelial cell linings. (Tobioka *et al.* 1996). Membrane-Associated Guanalytes Inverted Orientation (MAGI) -1, -2, -3, play a key role in the assembly and maintenance of TJs (Martin *et al.* 2011). MAGI -1 is widely expressed at and have been found to be localised to the epithelial and endothelial cell to cell TJs, and acts as a scaffolding molecule to stabilise cadherin-mediated adhesions and to recruit molecules in TJ. (Laura *et al.* 2002). Roles attributed to MAGI include organisation of protein complexes at the cell and synaptic junctions, regulation of cell polarity, connecting trans-cellular proteins with the cytoskeleton in TJs. (Martin *et al.* 2011). MAGI have also been reported to be involved in cancer cell signaling as a tumour suppressor regulator protein (Matsuda *et al.* 2013). Kranjec *et al.* highlighted the fact that MAGI-1 expression can induce cell growth arrest and apoptosis in Human Papilloma Virus (HPV) tumour cells. (Kranjec *et al.* 2014). MAGI-2 has been shown to play a critical role in inhibition of cell migration and proliferation function in hepatic cancer cells (Hu

et al. 2007). MAGI -2 may act as a tumour suppressor by its actions, which result in stabilising PTEN expression. This binding region is deleted in uterine leiomyomas, prostate cancer, and glioblastoma (Wu *et al.* 2000). MAGI -2 has also been identified as playing an important role in human prostate cancer. (Berger *et al.* 2011), human melanomas (Pleasance *et al.* 2010), cervical cancer (Chen *et al.* 2014) and in advanced adenocarcinoma of the lungs (Kitamura *et al.* 2014). MAGI -3 may relate to the pathogenesis of HTLV-1-associated diseases. (Ohashi *et al.* 2004). MAGI -3 is a possible target site for the Human Papilloma Virus 6 (HPV-6), which is responsible for some human cancers. (Thomas *et al.* 2002). Increased expression of MAGI -1 was identified as a suppressor of colorectal cancer and its metastatic activity. (Zaric *et al.* 2012). MAGI -3 inhibited LPA induced migration and invasion of colon cancer cells (Lee *et al.* 2011).

3.2. Aims

We aimed to look at the clinical correlation of expression of MAGI proteins in human colorectal cancer, with metastasis and patient survival. For this purpose, we analysed quantitative expression levels of MAGI -1, -2 and -3 on Q-PCR in human colorectal cancer tissue, compared normal colorectal tissue, about Dukes and TNM staging, with attention to local and distal metastasis. We also assessed and compared the level of MAGI -1, -2 and -3 expressions on Q-PCR with patient survival.

3.3. Materials and methods

3.4. Cell lines

HT-115, HRT-18 and RKO, human colorectal cancer cells with epithelial morphology were established from highly invasive human colorectal carcinoma, which expresses high amounts of the tumour marker carcinoembryonic antigen. The cells were obtained from the European Collection of Animal Cell Culture (ECACC), HECV, human endothelial cells were purchased from Interlab, Milan, Italy (Table 3.4.1). Different types of cells for both gastric and colonic cancer were chosen due to, firstly, their varying invasiveness properties and differential expression patterns of types of MAGI proteins i-e, MAGI -1, -2 and -3.

Table 3.4.1. Specification of colorectal cancer cell lines

Cell line	HHT -115	HRT -18	RKO
Species	Human	Human	Human
Tissue	Colon	Rectum	Rectum
Age	63	67	82
Gender	Male	Male	Female
Morphology	Epithelial	Epithelial-like	Epithelial
Growth mode	Adherent	Adherent	Adherent
Karyotype	2n=46	2n=46	2n=46
Tumorigenic	Specified	Yes	Yes
Country	UK	UK	UK

3.3.1. MAGI primers used

MAGI-1, MAGI-2 and MAGI-3 primers were designed in-house by using the Beacon designer programme, synthesised by Sigma-Aldrich UK, and were stored in -20^{0}C freezers. The required primers (MAGI -1, -2, -3) were taken out when needed and defrosted on room temperature, following which they were diluted to 1:100 concentration using PCR water. Subsequently, the diluted primers were stored in a designated primer fridge for future use, and the originals were returned to the freezer.

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

Once RNA had been isolated and quantified, RT-PCR was carried out to convert RNA into complementary DNA (cDNA) using DuraScript^{TM} RT PCR kit (Sigma-Aldrich, Dorset, UK). A fixed amount of 0.5 micrograms of RNA was converted to cDNA as per protocol. RNA concentrations were Standardised and used in Q-PCR.

3.3.3. Quantitative Polymerase Chain Reaction (Q-PCR)

Q-PCR is an exceedingly sensitive technique for measuring and quantifying minute amounts of genetic material. The c-DNA of HRT-18, RKO and HT-115 colorectal cancer cell lines was used. The thermo-cycler settings were set to 36 cycles as the standard for expression of MAGI.

3.4. Data analysis

The relationship between MAGI -1, -2 and -3 expression and tumour grade, TNM staging and nodal status was analysed using Mann-Whitney U tests. Survival analysis curve was performed using Kaplan-Meier survival analysis and Kruskal – Wallis tests. Differences

were considered to be statistically significant at $p<0.05$.

3.5. Results

3.5.1. Q-PCR analysis of MAGI expression in human colorectal cancer

The expression of MAGI -1, -2 and -3, on Q-PCR, were plotted on to the tables below (3.5.1.1, 3.5.1.2 and 3.5.1.3). Data were analysed using Mann-Whitney U tests, and differences were considered statistically significant where p value was equal to or less than 0.05. We analysed MAGI -1, -2 and -3 expressions in normal tissue and tumour tissue in our cohort, which showed consistent trends of lower expression of MAGI in tumour tissues.

Table 3.5.1.1. Correlation between expression of MAGI -1 and clinical parameters

	MAGI-1 GAPDH normalised		
	N	Median	Significance
Normal	72	1437	0.225
Tumour	90	1	
Paired Normal	60	1220	0.023
Paired Tumour	65	2	
Normal	72	1437	0.802
Differentiation 1	2	0.533	
Normal	72	1437	0.263
Differentiation 2 & 3	65	1	
Differentiation 1	2	0.533	0.671
Differentiation 2&3	65	1	
Node 0	36	1	0.914
Node 1&2	31	0.7	
Normal	72	1437	0.593
TNM1	9	0.17	
Normal	72	1437	0.659
TNM4	6	0.1	
TNM 1	9	0.17	0.061

TNM 2&3&4	59	2	
Normal	72	1437	0.802
T 1	2	42.7	
Normal	72	1437	0.263
T 2&3&4	65	1	
T 1	2	42.7	0.955
T 2&3&4	65	1	
Normal	72	1437	0.635
Dukes A	7	0.3	
Normal	72	1437	0.269
Duke-BC	62	2	
Dukes A	7	0.3	0.237
Dukes BC	62	2	
Treat Non	38	1	0.166
Chemo Radio	5	3.74	
Non Invasive	47	1	0.929
Invasive	25	1.7	
No Dis Met	47	1	0.815
Dis Met	19	1.7	
Alive	34	1.68	0.965
Died	21	0.71	

The clinical correlation in MAGI -1 expression and clinical parameters is depicted in Table 3.5.1.1. We investigated MAGI -1 expression in 162 (normal background = 72; tumour = 90) samples of which 126 were paired samples (n = 63 pairs) of patients with colorectal adenocarcinoma using real-time PCR. The p-value marked in red shows a significant reduction in expression of MAGI -1, in paired tumour tissue when compared to paired normal colorectal tissue (P-value 0.023). The other value which reaches near to statistical significance (0.061 - marked in green), shows that there was a marked reduction in expression levels of MAGI -1 mRNA transcripts on Q-PCR in the tumour tissue when compared with the normal colorectal tissue between the TNM1 and TNM 2,3 & 4 stages of tumours. However most of the results did not achieve statistical significance, but overall trends were of significance for their consistent pattern of the same theme, that was a reduced expression of MAGI -1 in tumour tissues when compared to the normal tissue. A marked reduction in expression levels of MAGI -1 mRNA transcripts on Q-PCR in Dukes A and Dukes B & C colorectal cancer tissue, when compared with normal colorectal tissue was noticed, which suggested an important correlation between cancer metastasis and reduced levels of MAGI -1. Also of note was a marked reduction in expression levels of MAGI -1 mRNA transcripts on Q-PCR in TNM1 and TNM4 colorectal cancer tissue, when compared with normal colorectal tissue. All of the above findings suggest an important correlation between cancer metastasis and reduced levels of MAGI -1.

Table 3.5.1.2. Correlation between expression of MAGI -2 and clinical parameters

	MAGI-2 GAPDH normalised		
	N	Median	Significance
Normal	71	5132	0.416
Tumour	94	3	
Paired Normal	61	4406	0.001
Paired Tumour	68	2	
Normal	71	5132	0.852
Differentiation 1	2	111	
Normal	71	5132	0.476
Differentiation 2 & 3	68	3	
Differentiation 1	2	111	0.943
Differentiation 2&3	68	3	
Node 0	39	2	0.594
Node 1&2	31	4.6	
Normal	71	5132	0.686
TNM1	9	0.6	
Normal	71	5132	0.739
TNM4	6	7.5	
TNM 1	9	0.6	0.829
TNM 2&3&4	62	4	

Normal	71	5132	0.852
T 1	2	1.43	
Normal	71	5132	0.476
T 2&3&4	68	4	
T 1	2	1.43	0.929
T 2&3&4	68	4	
Normal	71	5132	0.720
Dukes A	7	0.6	
Normal	71	5132	0.485
Duke-BC	65	4	
Dukes A	7	0.6	0.856
Dukes BC	65	4	
Treat Non	42	2	0.604
Chemo Radio	5	25	
Non Invasive	50	3	0.681
Invasive	26	2.9	
No Dis Met	50	2	0.951
Dis Met	19	12.9	
Alive	36	1.42	0.097
Died	22	7.7	

The clinical correlation in MAGI -2 expression and clinical parameters is depicted in table 3.5.1.2. We investigated MAGI -2 expressions in 165 (normal background = 71; tumour = 94) samples of which 130 were paired samples (n = 65 pairs) of patients with colorectal adenocarcinoma using real-time PCR. The only p-value to reach statistical significance (0.001 marked in red) was between MAGI-2 levels for Paired Normal and Paired Tumour samples. There was marked a reduction in expression levels of MAGI -2 mRNA transcripts on Q-PCR in a tumour and paired tumour tissue, when compared with normal and paired normal colorectal tissue, suggesting the possibility of an important correlation between cancer metastasis and reduced levels of MAGI -2. There was a marked reduction in expression levels of MAGI -2 mRNA transcripts on Q-PCR in Dukes A and Dukes B & C when compared with normal colorectal tissue, again suggesting the possibility of an important correlation between cancer metastasis and reduced levels of MAGI -2. There was a marked reduction in expression levels of MAGI -2 mRNA transcripts on Q-PCR in TNM1 and TNM4, when compared with normal colorectal tissue, suggesting the possibility of an important correlation between cancer metastasis and reduced levels of MAGI -2. However, despite these encouraging trends, we did not find positive expression of MAGI -2 in the colorectal cancer cell lines (HT-115, HRT-18 and RKO) which we used but in our experimental work (presented later in the Chapter).

Table 3.5.1.3 Correlation between expression of MAGI -3 and clinical parameters

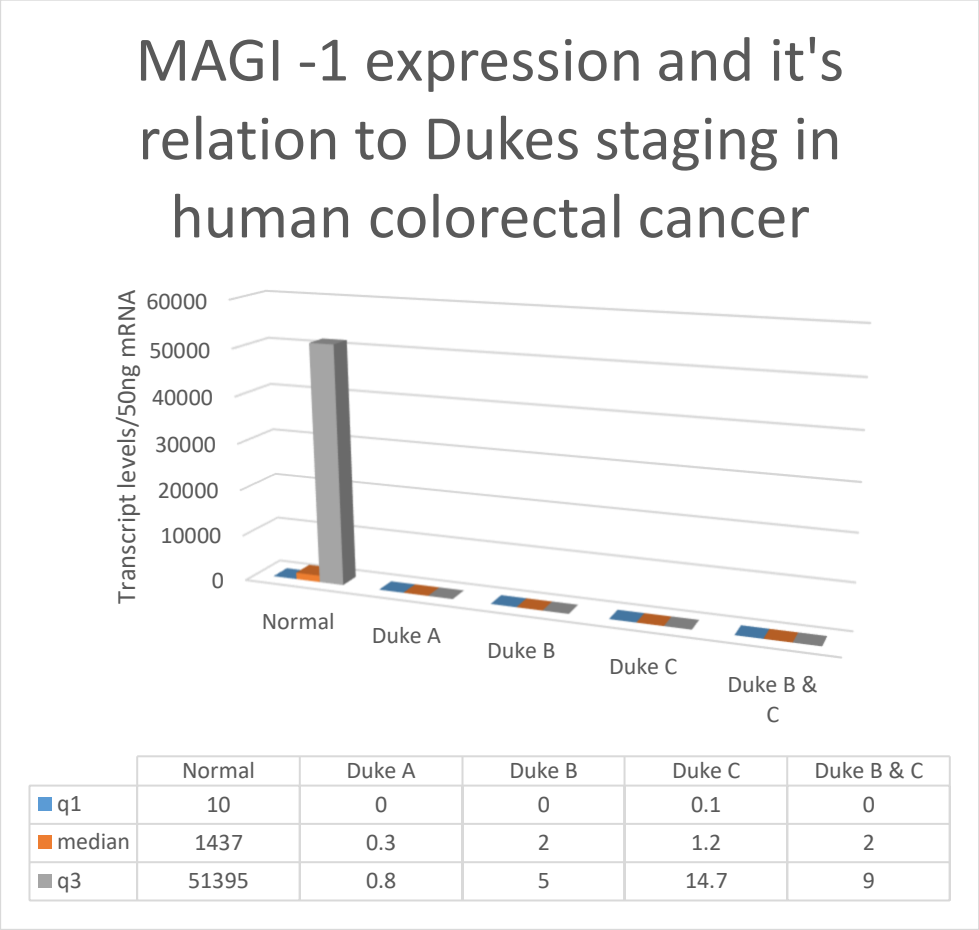
	MAGI-3 GAPDH normalised		
	N	Median	Significance
Normal	64	1	0.761
Tumour	74	0	
Paired Normal	53	2	0.849
Paired Tumour	53	2	
Normal	64	1	0.837
Differentiation 1	2	1840849	
Normal	64	1	0.693
Differentiation 2 & 3	54	0	
Differentiation 1	2	1840849	0.774
Differentiation 2&3	54	0	
Node 0	31	0	0.830
Node 1&2	25	0	
Normal	64	1	0.692
TNM1	7	914083	
Normal	64	1	0.837
TNM4	2	0.0436	
TNM 1	7	914083	No more data
TNM 2&3&4	49	0	
Normal	64	1	
T 1	2	1.04E+08	
Normal	64	1	
T 2&3&4	54	0	

The clinical correlation in MAGI -3 expression and clinical parameters is depicted in table 3.5.1.3. We investigated MAGI -3 expressions in 138 (normal background = 64; tumour = 74) samples of which 106 were paired samples (n=53 pairs) of patients with colorectal adenocarcinoma using real-time PCR. There were no statistically significant values with regards to the clinical correlation of MAGI -3 expressions in different subgroups of colorectal cancer. This may be due to the low level of MAGI -3 expression in our tissue samples.

3.6. Correlation between MAGI -1 expression and clinical parameters

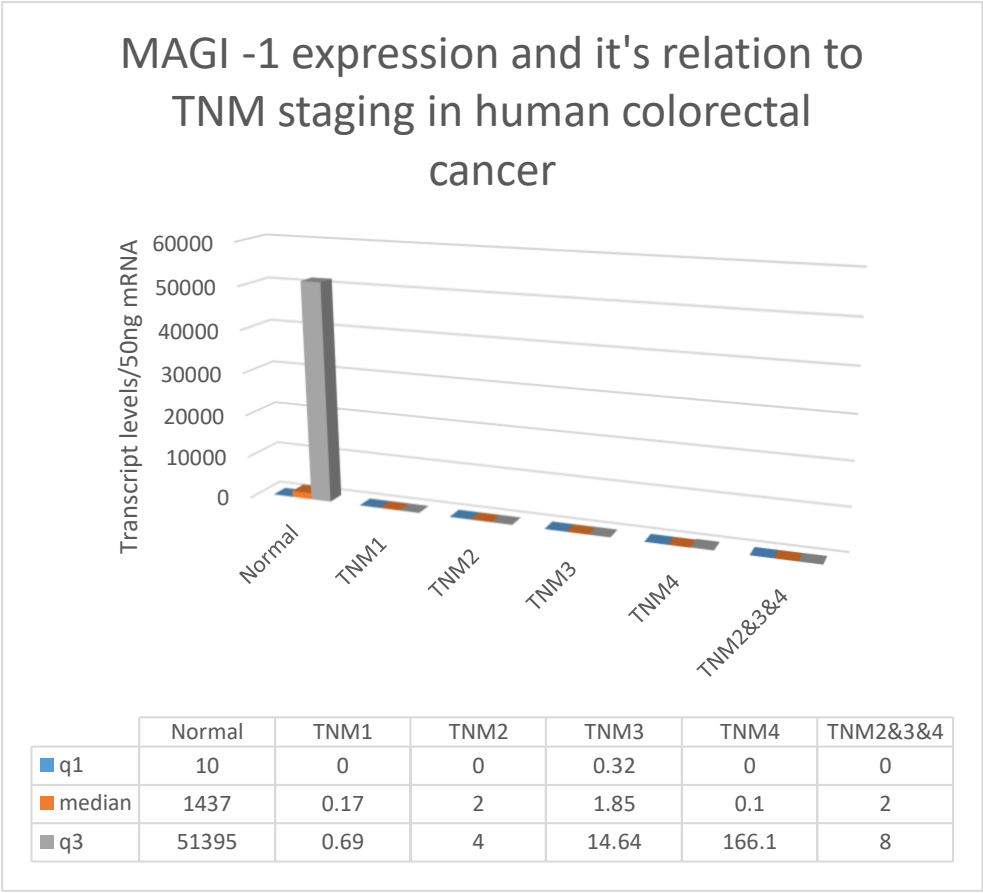
The correlation between expression levels of MAGI -1 on Q-PCR in human colorectal cancer tissues, with Dukes' and TNM staging and overall prognosis, was made. The data was analysed, and comparison was drawn. Marked reduction in expression levels of MAGI -1 mRNA transcripts were found on Q-PCR in Dukes A, B, C and B & C combined when compared with normal colorectal tissue (Figure 3.6.1). This suggested an important correlation between cancer metastasis according to Dukes' classification and reduced levels of MAGI -1. Marked reduction in expression levels of MAGI -1 mRNA transcripts were also found on Q-PCR in TNM1, TNM2, TNM3 and TNM 2&3&4 combined when compared with normal colorectal tissue (Figure 3.6.2), again suggesting an important correlation between cancer metastasis according to TNM classification and reduced levels of MAGI -1. Correlation of MAGI -1 expression in normal, non-invasive and invasive, disease-free, non-recurrence and recurrence cases of human colorectal cancer patients was made. There was marked a reduction in expression levels of MAGI -1 mRNA transcripts on Q-PCR in colorectal cancer tissues including non-invasive and invasive, disease-free, non-recurrence and recurrence types when compared to normal.

However, there was no overall significant difference between the expression of MAGI -1 on Q PCR between non-invasive and invasive, disease-free, non-recurrence and recurrence groups (Figure 3.6.3). Therefore, we suggest that MAGI -1 expression has little or no effect on overall prognosis of colorectal cancer patients, however, there is a clear correlation between reduced levels of MAGI -1 and colorectal cancer metastasis.



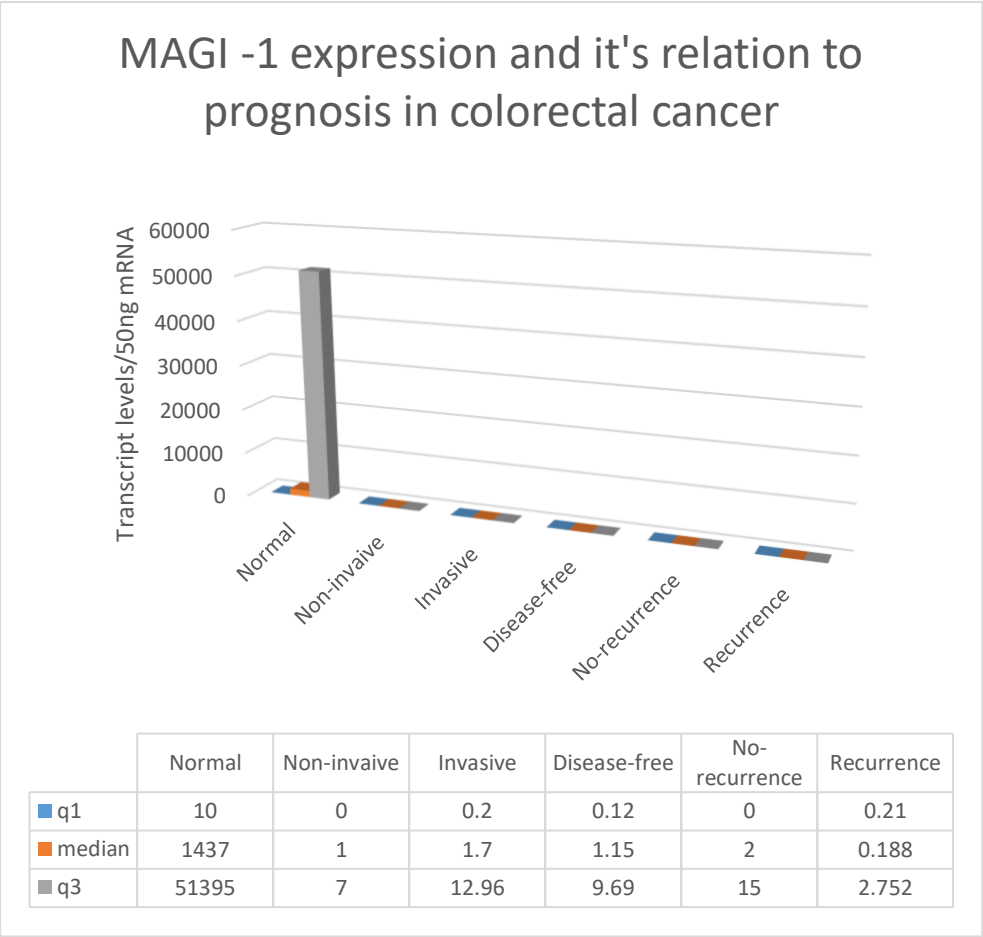
	Normal	Duke A	Duke B	Duke C	Duke B & C
q1	10	0	0	0.1	0
median	1437	0.3	2	1.2	2
q3	51395	0.8	5	14.7	9

Figure 3.6.1. Expression of MAGI -1 in Normal and Dukes A and Dukes B & C: There was marked a reduction in expression levels of MAGI -1 mRNA transcripts on Q-PCR in Dukes A and Dukes B & C colorectal cancer tissue when compared with normal colorectal tissue. The above findings suggest an important correlation between cancer metastasis and reduced levels of MAGI -1



	Normal	TNM1	TNM2	TNM3	TNM4	TNM2&3&4
q1	10	0	0	0.32	0	0
median	1437	0.17	2	1.85	0.1	2
q3	51395	0.69	4	14.64	166.1	8

Figure 3.6.2. Expression of MAGI -1 in Normal and TNM1, TNM2, TNM3 and TNM2&3&4: There was marked a reduction in expression levels of MAGI -1 mRNA transcripts on Q-PCR in TNM1, TNM2, TNM3 and TNM 2&3&4 colorectal cancer tissue when compared with normal colorectal tissue. The above findings suggest an important correlation between cancer metastasis and reduced levels of MAGI -1.

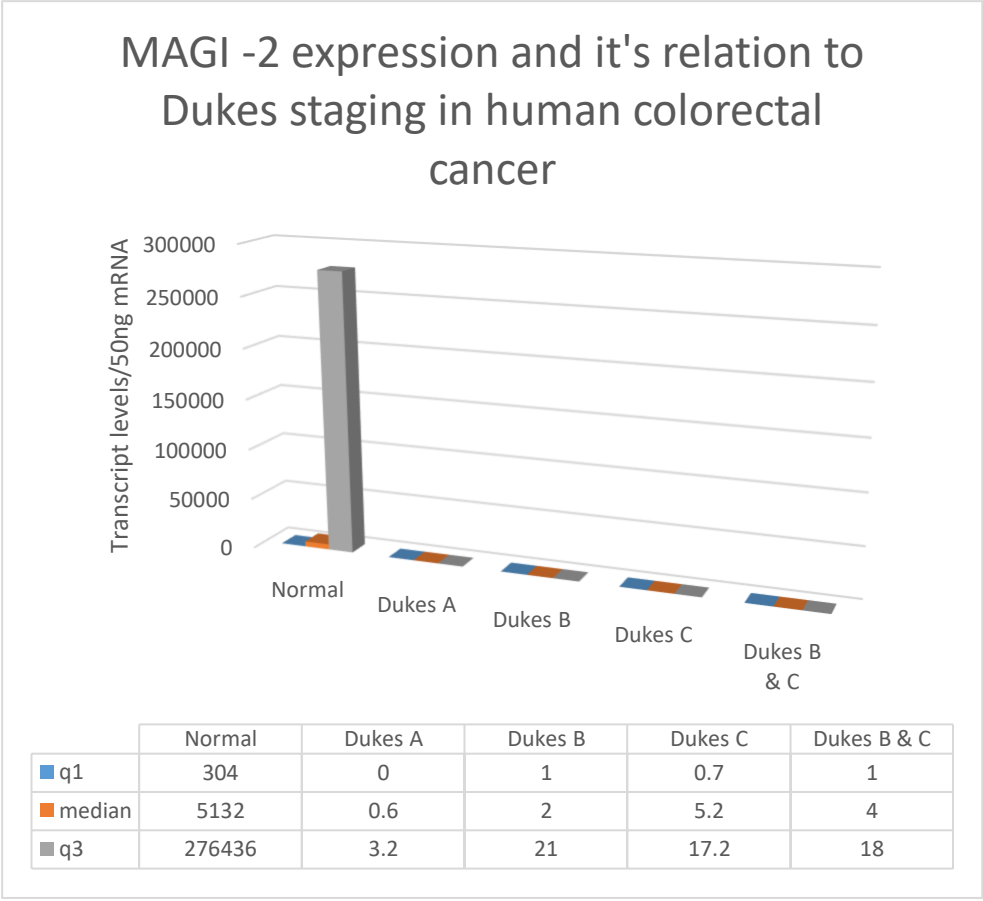


	Normal	Non-invaive	Invasive	Disease-free	No-recurrence	Recurrence
q1	10	0	0.2	0.12	0	0.21
median	1437	1	1.7	1.15	2	0.188
q3	51395	7	12.96	9.69	15	2.752

Figure 3.6.3. Relation of MAGI -1 expression in normal, non-invasive and invasive, disease-free, non-recurrence and recurrence cases of human colorectal cancer patients. There was a marked reduction in expression levels of MAGI -1 mRNA transcripts on Q-PCR in normal, non-invasive and invasive, disease-free, non-recurrence and recurrence cases, when compared to normal, but overall no significant difference between non-invasive and invasive, disease-free, non-recurrence and recurrence cases. Hence, the level of MAGI -1 expression had no or little effect on overall prognosis of the patients.

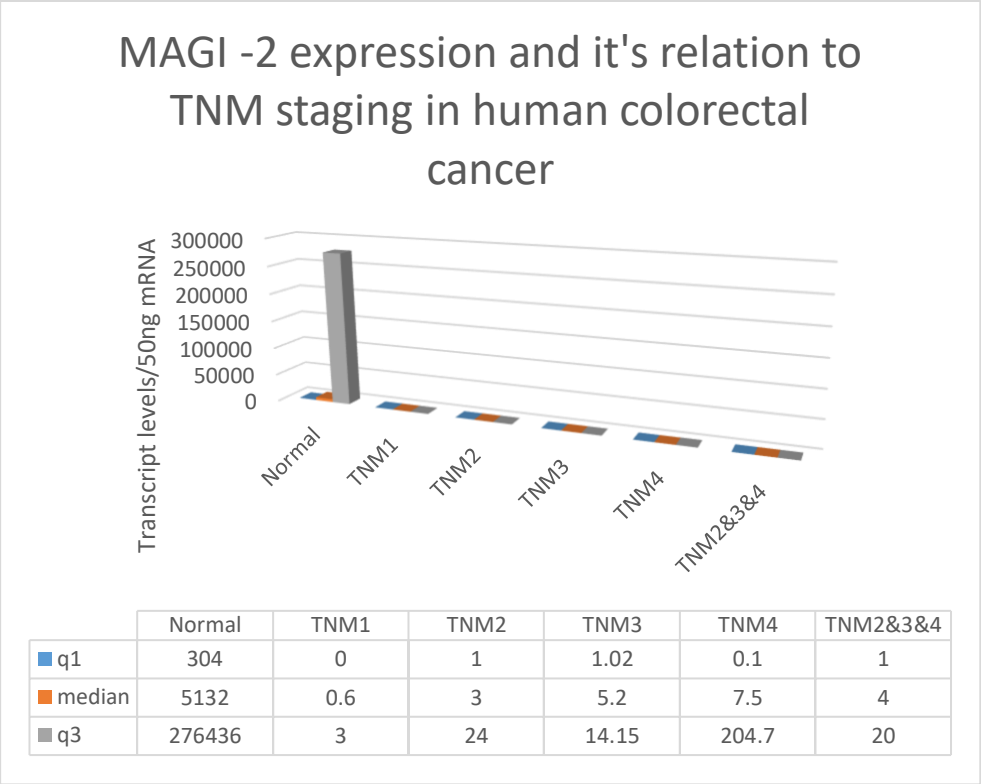
3.7. Correlation between MAGI -2 expression and clinical parameters

The correlation between expression levels of MAGI -2 on Q-PCR in human colorectal cancer tissues, with Dukes' and TNM staging and overall prognosis, was made. The data was analysed, and a comparison was drawn. Marked reduction in expression levels of MAGI -2 mRNA transcripts was found on Q-PCR in Dukes A, B, C and B & C combined when compared with normal colorectal tissue (Figure 3.7.4). This suggested an important correlation between cancer metastasis according to Dukes' classification and reduced levels of MAGI -2. Marked reduction in expression levels of MAGI -2 mRNA transcripts was also found on Q-PCR in TNM1, TNM2, TNM3 and TNM 2&3&4 combined when compared with normal colorectal tissue (Figure 3.7.5), again suggesting an important correlation between cancer metastasis according to TNM classification and reduced levels of MAGI -2. The relation of MAGI -2 expressions in normal, non-invasive and invasive, disease-free, non-recurrence and recurrence, cases of colorectal cancer patients was observed. Again, reduced levels of MAGI -2 mRNA transcripts on Q-PCR in colorectal cancer tissues, including non-invasive and invasive types, disease-free, non-recurrence and recurrence type; were found when compared to healthy ones. However, there was no overall significant difference found in the expression of MAGI -2 with Q PCR between non-invasive and invasive, disease-free, non-recurrence and recurrence groups (Figure 3.7.6.1). Therefore, we concluded that MAGI -2 expressions had no or little effect on overall prognosis of the patients, but there was a clear correlation between reduced levels of MAGI -2 and colorectal cancer metastasis.



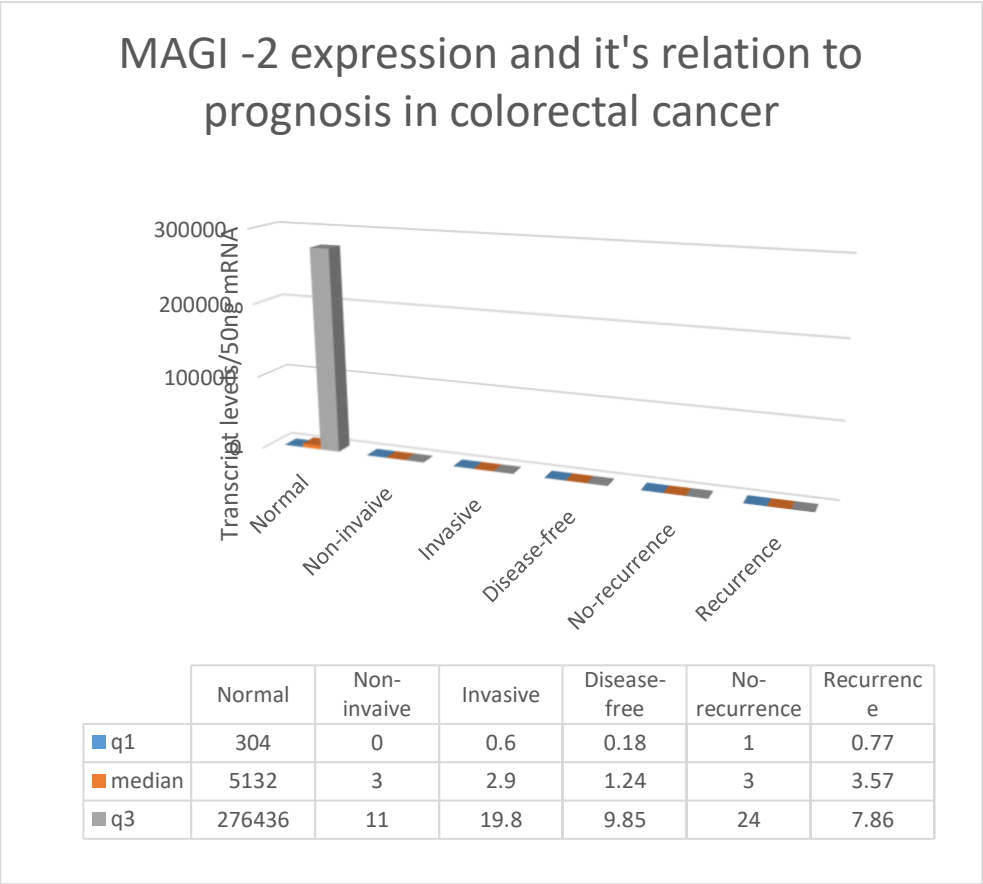
	Normal	Dukes A	Dukes B	Dukes C	Dukes B & C
q1	304	0	1	0.7	1
median	5132	0.6	2	5.2	4
q3	276436	3.2	21	17.2	18

Figure 3.7.1. Expression of MAGI -2 in Normal and Dukes A and Dukes B & C: There was marked a reduction in expression levels of MAGI -2 mRNA transcripts on Q-PCR in Dukes A and Dukes B & C colorectal cancer tissue when compared with normal colorectal tissue. The above findings suggest an important correlation between cancer metastasis and reduced levels of MAGI -2.



	Normal	TNM1	TNM2	TNM3	TNM4	TNM2&3&4
q1	304	0	1	1.02	0.1	1
median	5132	0.6	3	5.2	7.5	4
q3	276436	3	24	14.15	204.7	20

Figure 3.7.2. Expression of MAGI -2 in Normal and TNM1, TNM2, TNM3 and TNM2&3&4: There was marked a reduction in expression levels of MAGI -2 mRNA transcripts on Q-PCR in TNM1, TNM2, TNM3 and TNM 2&3&4 colorectal cancer tissue when compared with normal colorectal tissue. The above findings suggest an important correlation between cancer metastasis and reduced levels of MAGI -2.

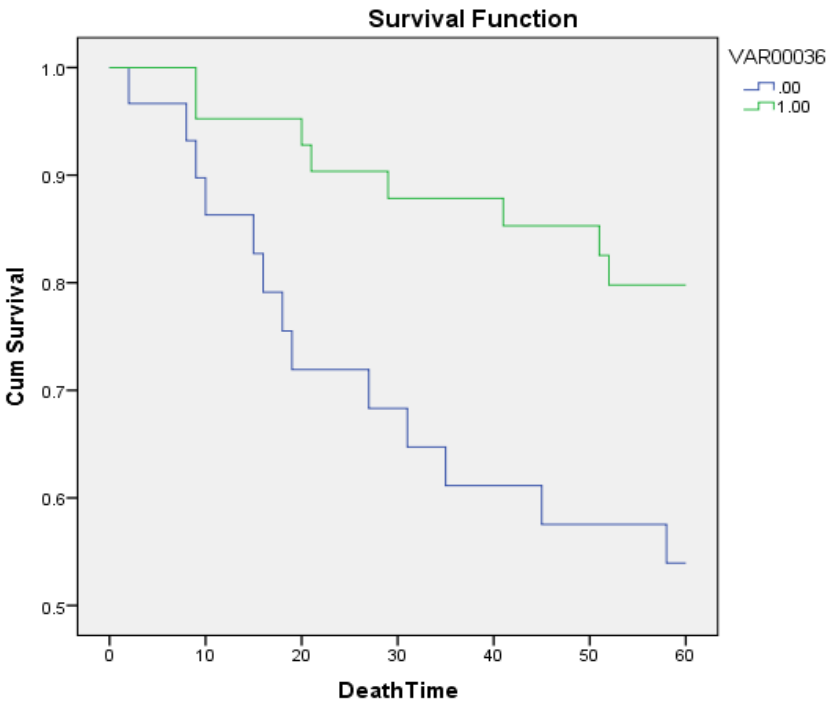


	Normal	Non-invaive	Invasive	Disease-free	No-recurrence	Recurrence
q1	304	0	0.6	0.18	1	0.77
median	5132	3	2.9	1.24	3	3.57
q3	276436	11	19.8	9.85	24	7.86

Figure 3.7.6.1 Relation of MAGI -2 expressions in normal, non-invasive and invasive, disease-free, non-recurrence and recurrence cases of human colorectal cancer patients. There was a marked reduction in expression levels of MAGI -2 mRNA transcripts on Q-PCR in normal, non-invasive and invasive, disease-free, non-recurrence and recurrence cases, when compared to normal, but overall no significant difference between non-invasive and invasive, disease-free, non-recurrence and recurrence cases. Hence the level of MAGI -2 expressions had no or little effect on overall prognosis of the patients.

3.8. Survival Curves

The difference between the expression of MAGI -1, -2 and -3 combined and individual expression of MAGI -1, -2 and -3, about overall survival, and disease-free survival were analysed using Mann – Whitney U and Kruskal – Wallis tests. Survival curves were plotted by using Kaplan – Meier survival analysis (Figures 3.8.1, 3.8.2, 3.8.3,.3.8.4, 3.8.5, 3.8.6, 3.8.7 and 3.8.8). Differences were considered statistically significant where the value of p was equal to or less than 0.05. We looked at the overall survival rate and disease-free survival in our cohort, which showed consistent trends of higher survival rate in patients with increased levels of expression of MAGI proteins. Combined analysis of all MAGI -1, -2, and -3 proteins showed significant statistical results in both overall and disease-free survival rates (Wilcoxon statistical significance of 0.015 and 0.045) in patients with increased expression of MAGI proteins (Figure 3.8.1 and 3.8.2). Overall survival of patients with increased expression levels of MAGI-1 showed higher survivals rates, close to statistical significance (0.074) (Figure 3.8.3).

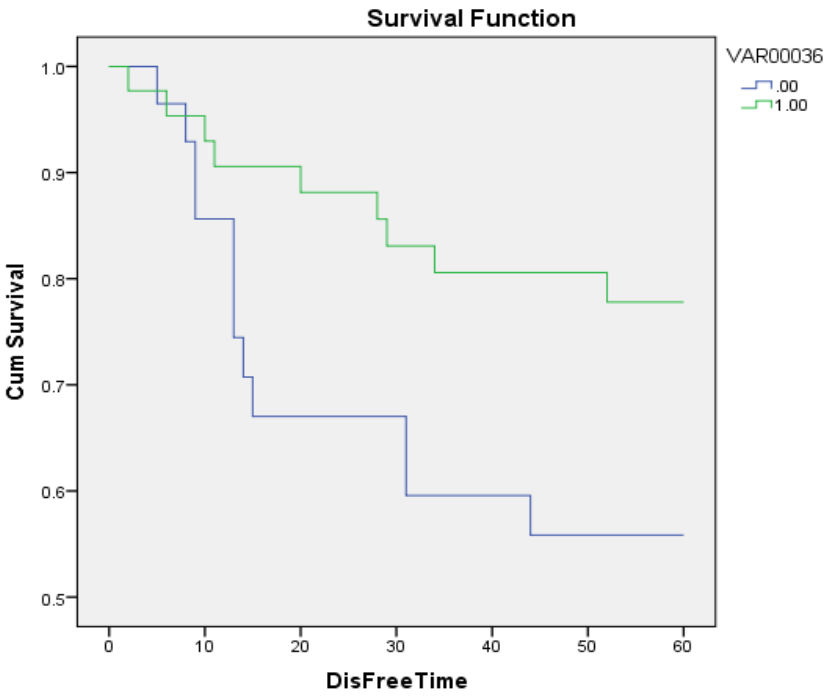


Overall Comparisons

Wilcoxon (Gehan) Statistic	df	Sig.
5.888	1	.015

a. Comparisons are exact.

Figure 3.8.1. MAGI-1,-2 & -3 COMBINED ANALYSIS, OVERALL SURVIVAL: Showing Kaplan-Meier survival curves in correlation to MAGI -1, -2, and -3 levels combined and overall patient's survival. Patients who had low levels of MAGI -1, -2 and -3 combined, had a relative reduced survival when compared to patients with higher levels of MAGI -1, -2 and -3 combined (P-value 0.015).

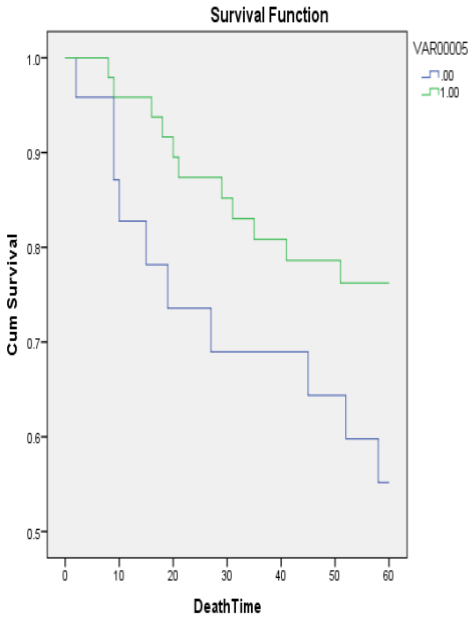


Overall Comparisons

Wilcoxon (Gehan) Statistic	df	Sig.
4.004	1	.045

a. Comparisons are exact.

Figure 3.8.2. MAGI-1,-2 & 3 COMBINED ANALYSIS, DISEASE FREE SURVIVAL: Showing Kaplan-Meier survival curves in correlation to MAGI -1, -2, and -3 combined and overall patient's disease-free survival. Patients who had low levels of MAGI -1, -2 and -3 combined, had a relatively reduced disease-free survival when compared to patients with higher levels of MAGI -1, -2 and -3 combined (P-value 0.045).

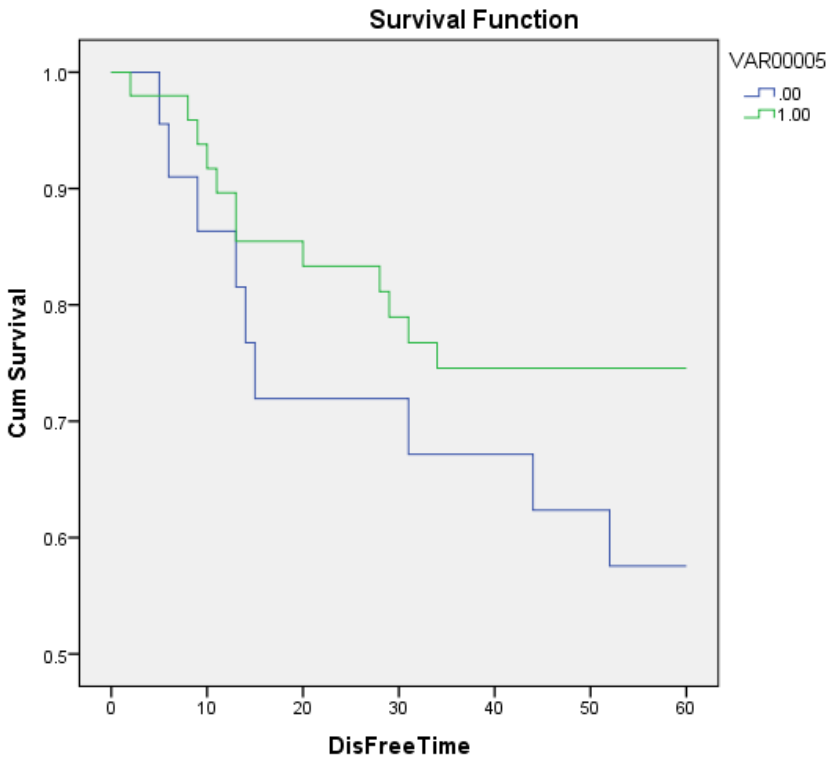


Overall Comparisons

Wilcoxon (Gehan) Statistic	df	Sig.
3.188	1	.074

a. Comparisons are exact.

Figure 3.8.3. MAGI -1 OVERALL SURVIVAL: Showing Kaplan-Meier survival curves in correlation to MAGI -1 and overall patient survival. Patients who had low levels of MAGI -1, had a relatively reduced overall survival when compared to patients with higher levels of MAGI -1.

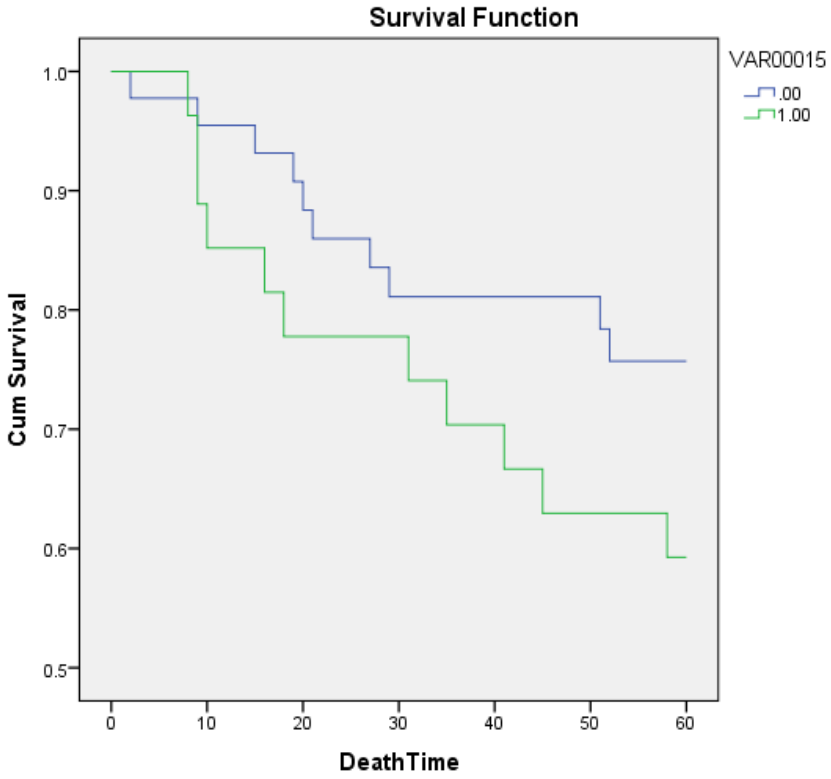


Overall Comparisons

Wilcoxon (Gehan) Statistic	df	Sig.
1.731	1	.188

a. Comparisons are exact.

Figure 3.8.4. MAGI -1 DISEASE-FREE SURVIVAL: Showing Kaplan-Meier survival curves in correlation to MAGI -1 and disease-free patient survival. Patients who had low levels of MAGI -1, had a relatively reduced disease-free survival when compared to patients with higher levels of MAGI -1.

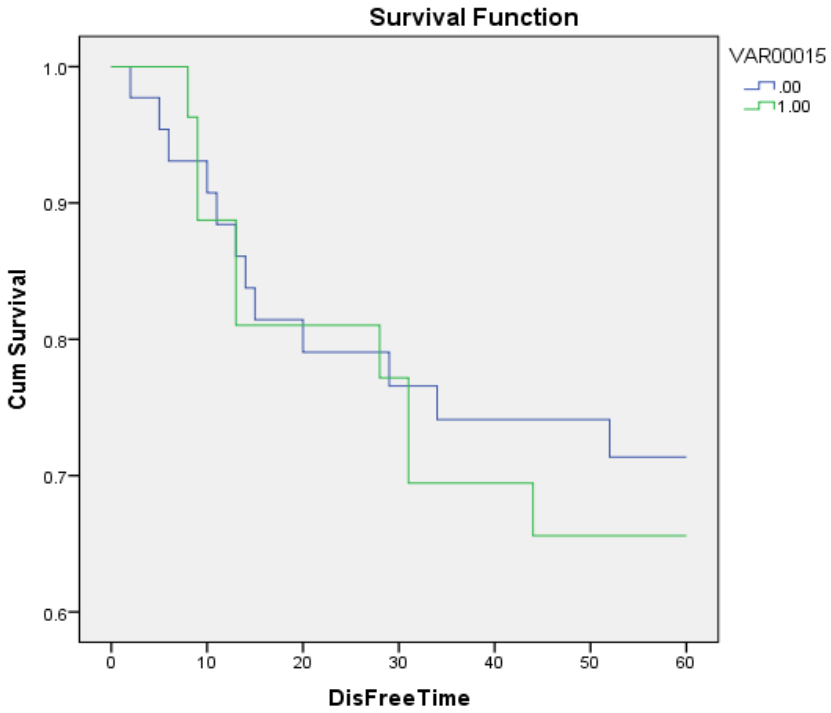


Overall Comparisons

Wilcoxon (Gehan) Statistic	df	Sig.
1.868	1	.172

a. Comparisons are exact.

Figure 3.8.5. MAGI -2 OVERALL SURVIVAL: Showing Kaplan-Meier survival curves in correlation to MAGI -2 and overall patient survival. Patients who had low levels of MAGI -2, had a relatively reduced overall survival when compared to patients with higher levels of MAGI -2.

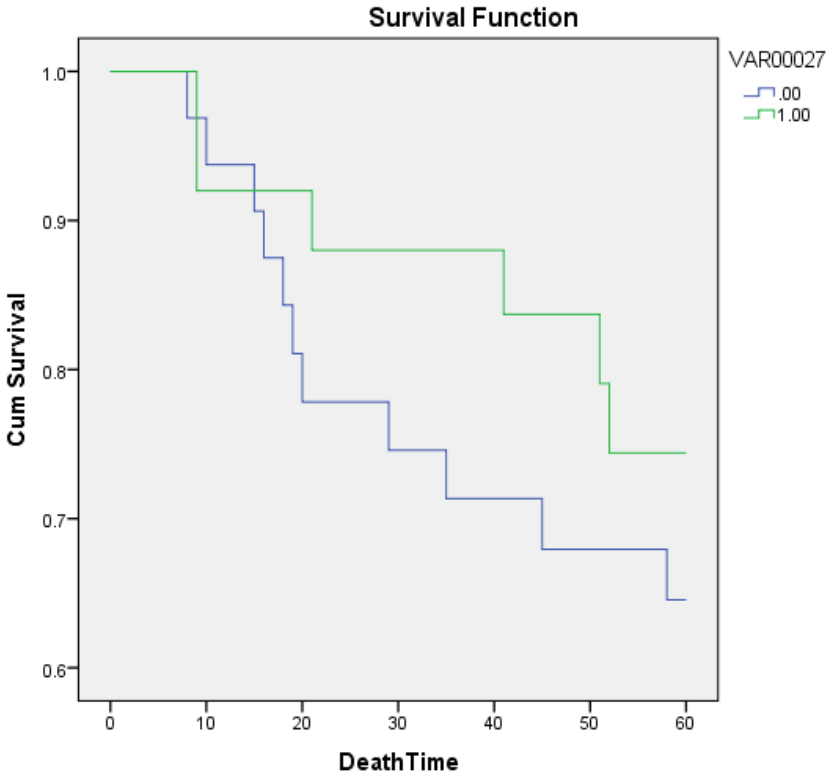


Overall Comparisons

Wilcoxon (Gehan) Statistic	df	Sig.
.141	1	.707

a. Comparisons are exact.

Figure 3.8.6. MAGI -2 DISEASE-FREE SURVIVAL: Showing Kaplan-Meier survival curves in correlation to levels of MAGI -2 and patients' disease-free survival. Patients who had low levels of MAGI -2 had a relatively reduced disease-free survival when compared to patients with higher levels of MAGI -2.

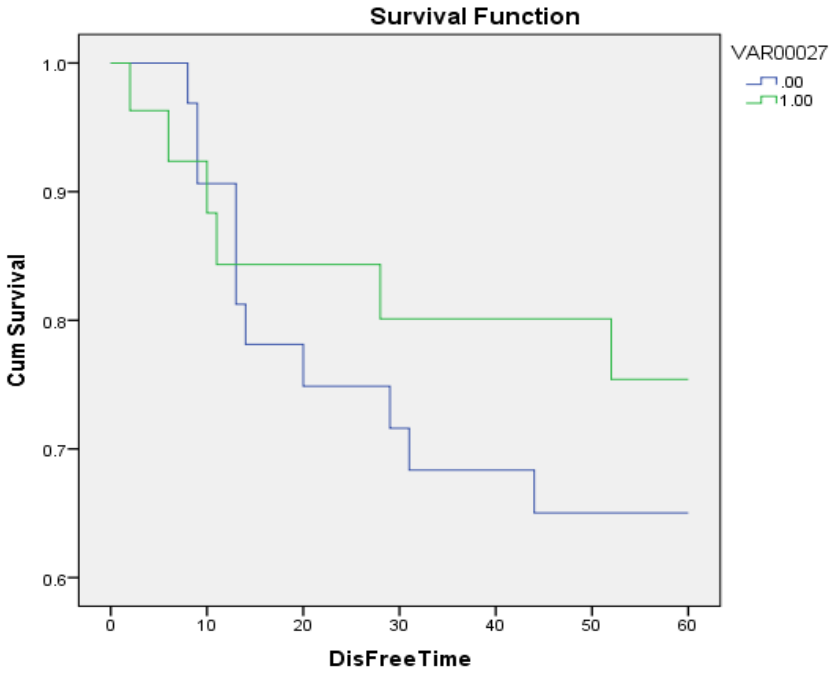


Overall Comparisons

Wilcoxon (Gehan) Statistic	df	Sig.
.979	1	.323

a. Comparisons are exact.

Figure 3.8.7. MAGI -3 OVERALL SURVIVAL: Showing Kaplan-Meier survival curves in correlation to MAGI -3 and patients overall survival. Patients who had low levels of MAGI -3, had a relatively reduced overall survival when compared to patients with higher levels of MAGI -3.



Overall Comparisons

Wilcoxon (Gehan) Statistic	df	Sig.
.602	1	.438

a. Comparisons are exact.

Figure 3.8.8. MAGI-3 DISEASE-FREE SURVIVAL: Showing Kaplan-Meier survival curves in correlation to MAGI -3 and patients' disease-free survival. Patients who had low levels of MAGI -3, had a relatively reduced disease-free survival when compared to patients with higher levels of MAGI -3.

Survival curves showed an increase in overall survival and disease-free survival in our cohort with consistent trends of higher survival rates with increased MAGI -1, -2 and -3 expression levels in patients. This was consistent with MAGI -1, -2 and -3 expression on Q-PCR in normal tissue and tumour tissue in our cohort, which showed consistent trends of higher expression levels of MAGI in the normal tissue in comparison to tumour tissue. Survival curves were also consistent with the increased expression of MAGI -1 and -2 in normal colorectal tissue when compared to a metastatic disease.

3.9. Discussion:

There is evidence in the literature that MAGI protein plays an important role in stabilising TJs, and thereby inhibiting invasion and migration of colorectal cancer cells. Landy *et al.* suggested that the dysregulation of TJs proteins precede the development of inflammatory bowel disease (IBD), and also leads to the development of IBD associated colorectal cancer (CRC). (Landy *et al.* 2016) Zaric *et al.* identified MAGI -1 over-expression as a suppressor of colorectal cancer and its metastatic activity. (Zaric *et al.* 2012) Lee *et al.* found over-expression of MAGI -3 to inhibit LPA induced migration and invasion of colon cancer cells, whereas by knocking down MAGI -3 continues the repeated effect of NHERF -2 over-expression (Lee *et al.* 2011). We found decent evidence in the literature that MAGI proteins (-1, -2, and 3) play an important role in cancer metastasis; however, there was limited information on their types (MAGI -1, MAGI -2 and MAGI -3), especially in human colorectal cancer metastasis. So we decided to check MAGI -1, -2 and -3 expression and their significance in human colorectal cancer metastasis using Q-PCR and the effect of individual types of MAGI (-1, -2 and -3)

expression on survival in patients. We started off by using quantitative PCR to demonstrate that expression levels of MAGI-1 were decreased in the paired tumour tissues than the normal tissues (statistically significant, p-value 0.023) (Table 3.5.1.1). Also, that expression levels of MAGI -1 were decreased in the TNM stage -2, -3 and -4 when compared with TNM stage -1. The results were consistent with the trend and also were near to achieving statistical significance (0.061) (Table 3.5.1.1). Although the numbers were small and did not achieve statistical significance, the trends were consistent with the low expression of MAGI-1 associated with colorectal cancer metastasis (Table 3.5.1.1). It was possible that the few statistically significant results were due to the low power of our sample, but the consistency in the trends certainly was remarkable and stood out. We had similar results for decreased expression levels of MAGI-2 in a tumour paired and normal tissue with statistical significance (0.001) (Table 3.5.1.2), but we did not have samples achieving statistical significance in MAGI-3 (Table 3.5.1.3), which was probably due to the small numbers and reduced number of samples. Survival curves showed an increase in overall survival and disease-free survival in our cohort with consistent trends of higher survival rates from increased MAGI expression levels in tissue. Combined analysis of MAGI -1, -2 and 3 (Figure 3.8.1 and 3.8.2) showed higher survival rates in both, overall and disease-free survival, which were of statistical significance (Wilcoxon statistical significance of 0.015 and 0.045). Survival curves of individual MAGI -1, -2 and 3 were consistent with a similar pattern, and showed a higher rate of survival with increased expression of individual MAGI type (MAGI -1 and -2) (Figures 3.8.1, 3.8.2, 3.8.3, 3.8.4, 3.8.5 and 3.8.6). Although individual MAGI type (MAGI -1, -2, -3) results did not reach statistical significance, the trends were consistent with increased overall survival and disease-free survival in tissues with increased expression. MAGI-1 overall survival was of note as it reached near statistical significance (0.074) (Figure 3.8.3).

mRNA transcripts of MAGI -1 and -2 expressions were found to be reduced about Dukes' and TNM classifications of cancer metastasis when compared with normal colorectal tissue (Figure 3.6.1, 3.6.2 and 3.7.4, 3.7.5). However, there was no overall significant difference found in the expression of MAGI -1 and -2 on Q PCR between non-invasive and invasive, disease-free, non-recurrence and recurrence groups (Figure 3.6.3 and 3.7.6.1). Therefore, we concluded that MAGI – 1 and -2 expression had no or little effect on overall prognosis of the patients, but there was a clear correlation between reduced levels of MAGI -2 and colorectal cancer metastasis. As we have mentioned before, from literature review and our analysis of MAGI protein expression in human colorectal cancer cell lines (presented later in the thesis) and also from the increased disease-free survival of our cohort, we had compelling evidence that MAGI proteins play an important role in the invasiveness and metastasis in human colorectal cancer, through their role in maintenance of TJ functions and strength. MAGI are a novel set of proteins and its types MAGI -1, -2 and -3, is a potential area of further research work, which was positively supported by the results of our initial experimental work. MAGI proteins have significant clinical relevance which makes them worthwhile of not only a fascinating and attractive area of research, but moreover has the potential for a future therapeutic agent.

CHAPTER 4:

MAGI - EXPRESSION IN COLORECTAL CANCER CELL LINES AND CREATION OF KNOCKDOWNS (KDS)

4.1.1. Introduction

MAGI (Membrane Associated Guanylate Kinase with the inverted arrangement of protein-protein interaction domains) are peripherally associated cytoplasmic proteins, active in tight junctions (TJ). MAGI proteins were first reported by Dobrosotskaya *et al.* in 1997 as prototypical members of membrane-associated guanylate kinase (MAGUK) family as part of *Drosophila* disc large tumour suppressor protein. (Dobrosotskaya *et al.* 1997). MAGUK family of proteins are mainly found in the assembly of multiple protein complexes on the inner surface of the plasma membrane at regions of the cell to cell contact points (TJs) in epithelial cells and synaptic junctions in neurons. The three types of MAGI proteins are MAGI -1, MAGI -2, and MAGI -3, based on their highest degree of to the central PDZ domains with the inverted orientation of the GuK domain and SH3 domain and their significant resemblance in protein to protein interactions. MAGI -1 is reported to be expressed widely expressed in different tissues and tumours, whereas MAGI -2 whereas MAGI -2 are reported to be predominantly expressed in the brain and renal tissue. MAGI -3 is reported to be expressed in various tissues, including foetal and adult, and also in tumours. (Wu *et al.* 2000). MAGI proteins are active in the organisation of protein complexes at the cell and synaptic junctions, regulation of cell polarity, connecting trans-cellular proteins with the cytoskeleton in TJs. (Martin *et al.* 2011). MAGI is expressed in cell-to-cell contact junctions, which are focal points for restraining of not only the disruption of junctional complexes but also the commencement of tumour cell invasion (Kotelevets *et al.* 2005). Knocking down of MAGI protein causes a loss of junctional compartmentalisation along the lateral membrane, and reduces the overall robustness of cell-to-cell adhesion (Stetak *et al.*

2010). Focusing on MAGI -1 in colorectal cancer, it is reported in the literature that over-expression in colorectal cancer cells, suppresses metastasis (Zaric *et al.* 2012).

4.1.2. Aims

We aimed to study the expression of MAGI -1, -2 and -3 in human colorectal cancer cells. We expressed MAGI -2 in human gastric cancer cell lines and used it as a control to lack of MAGI 2 expression in colorectal cancer cell lines. Furthermore, we aimed to create MAGI -1 knockdowns (KD), to study their effect on changes in cell morphology on microscopy and on the strength of TJs between cells by using immunofluorescence in human colorectal cancer cell lines. We also studied the effect of KD of MAGI -1 on colorectal cancer cell invasion and migration (described in next chapter (chapter 5)).

4.1.3. Materials:

4.1.3.1. Cell lines:

HT -115, HRT -18 and RKO, three different types of colorectal cancer cell lines were chosen and tested for expression MAGI -1, -2 and -3. MAGI -1 and MAGI -3 were found to have a positive expression; however, we did not have MAGI -2 expressions in colorectal cancer cell lines. Therefore, gastric cell lines (AGS and HGC -27) were used as controls for MAGI -2 expressions. The details of colorectal cancer and gastric cancer cell lines used were as follows (Table 4.1.3.1.1 & 4.1.3.1.2).

Table 4.1.3.1.1. Specification of colorectal cancer cell lines

Cell line	HT -115	HRT -18	RKO
Species	Human	Human	Human
Tissue	Colon	Rectum	Rectum
Age	63	67	82
Gender	Male	Male	Female
Morphology	Epithelial	Epithelial-like	Epithelial
Growth mode	Adherent	Adherent	Adherent
Karyotype	2n=46	2n=46	2n=46
Tumorigenic	Specified	Yes	Yes
Country	UK	UK	UK

Table 4.1.3.1.2. Specification of gastric cancer cell lines

Cell line	AGS	HGC - 27
Organism	Human	Human
Tissue	Stomach	Lymph node
Age	54	Unspecified
Gender	Female	Unspecified
Morphology	Epithelial	Epithelial
Growth mode	Adherent	Adherent

(The cells were obtained from the European Collection of Animal Cell Culture (ECACC))

4.1.4. MAGI primers used

MAGI-1, MAGI-2 and MAGI-3 primers were used, by Invitrogen (Paisley, UK), were stored in freezers in the home department. MAGI -1, -2, -3 primers were diluted to 1:10 concentration by adding PCR water, ready to use (Table 4.1.4.3).

Table 4.1.4.3. Primer sequences

Primers	Forward	Reverse
MAGI-1 (F11 R11)	CTCC AAGAAA CAGCA TTACC	CTGAA GTGG AACTT CTCTGG
MAGI-2 (F11 R11)	CAGTG ACTAC GCAA CCT ACA	GGATG TCT GGTTT CACA TCT
MAGI-2 (F12 R12)	TCAAGG ATGCA GGTCTT AGT	CTTATT GCTGG TCCA TCTTC
MAGI-3 (F12 R12)	GGGAAA TCA CAC AAACAA GT	TGAG ACAG TTCAA CAAT GGA

4.2. Methods.

4.2.1. Generation of mutant RKO, HT-115 and HRT-18 cell lines with the MAGI-1 knockdown.

The pEF6/V5-His TOPO® TA expression system (Invitrogen, Paisley, UK) provides a highly efficient one-step cloning technique which does not require ligase, post-PCR procedures, or PCR primers containing specific sequences. Such an approach allows the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for constitutive expression in mammalian cells after transfection. The line raised pEF6/V5-His TOPO® vector in the kit consists of single, overhanging 3' deoxythymidine (T) residues which allow *Taq* polymerase-amplified PCR products, containing a single deoxyadenosine (A) at the 3’ ends, to ligate efficiently. The EF-1 ✔ promoter in pEF6/V5-His TOPO® then enables the constitutive expression of the PCR product after the transfection of the plasmid.

The PCR products were cloned into pEF6/V5-His TOPO® plasmids, and the total 6µl cloning

reaction was mixed and stored on ice in advance to the One Shot Chemical Transformation (Invitrogen, Paisley, UK). The condition used for TOPO cloning reaction was as follows:

- PCR product - 4µl
- Salt solution - 1µl
- TOPO vector - 1µl

5µl of TOPO cloning reaction was added into each vial of One Shot TOP10 Chemically Competent *E. coli.* (Invitrogen, Paisley, UK) and mixed gently by stirring using the pipette tip. The vial was stored on ice for 5-30 minutes (based on the product size) before heat-

shocking at 42°C for 30 seconds followed by immediately transferring into ice. To each vial, 250µl of room temperature S.O.C medium was added, and the tubes were shaken at a 45° angle at 225rpm on a horizontal orbital shaker (Bibby Stuart Scientific, UK) at 37°C for 1 hour. After the incubation period, the transformed products from each vial were spread in different seeding density on a pre-warmed agar petri dish containing 100µg/ml ampicillin followed by incubating overnight at 37°C. Several colonies from each petri dish were randomly picked, and the PCR product insertion and orientation of the incorporated PCR product were checked by PCR using primer combination of T7F vs BGHR and T7F vs specific forward or reverse primer respectively. The reaction mix is outlined as follows:

PCR product insertion primer pair mix (used for overexpression sequences)

- GoTaq Green master mix - 8µl
- T7F plasmid specific forward primer - 1µl
- BGHR plasmid specific reverse primer - 1µl
- PCR water -6µl

For ribozyme transgene insertion and orientation checking, the molecule specific forward and reverse primer was replaced in the above mixtures with forward (RbToPF) and reverse (RbBMR) sequences to the common region of the ribozyme transgene. Each colony was inoculated into both reaction mixes using a sterile pipette tip, and the reaction mixes were placed in a 2720 Thermal Cycler (Applied Biosystems, Paisley, UK). The PCR reaction conditions were used as follows: 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 40 seconds and 72°C for 1 minute with a final extension step of 72°C for 10 minutes. Gel electrophoresis on a 2% agarose gel stained with a SYBR Safe DNA gel stain was utilised to visualise the conventional PCR reaction

products. Colonies with correctly orientated PCR product inserts were inoculated into universal tubes containing 5ml of selective LB broth (containing 100µg/ml ampicillin) followed by being incubated overnight at 37°C while being shaken with the 45° angle at 225rpm.

4.2.2. Plasmid amplification and extraction

Plasmid extraction was undertaken using the Sigma GenElute Plasmid MiniPrep Kit (Sigma, Dorset, UK) based on the protocol provided. The overnight incubated universal tubes containing the chosen colonies were centrifuged at 5,000RCF to acquire a bacterial pellet. The supernatant in each tube was discarded, and the bacterial pellet was re-suspended thoroughly using 200µl Resuspension Solution containing RNase A and transferred into a 1.5ml Microfuge tube. To the Microfuge tube was added 200µl of Lysis Solution followed by eight gentle inversions. This lysis stage was conducted within 5 minutes to avoid permanent plasmid denaturation. Then, 350µl of Neutralisation Solution was added, and the microfuge tube was gently inverted six times followed by centrifugation at 12,000g for 10 minutes to pellet the cell debris. The clear lysate in the Microfuge tube was transferred into a Mini-prep Binding Column which was placed into a micro-centrifuge Tube. Such Mini-prep Binding Column was washed with Column Preparation Solution in advance to maximise plasmid binding capacity. The clear lysate added micro-centrifuge Tube was then centrifuged for 1 minute at 12,000g to enable the plasmid binding to Mini-prep Binding Column. The flow-through liquid was discarded, and 700µl of Wash Solution (containing ethanol) was added to the Mini-prep Binding Column followed by centrifugation at 12,000g for 1 minute before discarding the flow-through liquid. The Microfuge tube was then centrifuged again at 12,000g for 1 minute to remove the remaining Wash solution before the Mini-prep Binding Column was transferred into a fresh Collection Tube. The plasmid was eluted by adding 50µl of

Elution Solution followed by spinning the fresh Collection Tube at 12,000g for 1 minute and was stored at -20°C for further use. Knockdowns (KDs) were created by using ribozymes containing purified plasmids for MAGI -1 gene KD. 2 different ribozymes were used for each WT cell line. Electroporation was used to introduce the plasmids into the cells.

4.2.3. Transfection by electroporation

The WT cancer cells were grown in T25 flasks to reach full confluence so that the TJs had formed and were well established. The culture medium was removed, and the flasks were washed with BSS solution twice. 1 ml of TRI Reagent (Sigma-Aldrich) was added to the flask, and the flask was kept in the incubator for 5 minutes. After 5 minutes the detached cells floated in the reagent. The leftover attached cells were detached from the surface by gently tapping on the flask. The cell lysate was transferred into the pre-labelled universal tube, and 4ml of the normal medium was added to make up the total volume of 5ml. The mixture was centrifuged at 1700rpm for 8 minutes to pellet the cells. The supernatant was aspirated, and the pellet was re-suspended in 2 ml of normal medium. 500 micro-litres of the stock was transferred into four pre-labelled electroporation sterile cuvettes for each cell type. These included WT, two different ribozymes containing purified plasmids for MAGI -1 gene (KD1 and KD2), and ribozymes containing empty plasmids (PEF). The WT and PEFs were used as controls. Once the vector was added, 30 seconds were given for dispersion. The cuvettes were sealed by placing the lid back on and were put into the electroporation chamber. Standard settings were used for electroporation. Cells were subjected to electrical pulse 1500 farads and 300 volts. The injured cells were transferred to T25 flasks with 5ml of normal growth medium and were incubated at 37C, 5% CO_2 and 95% humidity.

4.2.4. Stabilisation and maintenance of transfected cells

Following 48 hours of incubation of transfected cells in to normal medium, the medium was changed to selection medium, containing Dulbecco's Modified Eagle's Medium (DMEM) with 10% of FCS (Foetal Calf serum or Fetal Bovine Serum) and antibiotics from the stock (see above) and in addition 250 micrograms of Blasticidin (0.5microgram /ml). The transfected cells were kept in the selection medium for 5 to 7 days. This resulted in the selection of transfected cells and apoptosis of non-transfected cells. The medium was then changed to maintenance medium. Successful KDs were confirmed by microscopy, cell imaging, qPCR, agarose gel PCR and immunofluorescence.

4.2.5. RNA isolation using TRI reagent

Confluent T25 flasks of the desired cell lines were selected, and cell culture medium was aspirated. Flasks were then washed twice with the BSS. 1 ml of TRI reagent was added to the flasks. Cells were scrapped, and cell lysate was passed several times through the pipette to produce a homogenous lysate. The sample was transferred to a sterile Microfuge tube and allowed to stand at room temperature for 5 minutes. 0.2ml of chloroform TRI reagent were added to each tube and shaken for 15 seconds and were left to stand at room temperature for 2-15 minutes, then centrifuged at 12000 for 15 minutes at 4◦C. Three phases appeared. The bottom red phase was the protein. The In-between white phase was DNA and the clear top phase was the RNA. The clear top phase was carefully transferred to a fresh Microfuge tube. 0.5 ml of isopropanol was added to the Microfuge tube, mixed by shaking and left for 5-10 minutes at room temperature, then centrifuged at 12000 for 10 minutes at 4C. A pellet appeared at the base (not always clearly visible). The supernatant was discarded. The pellet was washed with 75% EtOH which was made up of RNA-ase free water and then centrifuged at 7500 for 5 minutes. 75% EtOH was removed, and the sample was air dried for 5-10 minutes.

The pellet was dissolved in 20 micro-litres of RNA-ease free water. RNA concentration was measured on the nano-photometer. The lid factor used for most samples was 50. A lid factor of 10 was used for more concentrated samples, and 100 was used for more diluted samples. Dilution of concentrated samples was achieved by gradually adding ten micro-litres of RNAase free water and measuring the new concentration. Few of the RNA samples were stored at -80C freezer and others were used for direct reverse transcription (RT).

4.2.6. RNA quantification

The concentration of RNA extracted from the cells was measured using a UV101 Biotech Photometer (WPA, Cambridge, UK), which was set to detect single strand RNA in micrograms per micro-litres at 260 nanometers. Samples were measured by using Starna glass cuvette (Optiglass limited, Essex, UK).

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

Once RNA had been isolated and quantified, RT-PCR was carried out to convert RNA into complementary DNA (cDNA) using DuraScript™ RT PCR kit (Sigma-Aldrich, Dorset, UK). A fixed amount of 0.5 micrograms of RNA was converted to cDNA as per protocol. Standardized RNA concentrations were achieved by adding RNA-ease free water. The dilution amount of RNA-ease free water was calculated by 0.5 divided by the RNA concentration measured by the nano-photometer from earlier. Once diluted to standardised concentration forms, the new RNA concentration was measured on the nano-photometer. The Abgene reverse transcription kit was used for RT PCR. The following volumes were used:

Volume reaction was 20 micro-litres:

- PCR water was added to RNA to make volume 10 micro-litres
- ten micro-litres of AB RT Mastermix 2X

All of above were added to a dome-capped Microfuge tube's and placed into a thermo-cycler (Applied GeneAmp PCR sys 2700). Each reaction was run as per the following protocol:

Stage 1: 25◦C for 5 minutes

Stage 2: 37◦C for 120 minutes

Stage 3: 85◦C for 5 minutes

Stage 4: held at 4◦C

The final product was diluted with PCR water in 1:3 resulting in a final volume of 80 micro-litres, which was then labelled and stored at -80◦C.

4.2.8. Real-time quantitative polymerase chain reaction (q-PCR)

q-PCR was undertaken to analyse the transcript expression level of target genes. Such a technique is more sensitive than conventional RT-PCR and capable of detecting small quantities of cDNA, providing a more reliable value of the template copy number in each sample. This method requires a sequence-specific DNA based fluorescent reporter probe which only quantifies the target transcript containing such a probe sequence to greatly improve the specificity of the detection. Amplifluor™ Uniprimer™ Universal system (Intergen company®, New York, USA) was available at the home department and was used in this study. The Amplifluor probe consisted of a 3' region specific to the Z-sequence, which acted on the target-specific primers and a 5' hairpin structure labelled with a fluorescent tag (FAM). While in the hairpin structure, such a fluorescent tag was

linked to an acceptor moiety (DABSYL) which effectively quenched the fluorescence and produced no fluorescence signal. Primers were designed to the specific target transcript using Beacon Designer Software. To each of the primer pairs specific to the target sequence, a Z-sequence was added to allow incorporation of the Amplifluor uni primer probe during PCR. Such Z-sequence incorporated a DNA transcript that acted as a template for the Amplifluor uni primer probe, and the hairpin structure in the probe was subsequently degraded and unfolded by DNA polymerase disrupting the stable structure between fluorophore and quencher to enable sufficient fluorescence emission to be detected. The principle of this process was demonstrated in figure 18. Pre-prepared cDNA samples were amplified and detected on a Step One Plus Real-Time PCR System (Applied Biosystems, Paisley, UK), using Precision FAST 2X q-PCR Master-Mix (Primerdesign Ltd, Chandler's Ford, UK), specific forward primer (10pM), reverse primer containing the Z sequence (1pM) and the FAM-tagged Uni-primer probe (10pM). Each sample was loaded into a 96-well plate and the reaction mixture. The q-PCR conditions used are shown below:

q-PCR Reaction mix:

- Precision FAST 2X q-PCR MasterMix - 5µl
- Specific forward primer - 0.3µl
- Specific reverse primer with Z-sequence - 0.3µl
- Amplifluor probe – 0.3µl
- PCR H_2O – 3.1µl
- Prepared cDNA sample - 1µl

q-PCR conditions:

- Step 1: Initial denaturation - 94°C for 10 minutes
- Step 2: Denaturation - 94°C for 10 seconds
- Step 3: Annealing - 55°C for 30 seconds
- Step 4: Extension - 72°C for 10 minutes

Step 2 - 4 were repeated for 100 cycles.

The fluorescent signal was detected at the annealing stage, and the fluorescent signal emitted during PCR correlated to the amount of DNA template that had been incorporated into Z- sequence. The geometric increase in fluorescent signal directly correlated to the exponential increase of DNA template, which was used to determine a threshold cycle for each reaction. The transcript copy number was determined by the time/cycle number when fluorescent signal reached a specific threshold. Expression of the target sequence was detected in conjunction with a range of standards of the known transcript, PDPL, that are used to generate a standard curve to enable the calculation of transcript copy number in each unknown sample (Figure 2.2.11.1 and 2.2.11.2). Finally, the transcript copy number was normalised by the detected copy number of GAPDH.

4.2.9. Agarose gel electrophoresis, DNA staining and visualisation

The amplified DNA products from the PCR were analysed using 2% agarose gel electrophoresis. The analysis was based on the separation of DNA products according to their size on electrophoresis. The procedure was as follows:

4.2.10. Formation of 0.8% agarose gel

3 grams of agarose (Melford chemicals, Suffolk, UK) was added to 150 ml of TBE solution in a flask. The mixture was heated in the microwave to boiling temperature, the agarose was fully dissolved, and the mixture became transparent in colour. Then the gel was left to cool down on room temperature just for a few minutes, and ten micro-litres of the cyber safe was added into it, following which the liquid gel was poured down into the electrophoresis cassette. Plastic combs were already inserted into the electrophoresis cassettes, and the gel was allowed to settle down at room temperature, which took between 30-40 minutes. The plastic combs were then carefully removed, leaving indentation slots for loading of the PCR products for later on.

4.2.11. TBE Buffer

Once the gel was set, TBE buffer was carefully pour into the electrophoresis tank, directly on top of the gel, filling up to the maximum fill mark on the tank.

4.2.12. Loading gel with PCR products

Five micro-litres of 100 Kb DNA ladder (Invitrogen, Paisley, UK) was loaded into the first indentation slot which was used as a reference guide in measuring of the DNA products once the electrophoresis was completed. Ten micro-litres of the PCR products were carefully loaded into the rest of the indentation slots on the gel.

4.2.13. Electrophoresis

The electrophoresis cassette was connected with the power supply by its leads, making sure that the flow of the charge/current was in the right direction, that is, from the positive to the negative electrode, which separated the DNA products out on the gel. A

consort EV 243 electrophoresis device was used. The following setting was applied.

Current : 110mA

Voltage: 90V

Time: 42 minutes.

4.2.13.1. Detection of spread of PCR products using UV light

The TBE was poured away from the gel, and the gel was placed inside the UV light container (UVTech. Cambridge, UK). The degree of spread of PCR products was captured by using UV camera in the UV Tech. The light settings were optimised.

4.2.13.2. Immunofluorescent Staining of Cells

The immunofluorescence technique is used for light microscopy with a fluorescent microscope. The technique uses specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets in a cell, which allows visualisation of the presence and distribution of the target molecule. 30,000 cells were seeded into each chamber of 8 chamber slides x 8 (Millipore). The cells were incubated at 37C in 5% CO2 overnight to achieve confluence and development of tight junctions. The media was aspirated, and cells were fixed in 4% formalin for 10 minutes. The cells were rehydrated in BSS for 20minutes at room temperature. Cells were then permeabilised with 1% Triton x 100 made (made in BSS) for 5 minutes. 5% serum or TBS diluent was prepared while the cells were getting fixed and washed, which was used to dilute the primary and secondary FITC/TRITC reagents as well as blocking any non-specific staining. Each good chamber was covered with 150µl to cover the whole layer. The serum used in the diluent was from the animal in which the secondary was made.

Non-specific binding was blocked by covering the cells with the diluent and incubated at RT for 1 hour. The reagents were added very carefully on the side walls of the chamber, thus preventing any accidental detachment of cells. It was washed three times with TBS. Incubation was done with primary antibody at the concentration of 1:100 (made in dilute) for 1 hour. The following antibodies were used.

Primary antibodies used were as follows:

MAGI-1	SC – 25663	Rabbit (Santa Cruz Biotechnologies)

Secondary antibodies used were as follows:

Anti-rabbit IgG	Sigma Aldrich	Rabbit (for MAGI-1)

50µl of the primary antibody (Rabbit) was added per 5 ml of solution, and secondary antibody concentrations were achieved by adding 5µl per 5ml solution for the anti-rabbit antibody. The negative control was diluted with no primary antibody added. The wash was repeated for another three times with TBS. Appropriate sections were incubated with FITC / TRITC solution (stock = 4mg/ml, used 0.5µl per 100µl, and gave a final concentration of 2µg/100µl). TBS wash was repeated times 3. The plastic frame from the slide was removed, and a coverslip was mounted over the cells using FluorSave™, avoiding any air bubbles. Slides were placed on a tray, wrapped with foil to prevent any fading, and store in the fridge until were viewed under the fluorescent microscope.

4.3. Expression pattern of MAGI -1, -2 and -3 proteins in colorectal cancer cell lines

Our experiments were consistent with the positive expression of MAGI-1 and MAGI-3, but lacked expression of MAGI-2, in colorectal cancer cell lines (HT -115, RKO and HRT -18) on gel electrophoresis, which had profound implications on our work. Gastric cancer cell lines (AGS and HGC -27) were used as controls for expression of MAGI -2 (Figure 4.3.1). Therefore, our further work became more focused on studying the role of MAGI -1 in invasiveness of human colorectal cancer. If time had allowed, we would also have continued studying the role of MAGI -3 in invasiveness of human colorectal cancer cell, hence studying all three known types of MAGI proteins.

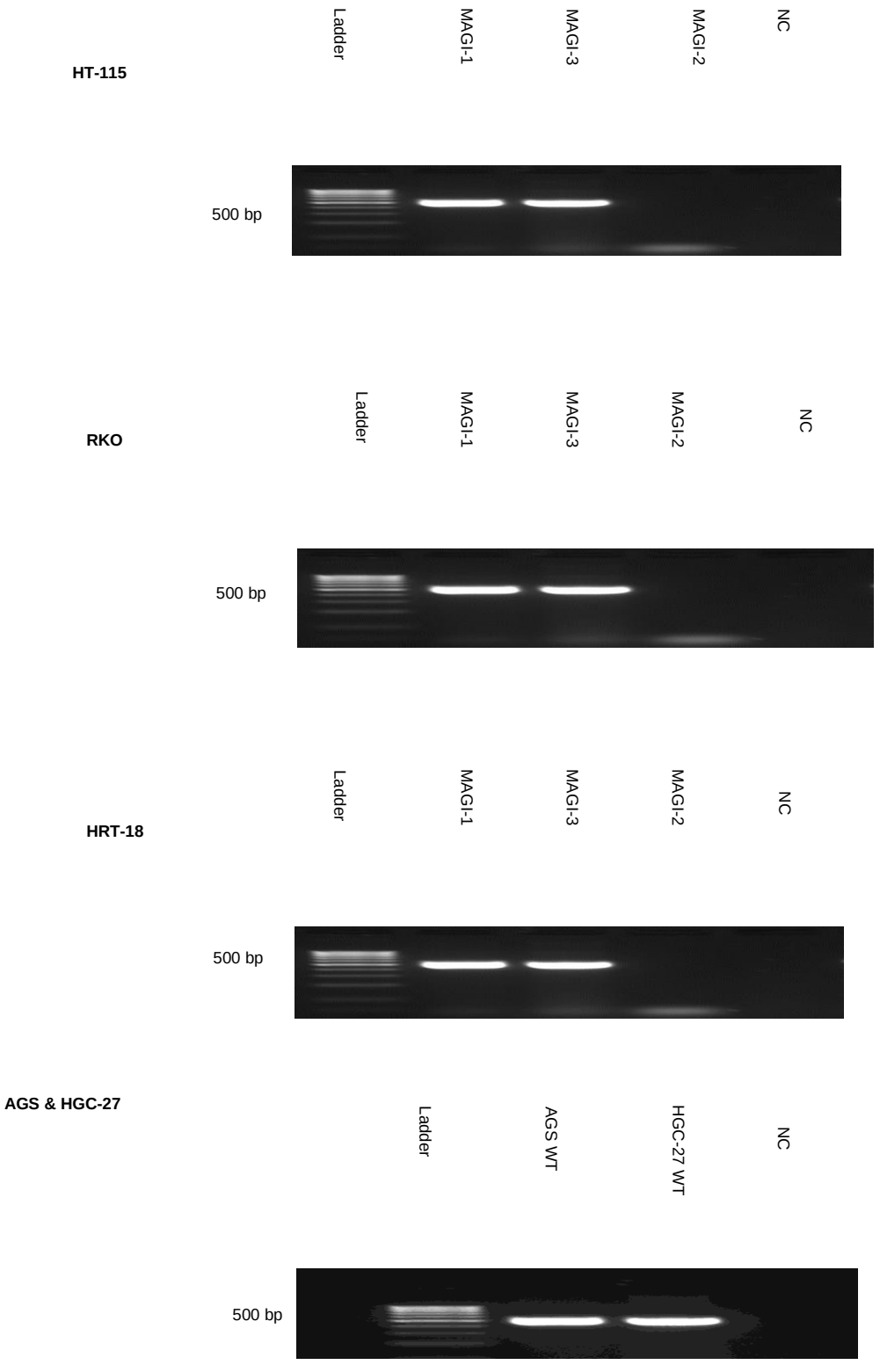


Figure 4.3.1. Expression of MAGI -1-2-3 in colorectal cancer cell lines (HT-115 WT, RKO WT, HRT-18 WT) which shows lack of MAGI -2 expressions. Gastric cell lines (AGS & HGC-27 WT), which expressed MAGI -2, were used as controls.

4.4. Evidence of MAGI -1 KDs on agarose gel electrophoresis

Successful MAGI -1 KDs were confirmed agarose gel electrophoresis in HT-115 and RKO cell types of colorectal cancer cell lines, but not in HRT-18 cell lines, which continued to show MAGI -1 expression (Figure 4.4.1) and were used as controls for HT-115 and RKO cell lines. The failure of KDs in HRT -18 cell lines could be due to individual variations characteristics of this cell line but provided us with the advantage of using it as controls.

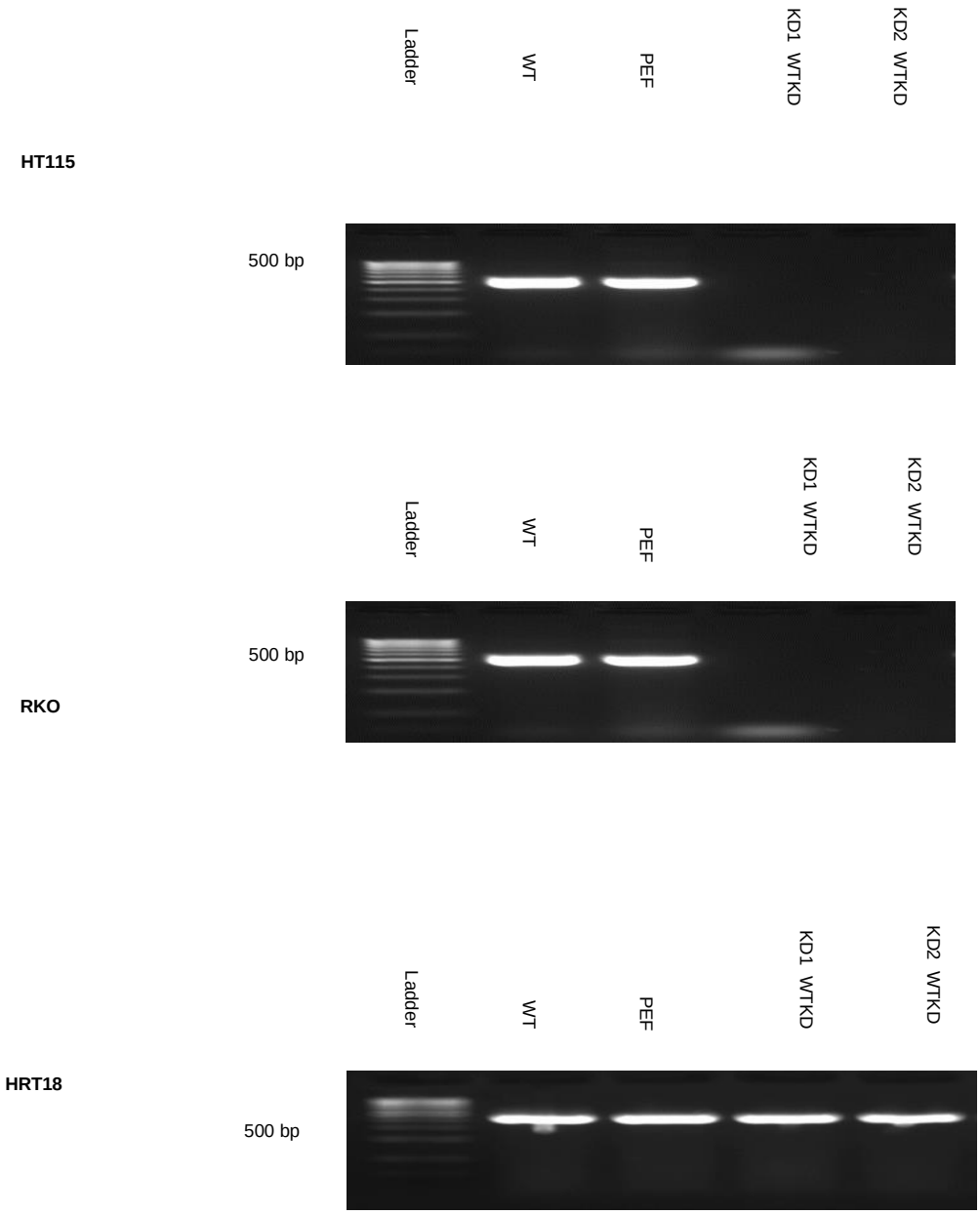
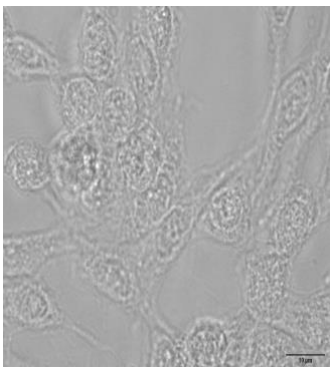


Figure 4.4.1. Expression of MAGI -1 in HT-115 WT and HT-115 MAGI-1 KDs, in RKO WT and RKO MAGI-1 KDs, in HRT-18 WT and HRT-18 MAGI-1 KDs

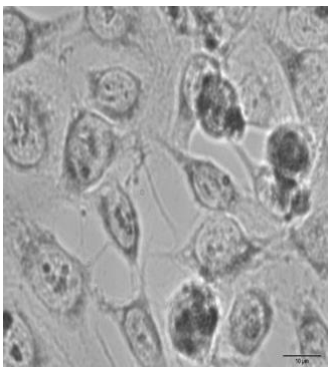
4.5 Colorectal cancer cell lines morphology and changes in morphology after MAGI-1 KD

The cell morphology and growth pattern of all three colorectal cancer cell types were visualised using a high-resolution microscope, and images were captured. Changes in the growth pattern and cell morphology were noticed between the WT and KDs of individual colorectal cancer cell lines (Figure 4.4.2).

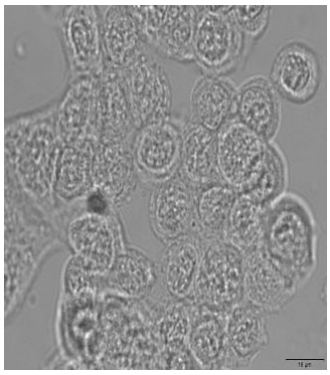
RKO WT



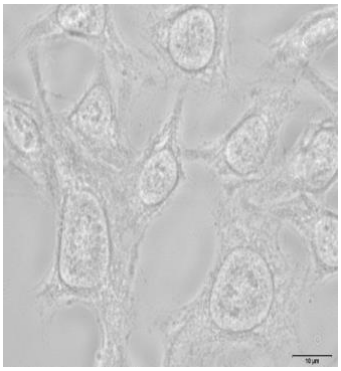
RKO MAGI -1KD



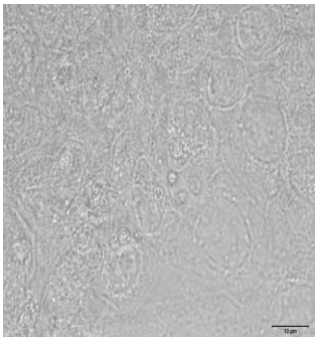
HT-115 WT



HT-115 MAGI -1 KD



HRT-18 WT



HRT-18 MAGI – KD

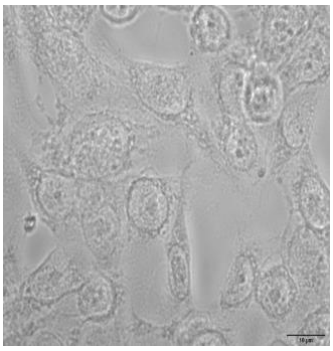
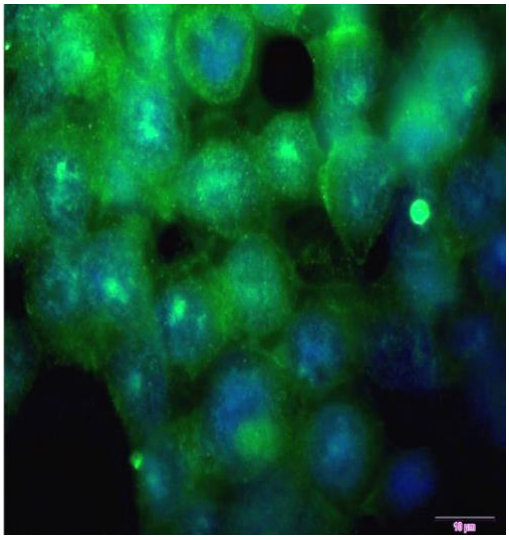


Figure 4.4.2. Morphology comparison between RKO-WT and RKO MAGI -1 KDs, HT-115-WT and HT-115 MAGI -1 KDs, HRT-18-WT and HRT-18 MAGI -1 KDs on high-resolution microscopy.

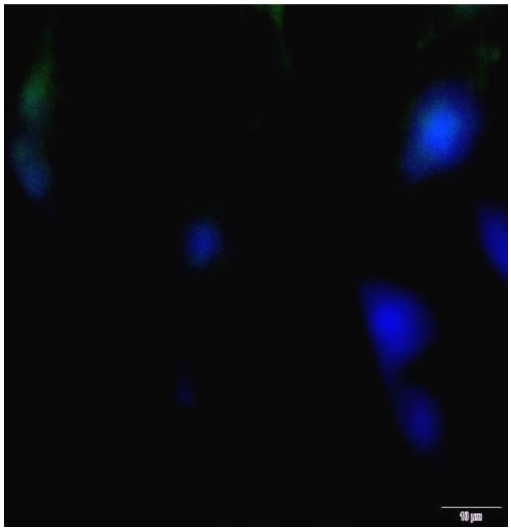
There were notable changes macroscopically and microscopically to the cell morphology and growth patterns between the three colorectal cancer cell types (HT-115, RKO & HRT-18). More importantly, significant changes in the growth pattern were noted between the wild-type colorectal cancer cell lines and their corresponding KDs. The HRT-18 cells had a slower growth rate than the other two cell lines (HT-115 & RKO) and were more adhesive in character. In the flasks, on naked eye appearance, the WT were quicker to grow and would spread in a uniform pattern on the wall of the flask, whereas the KDs would grow in clumps at a slower rate, across the board for all the three cell lines (HT-115, RKO & HRT-18). This was assumed secondary to the transfection and due to use of selection medium in the initial phase. There were also significant morphological differences between the wild types and the KD cell lines. The cells grown following MAGI -1 gene were knocked down, appeared to be spiky and in clusters, whereas the WT were noticed to be more rounded and uniform distribution. The differences were noted to be less apparent in the HRT-18 cell lines, possibly be due to their slower growth and more adhesive nature.

4.5. Absence of TJs on Immunofluorescence between cells as evidence of successful KDs

The slides were prepared with cells and were viewed under the fluorescent microscope. Immunofluorescence staining of TJs between cells and viewing them on the microscope demonstrated the presence of TJs in WTs and their absence in MAGI -1 KDs in HT115 and RKO, but to a lesser degree in HRT18 cell lines (Figures 4.5.1, 4.5.2 and 4.5.3). Immunofluorescence staining of TJs between cells for ZO -1 -2 & -3 were used as controls, which had no or minimal loss of TJs by knocking down MAGI -1 gene (Figures 4.5.4, 4.5.5 and 4.5.6). Individual cell lines HT-115 WT and HT-115 MAGI-1 KDs showed cells absence of TJs in the KDs. RKO WT and RKO MAGI-1 KDs also showed the absence of TJs in the KDs. HRT-18 WT and HRT-18 MAGI-1 KDs showed weakness, but not complete loss of TJs in the KDs (Figures 4.5.1, 4.5.2 and 4.5.3). ZO-1, ZO-2, and ZO-3 were used as controls. HRT-18 WT and HRT-18 MAGI-1 KD cells on fluorescent microscopy demonstrating some minimal loss of TJs in ZO-1s. RKO WT and RKO MAGI-1 KD cells on fluorescent microscopy demonstrating no or minimal loss of TJs in ZO-1s. HT-115 WT and HT-115 MAGI-1 KD cells on fluorescent microscopy demonstrating no loss of TJs in ZO-1s (Figures 4.5.4, 4.5.5 and 4.5.6).

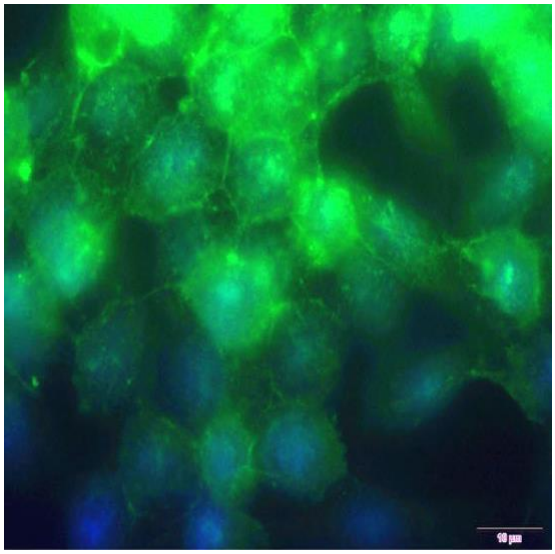


HT-115 MAGI -1 WT

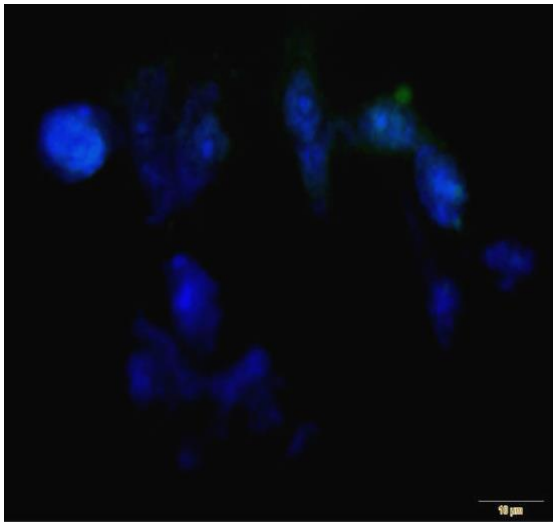


HT-115 MAGI -1 KD

Figure 4.5.1. Immunofluorescence of HT-115 WT and HT-115 MAGI-1 KD cells on fluorescent microscopy destrating presence of TJs in WTs, but in contrast shows absence of TJs in the KDs.

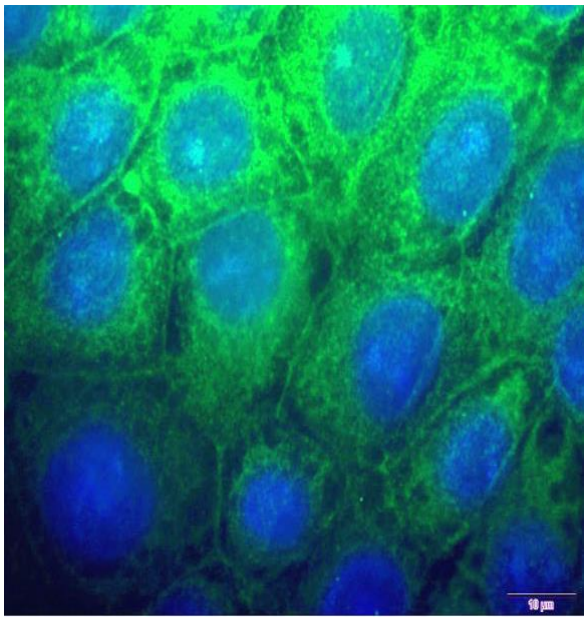


RKO MAGI -1 WT



MAGI-1 KD

Figure 4.5.2. Immunofluorescence of RKO WT and RKO MAGI-1 KD cells on fluorescent microscopy demonstrating presence of TJs in WTs, but in contrast shows absence of TJs in the KDs.



HR-18 MAGI -1 WT

HRT-18 MAGI -1 KD

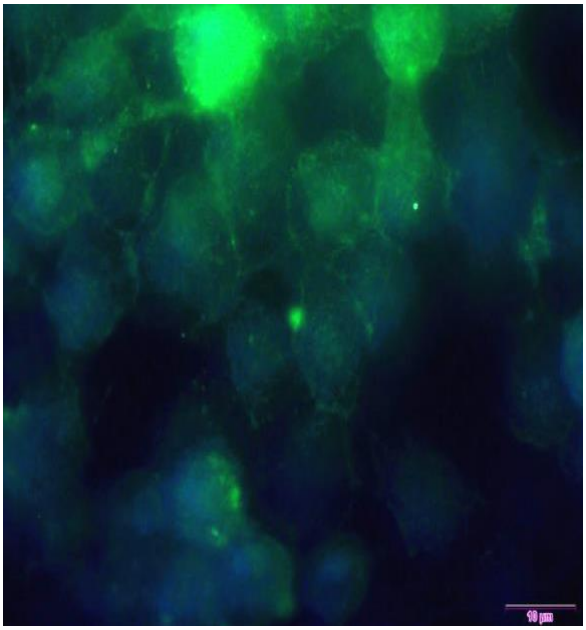
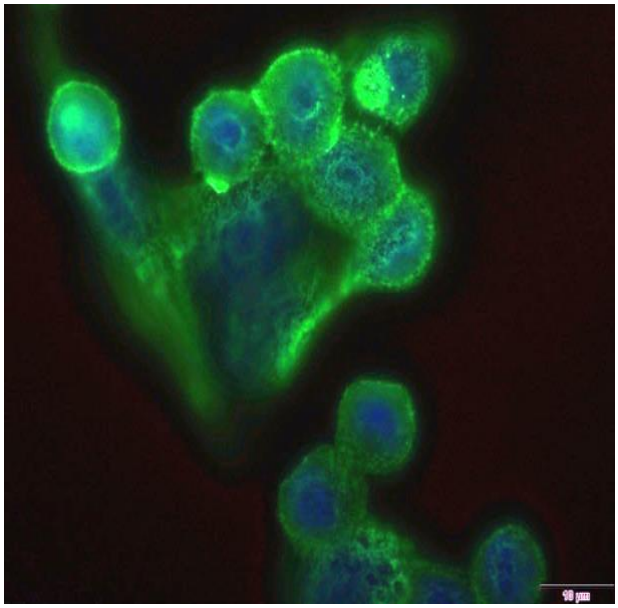
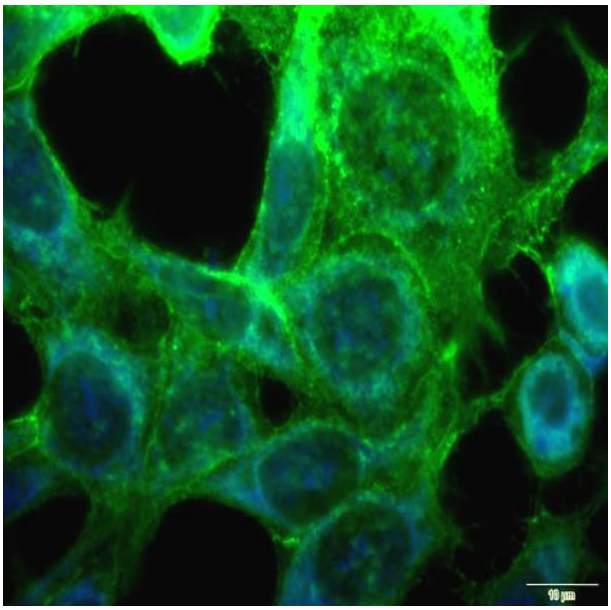


Figure 4.5.3. Immunofluorescence of HRT-18 WT and HRT-18 MAGI-1 KD cells on fluorescent microscopy demonstrating presence of TJs in WTs but in contrast shows only weakness, but not loss of TJs in the KDs.

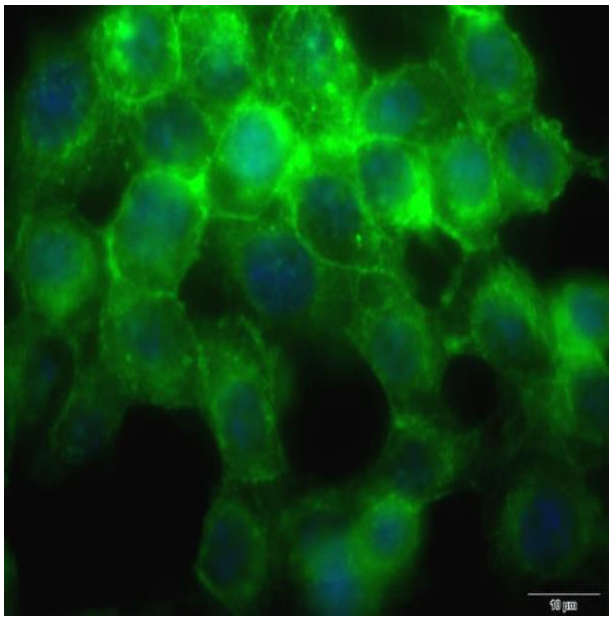


HT-115 ZO -1 WT

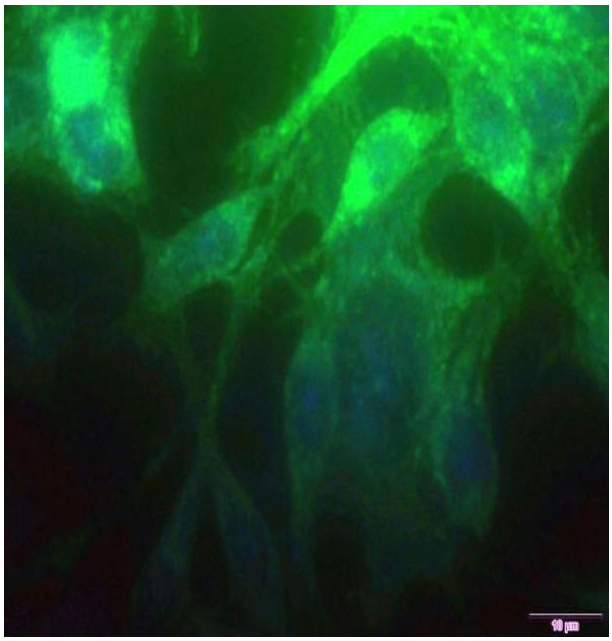


HT-115 ZO-1 MAGI -1 KDs

Figure 4.5.4. Immunofluorescence of HT-115 WT and HT-115 MAGI-1 KD cells on fluorescent microscopy demonstrating presence of TJs in WTs but also in contrast shows no loss of TJs in ZO-1s.

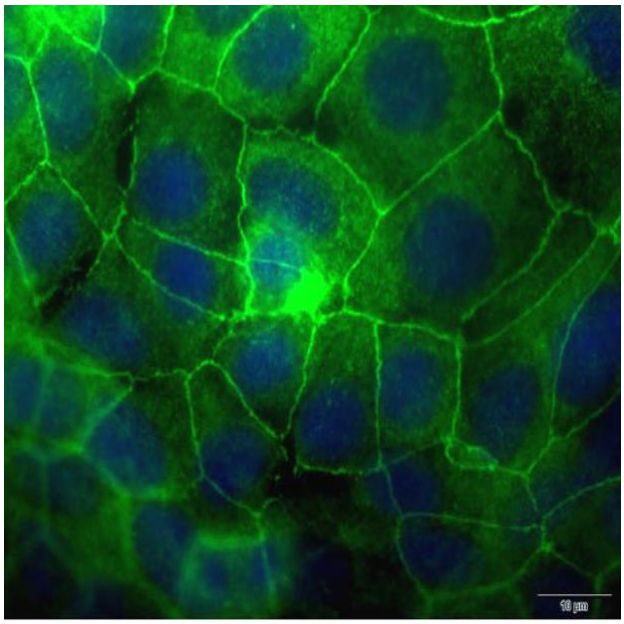


RKO ZO-1 WT

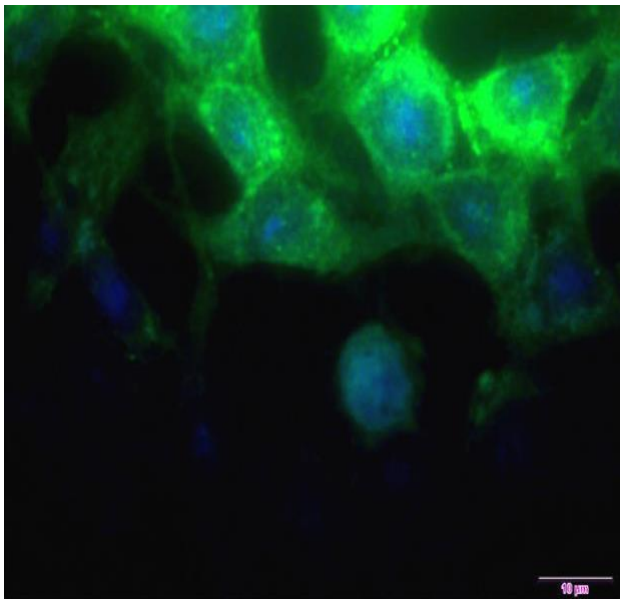


RKO MAGI-1 KDs

Figure 4.5.5. Immunofluorescence of RKO WT and RKO MAGI-1 KD cells on fluorescent microscopy demonstrating presence of TJs in WTs but also in contrast shows no or minimal loss of TJs in ZO-1s.



HRT-18 ZO -1 WT



HRT-18 MAGI -1 KDS

Figure 4.5.6. Immunofluorescence of RKO WT and RKO MAGI-1 KD cells on fluorescent microscopy demonstrating presence of TJs in WTs but also in contrast show minimal loss of TJs in ZO-1s.

4.6. Discussion:

MAGI -1 is widely expressed and found to be localised in epithelial and endothelial cell-to-cell Tight Junctions (TJ), and acts as a scaffolding molecule to stabilise cadherin-mediated adhesions and to recruit molecules in TJ. (Laura *et al.* 2002). Stromberg *et al.* reported that invasiveness of colorectal cancer cells had a co-relation with cell adhesion proteins and polymorphism in their genes. (Stromberg *et al.* 2008). Landy *et al.* suggested that the dysregulation of TJs proteins preceded the development of IBD associated colorectal cancer (CRC). (Landy *et al.* 2016). Zaric *et al.* reported an increased expression of MAGI -1 was seen to suppress migration and invasion of the cancer cells. It was also seen that knocking down MAGI -1 decreased the localisation of E-cadherin at the cell to cell junction, increased disruption of stress fibres and focal adhesion points resulting in increased migration and invasion. Inversely, over-expression of MAGI -1 suppressed the cancer growth and metastasis in colorectal cancer cells. (Zaric *et al.* 2012)

We had a lack of MAGI -2 expression in on qPCR and Agarose gel electrophoresis in colorectal cancer cell lines (HT-115, RKO, HRT18), which was consistent with the literature, as MAGI -2 is not found to be expressed in human colorectal cancer in the literature. Human gastric cancer cell lines (AGS, HGC -27) were used as controls for expression of MAGI -2. This had a profound implication on our work, and the further focus of our work shifted to concentrate on the role of MAGI -1 proteins in invasiveness of human colorectal cancer cell lines. However, MAGI -3 also remained of high interest to us, but due to the time limitation, further experiments was not carried out on MAGI -3, and as mentioned before, the focus of our work was concentrated on MAGI -1. In fact, MAGI -2 and MAGI -3 remains an extremely high area of interest for future, and if time would have allowed, we would certainly have had explored these two. The reason for us

choosing RKO, HT-115 and HRT-18 cell lines in colorectal cancer cell types was because of their high invasive and metastatic potential.

To study the effects of aberrant expression of MAGI -1 in human colorectal cancer, we created a different type of cell lines with different expression levels of MAGI -1 proteins. We did this by generating colonies of human colorectal cancer cell lines in which we knocked down MAGI -1 genes. Hence these cell colonies were not able to produce MAGI-1 proteins. We were able to prove our success in the creation of KDs by the lack of MAGI -1 expression in RKO and HT-115 cell lines following Q-PCR and agarose gel electrophoresis. The experiments were repeated which showed consistency in the results. However, HRT-18 KD cells continued to have some expression of MAGI-1, which was either due to their slow growing and more adhesive nature when compared to the other two cell lines or due to unsuccessful MAGI-1 KD in them. The similar differences in the behaviour were observed between the all three colorectal cancer cell lines (RKO, HTT-115 & HRT-18) in morphology and functional studies (discussed in following chapters). The changes were distinctive macroscopically and microscopically to the cell morphology and growth patterns between the individual colorectal cancer cell lines (HT-115, RKO & HRT-18). Once the KD cell lines were generated, significant changes in morphology and growth pattern between the wild types colorectal cancer cell lines and their corresponding KDs were noted. To the naked eye in the flasks, the WT cells were quicker to grow and would spread in a cluster pattern on the wall of the flask, whereas the KD cells would grow in uniform pattern and at a slower rate, across the board for all the three cell lines (HT-115, RKO & HRT-18) used in our experiments. This was assumed secondary to the transfection and due to use of selection medium in the initial phase. The cells were grown following MAGI -1 gene knocked down, and appeared to be spiky

and uniform, whereas the WT were noticed to be more rounded and more of cluster distribution. The differences were noted to be less apparent in the HRT-18 cell lines, could be due to their slower to grow and were more adhesive nature. Immunofluorescence staining showed the presence of TJs in WT cells and their absence in HT115 and RKO MAGI -1 KD cells. This was noticed to a lesser degree in HRT18 MAGI -1 KD cell lines. ZO -1 -2 & -3 were used as controls, which had no or minimal loss of TJs by knocking down MAGI -1 gene.

The results of functional tests which we carried out later (discussed in the following chapters) were consistent with our initial findings of MAGI -1 expression in different colorectal cancer cell lines (HT -115, RKO and HRT -18).

4.7. Conclusion:

We concluded that human colorectal cancer cells lacked expression of MAGI -2. We had evidence of successful MAGI -1 KDs from differences in the morphology of cells between WTs and KDs. We also had evidence of successful KDs from lack of expression of MAGI -1 in the KDs. We had evidence to suggest that reduction in MAGI -1 expression has a profound effect on the strength of presence of TJs in human colorectal cancer.

ROLE OF MAGI PROTEINS IN INVASIVENESS OF HUMAN COLORECTAL CANCER CELLS.

CHAPTER 5: INFLUENCE OF TARGETING MAGI -1 ON ADHESION, PROLIFERATION AND MIGRATION IN HUMAN COLORECTAL CANCER CELLS

5. Introduction

MAGI-1 is widely expressed in the epithelial and endothelial cell-to-cell tight junctions (TJs), where it acts as a scaffolding molecule to stabilise cadherin-mediated adhesions and to recruit molecules in TJ. (Dobrosotskaya *et al.* 1997, Laura *et al.* 2002). Roles attributed to MAGI include organisation of protein complexes at the cell and synaptic junctions, regulation of cell polarity, connecting trans-cellular proteins with the cytoskeleton in TJs. (Martin *et al.* 2011) Stetak *et al.* reported that the knockdown of MAGI protein causes a loss of junctional compartmentalisation along the lateral membrane, and reduces the overall robustness of cell-to-cell adhesion. (Stetak *et al.* 2010).

Metastatic spread of cancer is not uncommon, and it directly impacts on the patient's survival. Cancer metastasis is a series of complex events, involving the initial dissociation of malignant cells from a primary tumour that happens by the loss of cell adhesion between cells, which subsequently leads to invasion of the cells to the surrounding stroma. Hence the process of angiogenesis begins involving specific proteins expression and suppression, degrading basement membrane and extracellular matrix and also affecting the cell motility and migration (Brooks *et al.* 1996). For cancer cells to metastasize successfully, TJs must be disrupted. TJs is a critical structural adhesive component that exists, firstly between the cancer cells which prevent detachment, secondly between the endothelial and epithelial cell linings which prevent intravasation and extravasation of cancer cells (Martin *et al.* 2011).

5.1 Aims

We aimed to study the effect of MAGI -1 KD on adhesion, proliferation and migration in colorectal cancer cell lines. We also looked at the effect of MAGI -1 KDs on barrier function.

5.2 Materials and methods

5.2.1 Cell Lines:

HT -115, HRT -18 and RKO; human colorectal cancer cell lines were used. The cells were obtained from the European Collection of Animal Cell Culture (ECACC). (Table 5.2.1.1).

Table 5.2.1.1. Specification of colorectal cancer cell lines

Cell line	HHT -115	HRT -18	RKO
Species	Human	Human	Human
Tissue	Colon	Rectum	Rectum
Age	63	67	82
Gender	Male	Male	Female
Morphology	Epithelial	Epithelial-like	Epithelial
Growth mode	Adherent	Adherent	Adherent
Karyotype	2n=46	2n=46	2n=46
Tumorigenic	Specified	Yes	Yes
Country	UK	UK	UK

5.2.2 Electrical cell impedance sensing (ECIS)

Electrical cell impedance sensing (ECIS) is a technique for analysing cell behaviour based on work of Giaever and Keese, who found that it was possible to monitor the growth, migration and adhesion of cells growing on a small electrode by monitoring changes in impedance of the cells, that is, resistance to an alternating current) (Giaever and Keese 1984). The ECIS apparatus was supplied by Applied Biophysics (New York, USA). A 96 well plate array specific to the ECIS machine was used (IBIDI, Germany). Each well inside the 96 well plate has a unique design. It is built around two small gold electrodes, in the bottom of the wells in which the seeded cells are allowed to grow within the incubator. These electrodes work as sensing the impedance or resistance between the cells and the cell and the bottom of the array. Each Individual well is a part of plate array, which consists of a plastic housing encasing an integrated circuit incorporating all the electrodes included in the array. An array can contain various numbers of wells and types of plates, but a 96 well plate is the most widely used configuration. The plate slots into a dock, in the incubator, which connects it to the ECIS machine. The incubator in the apparatus does not provide carbon dioxide; therefore, DMEM used has to contain HEPES to compensate. (Figure 5.2.2.1)

5.2.2.1 ECIS: Procedure

The protocol for the procedure was available in the home department (Jiang *et al.*), which had been used extensively in the past for study of tumour cell behaviour (Keese, Wegener *et al.* 2004; Jiang 2012) (Figure 5.2.2.1.). A series of these experiments were performed in 96 well ECIS plates. All the 96 plates used were new, straight out of the packet inside the tissue culture hood, maintaining conditions as sterile as possible. 200 micro-litres of the normal medium was added to each of the 96 wells on the plate. The

top cover of the 96 well plate were placed and secured in position by manual pressure while was being transferred from tissue culture to the ECIS machine. ECIS machine which connected to a computer, which runs the ECIS software and displays the graph by the cell impedance changes.

HRT-18 WT, HT-115 WT and RKO WT were the wild types used with their respective HRT-18 PEF, HT115-PEF and RKO PEF. Amongst the transfected types, two knockdowns were created and verified for each cell type, labelled as HT-115 KD1 and KD2, RKO KD1 and KD2 and HRT-18 KD1 and KD2. The cell flasks were trypsinised and centrifuged at 17000 revolutions per minute (RPM) for 5 minutes in universal containers (UCs), following which they were re-suspended in 1 ml of medium. Cell dilution was measured by using the following.

http://www.celeromics.com/en/support/cell-dilutio-calculator.php

Each well was seeded with 100,000 cells in 200 micro-litres. Once final dilution volumes were measured, they were added to the ECIS plate, and the plate was taken back and put into the ECIS machine. Once the plate was fixed firmly into position inside the ECIS machine, the plate electrode connection with the machine electrodes was checked, which showed all the wells lit up in the green colour, meaning a good connection between electrodes had established. The incubator settings were set to 37°C and 95% humidity. The experiment was then started and delayed wounding was enabled from options and the setting was set for eight hours for colorectal cancer cell lines and 12 hours for gastric cancer cell lines. The current used for wounding was 3000 micro-amperes at a frequency of 4000 hertz with the time set to 20 seconds. The cell's

impedance and resistance measurements were displayed on the screen off the monitor and were recorded for the all-new addition of the experiment and were also saved for a later reference. The average of the percentage of increase in impedance of the cells in the initial hours was calculated to study cell adhesion. Similarly, the average percentage of increase after a successful wounding to the cells was used to calculate the conventional migration essay. Once the experiment was completed, used plates were carefully washed with bleach, and disposed of in the yellow clinical waste bins. In the end, the ECIS program was shut down, and the computer was switched off. The incubator was cleaned from inside with 70% ethanol. That experiment was repeated three times for each colorectal cancer and gastric cancer cell line.

The problems encountered during the ECIS experiments were to make sure that there were no quality control issues. It was realised that contamination could affect the quality of the readings generated inside the wells. Great care was taken to prevent any infection or contamination of the cells inside the wells. We were also aware that sometimes air bubbles inside the wells could lead to defective functioning of the electrode sensors, so great care was taken to avoid it. Several repetitions of the ECIS experiment were undertaken to make sure that the readings were consistent and any errors were filtered out and were not included in the final analysis.

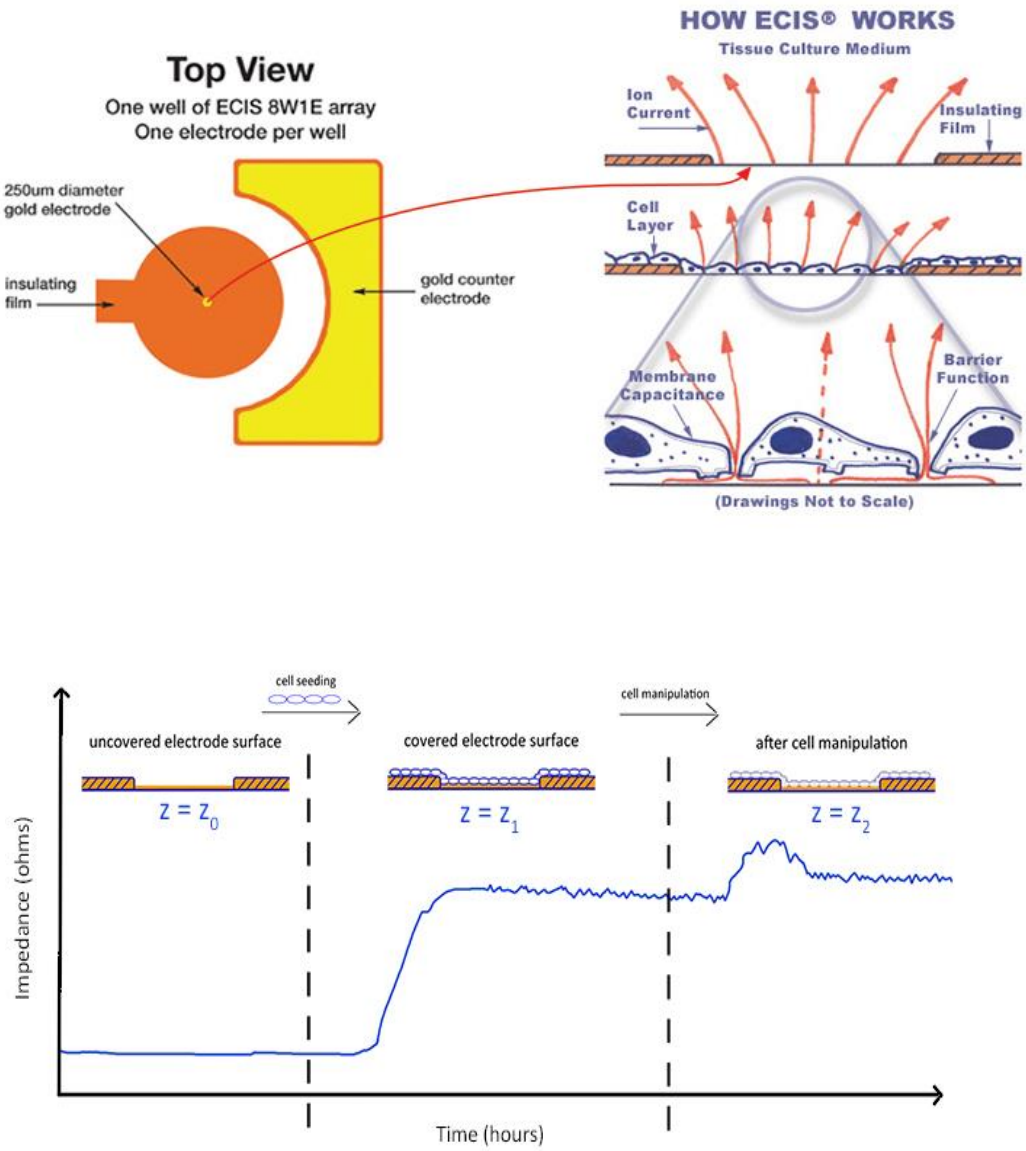


Figure 5.2.2.1. Electric cell impedance sensing (ECIS) and its mode of operation.

The ECIS machine provides detailed readings on cell adhesion and growth, which is recorded in real time. Also, there is provision for inflicting the cells with a uniform wound by electroporation, thereby providing further detail of their migratory function. The home department not only has the largest array of ECIS machines in the country but also has published one of the most useful texts regarding its uses (Jiang 2012).

(http://www.biophysics.com/ecis-theory.php).

5.2.3 Trans-epithelial resistance (TER)

Trans-epithelial or trans-endothelial resistance (TER) is an excellent method for assessing barrier function of the reconstructed epithelial of the grown cell lines. It has become a method of assessing cell-to-cell tight junction function in vitro. The cells are cultured over a porous membrane and grown to become dense, forming cell-to-cell tight junctions and establishing cellular polarity. With the help of the chopstick type of electrodes in the apparatus, the electrical resistance across the barrier between the cell layers can be determined by applying a small AC voltage with varying frequency.

5.2.4 TER: Procedure

The cells were trypsinised and re-suspended in DMEM, and cell count performed and cells diluted to 100,000 per 500 micro-litres. The cell mixture was added to the transparent 0.4 micro-metre pores sized inserts (Greiner bio-one, Stonehouse, UK). The 0.4 micro-litre sized pores membrane acts as a medium for selective diffusion. The inserts were placed inside 24 wells plates. 400 micro-litres of the cell mix was added to the inserts and 1000 micro-litres to the wells, containing the inserts. The cells were grown to confluence. The medium is replaced with fresh DMEM containing 15Mm Hepes, L-glutamine. (Lonza Laboratories, Verviers, Belgium). Medium alone was added to the base of the well (controls) (Martin *et al.* 2008). The classical way of measuring TER is by the use of handheld electrodes (chopstick-type electrodes). The electrode set up allows cell layer impedance measurements. We used the same method by using hand held static electrodes (WPI, FL, USA) and EVON volt-ohmmeter (EVON, World Precision Instruments, Aston, Herts, UK) (Martin *et al.* 2004a). Both electrodes were placed inside the well, one inside the insert and the other one outside the well. The resistance was measured across the colorectal cancer cell lines HRT-18 WT, HT-115 WT and RKO WT

were the wild types used with their respective KDs at intervals from 0 to 240 minutes. At the end of each experiment, the medium was removed, and the cells were stained with crystal violet and were examined under the microscope to ensure that the cell layers had remained attached throughout the experiment. TER experiments give us a relative estimation of cell functions such as migration, adhesion and resistance and comparison being made between the wild types and transfected cell lines, which are discussed in detail in the results section.

5.3 Results

5.3.1 Cell-to-cell tight junction function and its relation to MAGI expression

Trans-epithelial (TER) was used to assess barrier function of the of the cell lines. It confirmed cell-to-cell TJ function status *in vitro*. HT -115 cell lines (WT, PEF and two MAGI -1 KDs) showed a significant reduction in TER in the KDs when compared to the WTs and PEFs. This may suggest weakness of TJs secondary to decreased expression of MAGI -1 in KDs (p-value 0.037 in KD1 and 0.002 in KD2) (Figure 5.3.1).

Similarly, the RKO cell lines (WTs, PEF and MAGI -1 KDs) showed significant reduction in trans epithelial resistance between cells in the KDs when compared to the WTs and PEFs, suggest weakness of TJs secondary to decreased expression of MAGI -1 in KDs (p-value 0.001 in KD1 and 0.001 in KD2) (Figure 5.3.1.2).

However, HRT -18 cell lines (WT, PEF and MAGI -1 KDs) showed no reduction in trans epithelial resistance between cells in the KDs when compared to the WTs and PEFs, suggesting no loss of strength in TJs (p-value 0.13 KD1 and 0.09 KD2) (Figure 5.3.1.1). This was possibly due to the unsuccessful creation of MAGI -1 KDs and were used as controls for HT115 and RKO cell lines.

Overall, the results were consistent with a reduced cell-to-cell tight junction function in the HT-115 and RKO KDs in colorectal cancer cell lines due to a decrease in MAGI expression when compared with the PEFs and WTs. TER across the HRT18 PEF and KDs were not reduced because of possible unsuccessful KDs and not significantly low MAGI expression, which was consistent with the ECIS results.

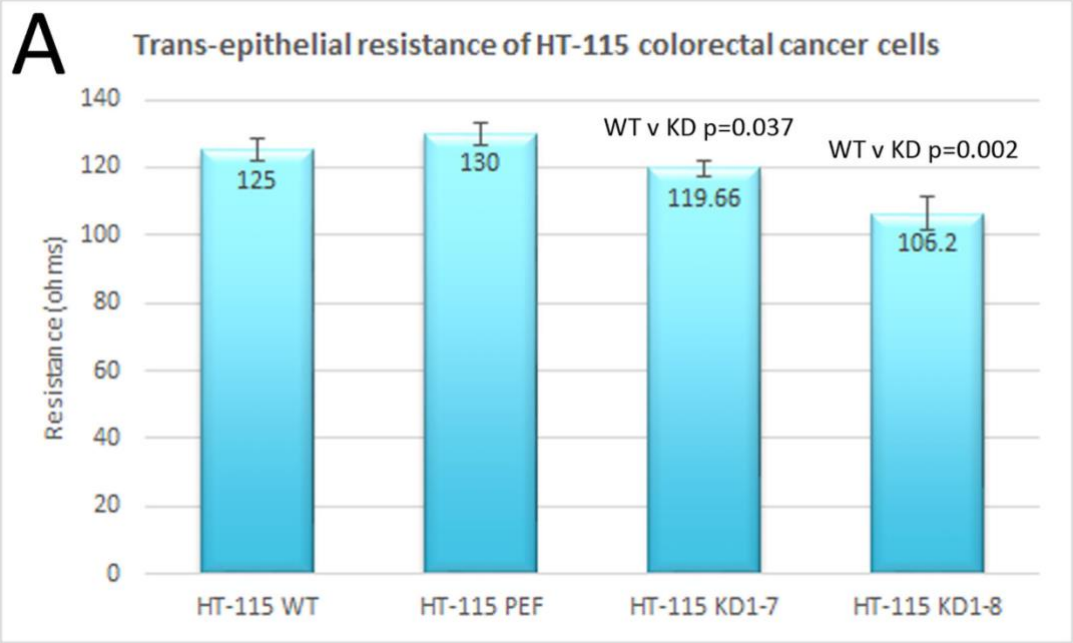


Figure 5.3.1. HT -115 WT, PEF and two MAGI -1 KDs cell lines showing significant reduction (p-value KD1 = 0.037 and p-value KD2 = 0.002) in trans epithelial resistance between cells in the KDs when compared to the WTs and PEFs suggesting weakness of TJs secondary to decreased expression of MAGI -1 in KDs.

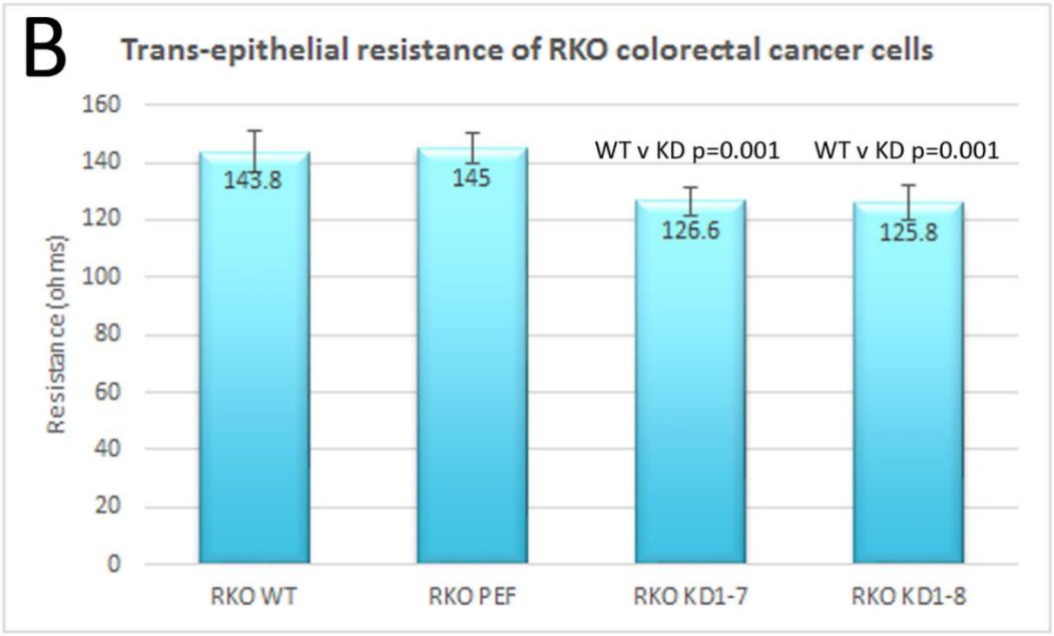


Figure 5.3.1.2. RKO WT, PEF and two MAGI -1 KDs cell lines showing significant reduction (p-value KD1 = 0.001 and p-value KD2 = 0.001) in trans epithelial resistance between cells in the KDs when compared to the WTs and PEFs suggesting weakness of TJs secondary to decreased expression of MAGI -1 in KDs.

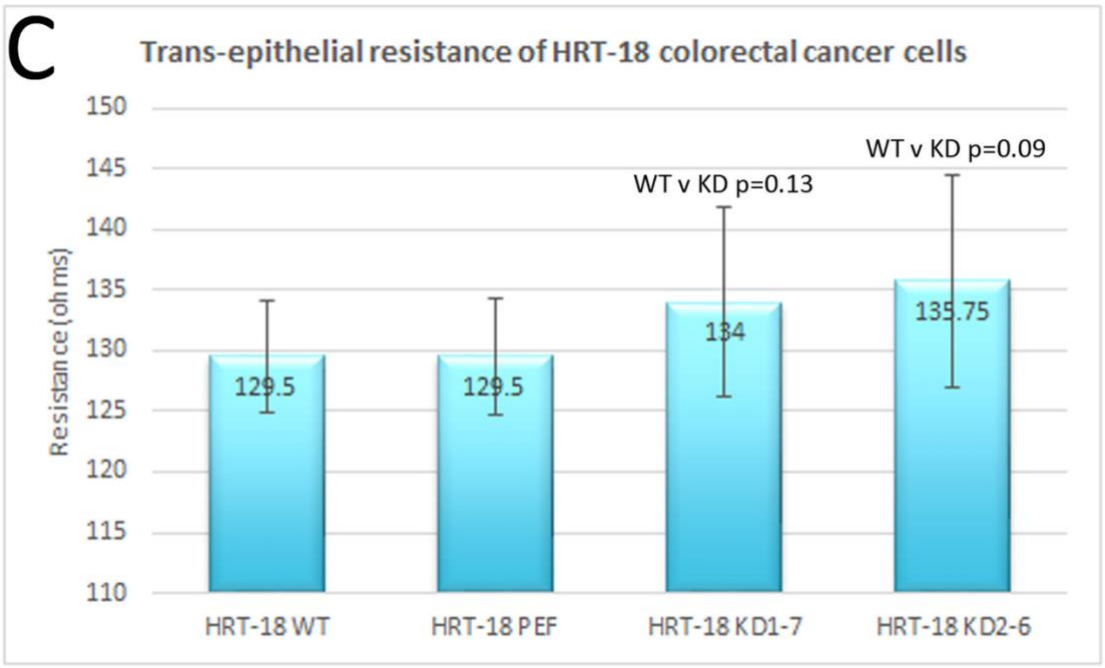


Figure 5.3.1.1. HRT -18 WT, PEF and two MAGI -1 KDs cell lines showing no reduction (p-value KD1 = 0.13 and p-value KD2 = 0.09) in trans epithelial resistance between cells in the KDs when compared to the WTs and PEFs suggesting no loss of strength in TJs. This was due to the unsuccessful creation of MAGI -1 KDs and was used as controls for HT115 and RKO cell lines.

5.3.2 ECIS results

HT -115 cell lines (WT and two MAGI -1 KDs) showed reduced cell adhesion and electrical impedance in KDs when compared to the WTs, which suggested a decrease in the strength of TJs secondary to decreased expression of MAGI -1 in KDs (Figure 5.3.2.1).

Similarly, RKO cell lines (WT and two MAGI -1 KDs) showed reduced cell adhesion and electrical impedance in KDs when compared to the WTs, which suggested a decrease in strength of TJs secondary to the decreased expression of MAGI -1 in KDs (Figure 5.3.2.2).

However, HRT18 cell lines (WTs and two MAGI -1 KDs) showed no reduction in cell adhesion and electrical impedance between KDs and PEFs, which possibly was due to the unsuccessful creation of KDs, were used as controls (Figure 5.3.2.3).

This means that knocking down MAGI -1 in colorectal cancer cell lines leads to increased proliferation and migration, hence increase the metastatic potential of cancer cells.

Overall, our ECIS results were consistent with our previous experiments, showing a decrease in cell impedance in KDs when compared with WTs in HT-115s and RKOs, but not in the HRT18s, which was possibly due to the less successful creation of HRT18 KDs. The decrease in cell impedance was due to the loss of TJs function secondary to the reduced expression of MAGI -1 in the KDs.

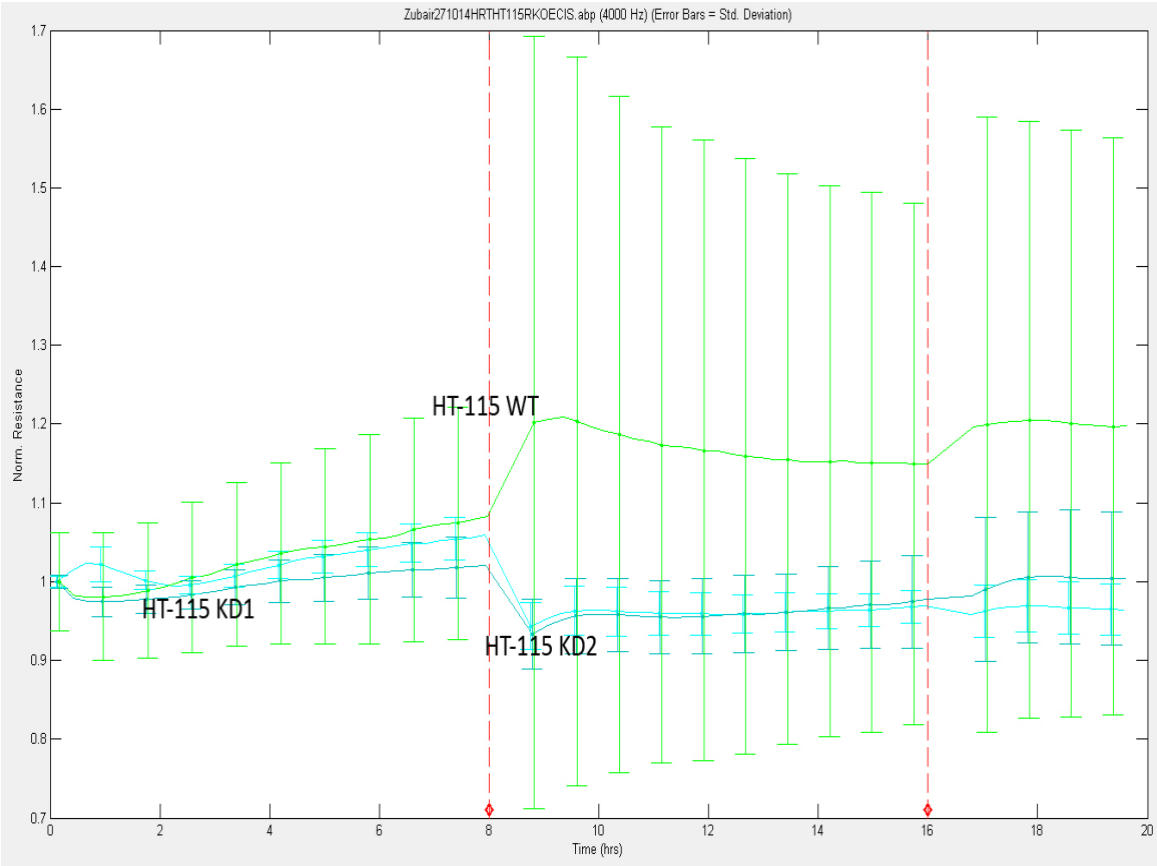


Figure 5.3.2.1. Shows ECIS results depicted on a graph for HT -115 WT and two MAGI -1 KDs cell lines. Electrical resistance is measured on the Y-axis and time is measured on the X-axis. Cells were wounded at 8 hours and 16 hours by electric shock. There was reduced cell adhesion and electrical impedance in MAGI -1 KDs in comparison to WTs, which suggested a decrease in the strength of TJs secondary to decreased expression of MAGI -1 in KDs. This leads to increased proliferation and migration, hence increase in metastasis of cancer cells.

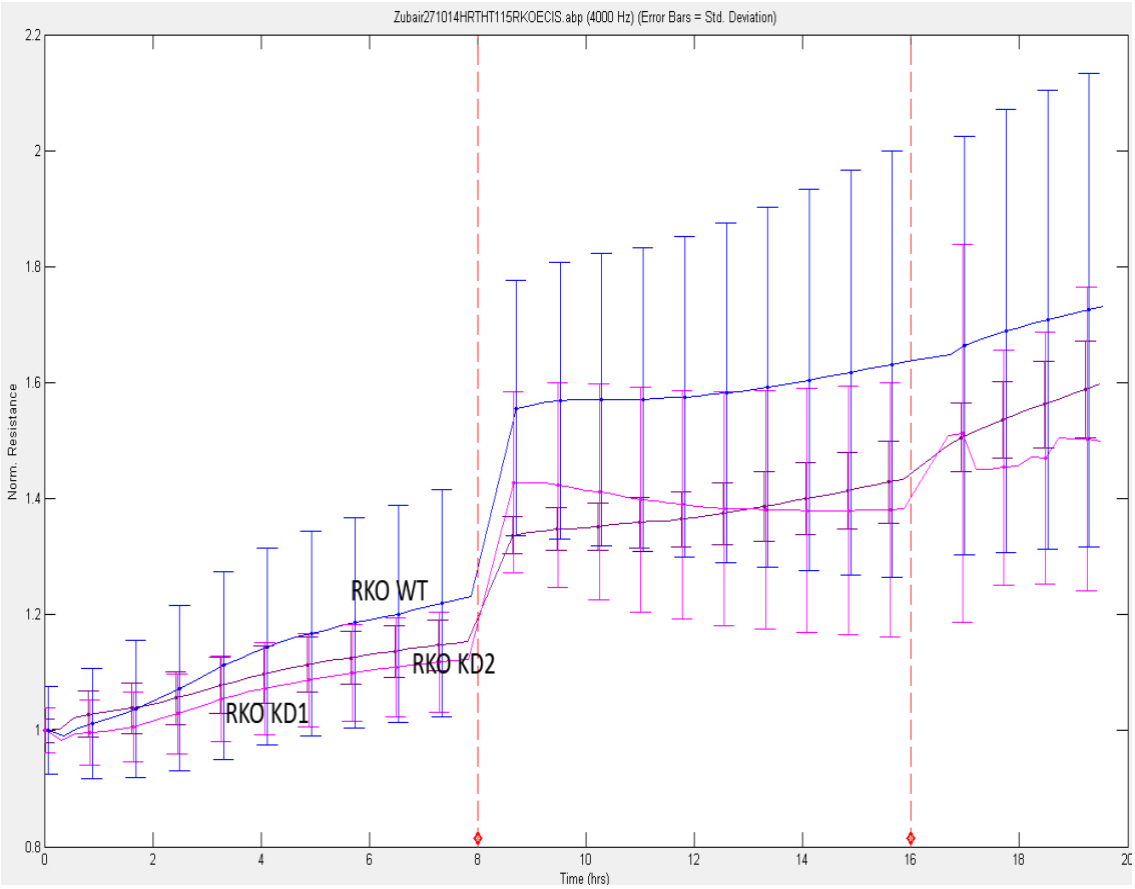


Figure 5.3.2.2. Shows ECIS results depicted on a graph for RKO WT and two MAGI -1 KDs cell lines. Electrical resistance is measured on the Y-axis and time is measured on the X-axis. Cells were wounded at 8 hours and 16 hours by electric shock. There was reduced cell adhesion and electrical impedance in MAGI -1 KDs in comparison to WTs, which suggested a decrease in the strength of TJs secondary to decreased expression of MAGI -1 in KDs. This leads to increased proliferation and migration, hence increase in metastasis of cancer cells.

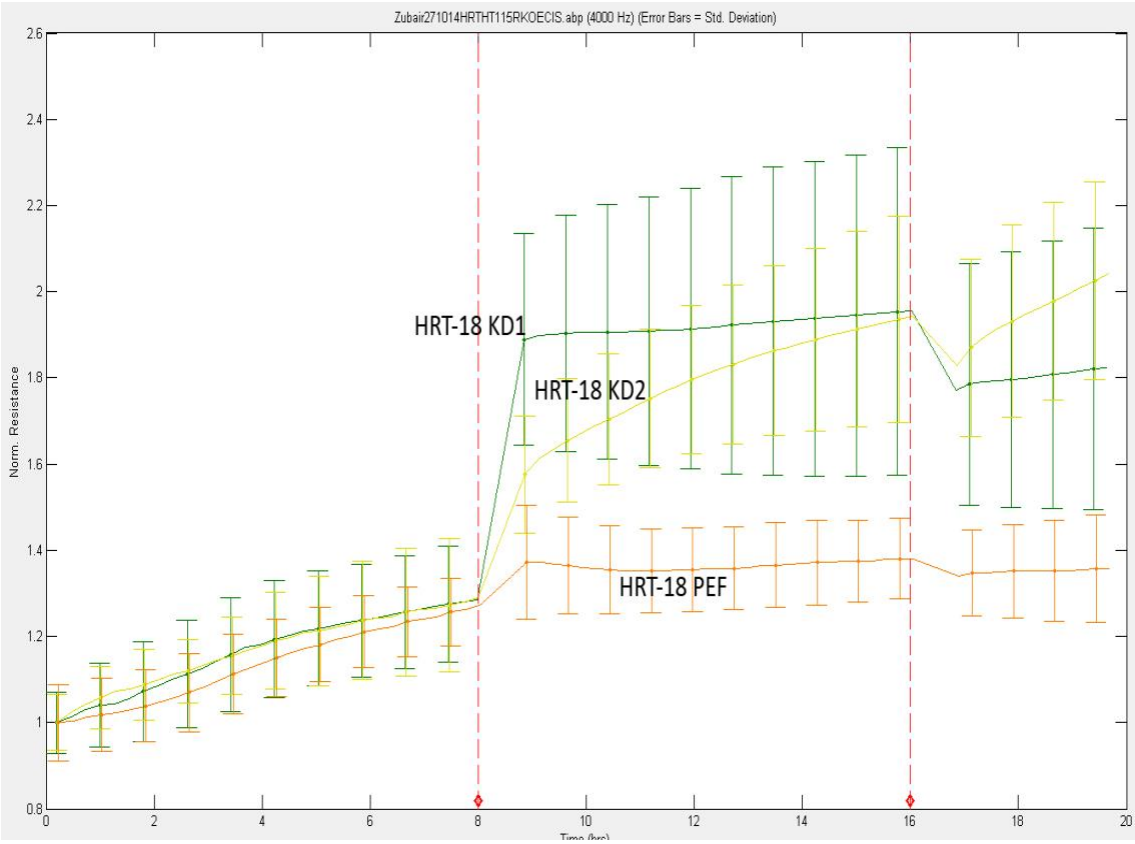


Figure 5.3.2.3. Shows ECIS results depicted on a graph for HRT18 and two MAGI -1 KDs cell lines. Electrical resistance is measured on the Y-axis and time is measured on the X-axis. Cells were wounded at 8 hours and 16 hours by electric shock. There was no significant reduction in cell adhesion and electrical impedance between cells in the MAGI -1 KDs when compared to the PEFs, which was due to unsuccessful KDs and were used as controls. Hence again proving that MAGI -1 expression reduces proliferation and migration of cancer cells, hence reduces the metastatic capability of cancer cells.

5.4 Discussion

Increased leakiness in TJs during the metastatic process has also been related to the oncogene mutations in cancer cell lines. It was reported by Soler *et al.* that the transepithelial resistance (TER) and para-cellular influx rate of TJs were effected in colonic cancer cells when compared to normal colonic cells, suggestive of increased permeability and decreased barrier function, hence leakier colon cancer epithelial cells than that of normal colonic epithelial cells. (Soler *et al.* 1999, Mullin *et al.* 2005). Landy *et al.* suggested that the dysregulation of TJs proteins precedes the development of inflammatory bowel disease (IBD) and also lead to the development of IBD associated colorectal cancer (CRC). (Landy *et al.* 2016)

MAGI -1 is widely expressed and is found to be localised at TJs, which acts as a scaffolding molecule to stabilise cadherin-mediated adhesions. (Laura *et al.* 2002) Endothelial cell-selective adhesion molecule (ESAM) regulates MAGI-1 recruitment to the cell contacts and subsequently promotes actin polymerisation and mature cell-to-cell adhesion (Kimura *et al.* 2010). Stetak *et al.* reported that the knockdown of MAGI protein causes a loss of junctional compartmentalisation along the lateral membrane, and reduces the overall robustness of cell-to-cell adhesion. (Stetak *et al.* 2010). Zaric *et al.* reported that increased expression of MAGI -1 was seen to induce stabilisation of cell to cell junctions. Increased expression of MAGI -1 was seen to suppress migration and invasion of the cancer cells. It was also seen that knocking down MAGI -1 decreased the localisation of E-cadherin at the cell to cell junction, increased disruption of stress fibres and focal adhesion points resulting in increased migration and invasion. Also, that the MAGI -1 over-expression in colorectal cancer cells suppressed the cancer growth and lungs metastasis in vivo in an orthotopic transitional intestinal model. In conclusion,

Zaric *et al.* identified the increased expression of MAGI -1 to suppress migration and invasion of the cancer cells, whereas knocking down MAGI -1 resulted in increased migration and invasion. (Zaric *et al.* 2012) Our experimental work was different to Zaric *et al.* as they used SW40, SW680, HCT116, T84 and HT29, which were different cell lines to ours, with different characteristics and varying metastatic potential. Also, that we used different methods than Zaric *et al.*, that is, we used ECIS and TER to study cancer cell behaviour. Soler *et al.* reported that the transepithelial resistance (TER) of TJs was effected in colonic cancer cells when compared to normal colonic cells, suggestive of increased permeability and decreased barrier function, hence leakier colon cancer epithelial cells than that of normal colonic epithelial cells. (Soler *et al.* 1999).

In our experiments, we used TER to assess and compare the barrier function of the reconstructed epithelial lining of the grown WT and MAGI -1 KD cell lines. ECIS was used to assess cell behaviour based on growth, migration and adhesion by monitoring changes in electrical impedance of the cells. The TER experiments were consistent with findings of reduced TJs function in the HT115 and RKO KDs colorectal cancer cell lines due to decreased MAGI -1 expression in comparison to PEFs. HT -115 WT, PEF and two MAGI -1 KDs cell lines showed significant reduction (p-value KD1 = 0.037 and p-value KD2 = 0.002) in trans epithelial resistance between cells in the KDs when compared to the WTs and PEFs, suggesting weakness of TJs secondary to decreased expression of MAGI -1 in KDs. RKO WT, PEF and two MAGI -1 KDs cell lines also showed significant reduction (p-value KD1 = 0.001 and p-value KD2 = 0.001) in trans epithelial resistance between cells in the KDs when compared to the WTs and PEFs, suggesting weakness of TJs secondary to decreased expression of MAGI -1 in KDs. However, HRT -18 WT, PEF and two MAGI -1 KDs cell lines showed no reduction (p-value KD1 = 0.13 and p-value KD2 = 0.09) in trans epithelial resistance between cells in the KDs when compared to the

WTs and PEFs, suggesting no loss of strength in TJs. This was probably due to the unsuccessful creation of MAGI -1 KDs and was used as controls for HT115 and RKO cell lines.

In ECIS experiments, HT -115 cell lines (WT and two MAGI -1 KDs) showed reduced cell adhesion and electrical impedance in KDs when compared to the WTs, which suggested a decrease in the strength of TJs secondary to decreased expression of MAGI -1 in KDs. Similarly, RKO cell lines (WT and two MAGI -1 KDs) showed reduced cell adhesion and electrical impedance in KDs when compared to the WTs, which suggested a decrease in strength of TJs secondary to decreased expression of MAGI -1 in KDs. However, HRT18 cell lines (WTs and two MAGI -1 KDs) showed no reduction in cell adhesion and electrical impedance between KDs and PEFs, which possibly, was due to the unsuccessful creation of KDs, were used as controls. This meant that knocking down MAGI -1 in colorectal cancer cell lines leads to increased proliferation and migration, hence increase the metastatic potential of cancer cells. Overall, our ECIS results were consistent with our previous experiments, showing a decrease in cell impedance in KDs when compared with WTs in HT-115s and RKOs, but not in the HRT18s, which was possibly due to the less successful creation of HRT18 KDs. The decrease in cell impedance was due to the loss of TJs function secondary to the reduced expression of MAGI -1 in the KDs.

The TER findings showed a decrease in cell impedance in MAGI -1 KDs in comparison to the wild types in HT115 and RKO cell lines, which implied an increase in cell migration and invasion potential secondary to break down of the TJs. Again, the findings between TER and ECIS were similar in the HRT18 colorectal cancer cell lines, that there was no reduction in cell resistance and cell impedance. This was due to possible failure of the

creation of successful MAGI -1 KDs in HRT 18 cell lines. Hence these were also used as controls.

5.5 Conclusion

We had convincing evidence from our experiments that MAGI -1 plays an important role in the TJ functions and knocking it down results in a decrease in epithelial barrier function and also results in decreased cell adhesion and increase in cell proliferation migration, hence increasing the invasive potential of the colorectal cancer cells.

ROLE OF MAGI PROTEINS IN INVASIVENESS OF HUMAN COLORECTAL CANCER CELLS.

CHAPTER 6: DISCUSSION

CHAPTER 6: DISCUSSION

Cancer is the leading cause of human morbidity and mortality worldwide. 14 million new cases were diagnosed in 2012, with more than 8 million cancer-related deaths reported in one year. (Cancer statistics WHO 2012) Metastatic spread is not uncommon and directly impacts on patient's survival. Metastasis is a complex multi-step process involving cell and cell-matrix interactions in which various types of cell adhesion proteins are involved. Body cavities, lumens and surfaces are lined by epithelial cells. These epithelial cells are exposed to repeated insults by the environmental agents and carcinogens, which cause repeated damage. To cope with the repeated, ongoing damage, the body constantly renews these epithelial cell linings, which have a very high regeneration and proliferation rate. This leads to a high probability of acquiring genomic mutation, which is probably why nearly ninety percent of cancers arise from epithelial cells. There is good evidence in the literature on the loss of TJs and spread of cancer. One of the important early steps in cancer metastases is interaction and penetration of the vascular endothelium by the cancer cells. The TJs in the vascular endothelium act as a barrier to molecules and cells. TJs in the epithelium act as an adhesive, which prevents cells from detachment. (Hollande et al. 2001). There has been enough evidence to prove that TJs are the first line of defence, which cancer cells must overcome to metastasise. (Martin et. al. 2002, Hoevel et. al. 2002, Ren et. al.1990, Satoh et. al.1996, Martin et. al. 2010, Kominsky et. al. 2003, Sauer et. al. 2005, Seok et. al. 2007, Usami et. al. 2006, Krämer et. al. 2000, Osanai et. al. 2006) Increased leakiness in TJs during the metastatic process has also been related to the oncogene mutations in cancer cell lines. It was also reported by Soler et al. that the Trans-Epithelial Resistance (TER) and para-cellular influx

rate of TJs were effected in colonic cancer cells when compared to normal colonic cells, suggestive of increased permeability and decreased barrier function, hence the leakier colon cancer epithelial cells than that of normal colonic epithelial cells. (Soler et al. 1999, Mullin et al. 2005) Stromberg et al. reported that invasiveness of colorectal cancer cells had a co-relation with cell adhesion proteins and polymorphism in their genes. (Stromberg *et al.* 2008). Landy *et al.* suggested that the dysregulation of TJs proteins precede the development of inflammatory bowel disease (IBD) and also lead to the development of IBD associated colorectal cancer (CRC). IBD results from the compromised integrity of epithelial barrier. Intestinal epithelium acts as a static barrier and has complex mechanisms to control and regulate bacterial interactions with the mucosal surface, during which apical tight junction proteins are critical. Alterations in tight junction proteins compromise epithelial barrier function in inflammatory bowel diseases. (Landy *et al.* 2016)

MAGI is an important novel set of proteins associated with TJs. Many important roles are attributed to MAGI, especially regarding maintenance of the integrity of the tight junctions, influencing the spread and metastasis of cancer cells. We found that there was a particular area of deficiency on MAGI proteins and its types in colorectal cancer cell lines. Zaric *et al.* concluded that over-expression of MAGI -1 was identified as a suppressor of colorectal cancer and its metastatic activity. The Increased expression of MAGI -1 was seen to suppress migration and invasion of the cancer cells, whereas knocking down MAGI -1 resulted in increased migration and invasion. (Zaric *et al.* 2012) Our study had significant differences from Zaric *et al.* study, who looked at the tumour suppressor activity of MAGI -1 in colorectal cancer cells. The key difference being in the cell lines as Zaric *et al.* used SW40, SW680, HCT116, T84 and HT29. Also, that we used

different methods than Zaric *et al.*, that is we used ECIS and TER to study cancer cell behaviour. Increased leakiness in TJs during the metastatic process has also been related to the oncogene mutations in cancer cell lines. It was also reported by Soler et al. reported that TER and para-cellular influx rate of TJs were effected in colonic cancer cells when compared to normal colonic cells, suggestive of increased permeability and decreased barrier function, hence leakier colon cancer epithelial cells than that of normal colonic epithelial cells. (Soler et al. 1999, Mullin et al. 2005).

6.1. Expression of MAGI -1, -2, -3 in colorectal cancer cells

In our experimental work on MAGI proteins in colorectal cancer cell lines, we had constant lack of MAGI -2 RNA expression on PCR (figure 4.3.1) in colorectal cancer cell lines (RKO, HT115, HRT 18), which was consistent with the literature, as there is nothing reported to date about MAGI -2 expressions in colorectal cancer cell lines. MAGI -1 and MAGI -3 were used as positive controls, which were expressed and gastric cell lines (AGS and HGC -27) were used as controls for MAGI -2 expressions. With the use of quantitative PCR (table 3.5.1.1, 3.5.1.2, 3.5.1.3), we demonstrated that expression levels of MAGI-1 were decreased in the paired tumour tissues than the normal tissues (statistically significant, p-value 0.023). Also, that expression levels of MAGI -1 were decreased in the TNM stage -2, -3 and -4 when compared with TNM stage -1. The results were consistent with the trend and also were near to achieving statistical significance (0.061). It is possible that few statistically significant results were due to the low power of our sample, but the consistency in trends was remarkable and stood out. We had similar results for decreased expression levels of MAGI-2 in a tumour paired and normal tissue with statistical significance (0.001), but we did not have samples achieving statistical

significance in MAGI-3, which was probably due to small numbers and a reduced amount of samples. Our findings were consistent with the literature, as it is well reported that increased expression of MAGI -1 is seen to suppress migration and invasion of the cancer cells. It is also reported that MAGI -1 overexpression of MAGI -1 is identified as a suppressor of colorectal cancer and its metastatic activity. (Zaric *et al.* 2012) MAGI -3 in literature is said to inhibit LPA induced migration and invasion of colonic cancer cells. (Lee *et al.* 2011). Also, we did not come across any publication that reported MAGI -2 expressions in colorectal cancer cell line, which was again consistent with our work.

6.2. MAGI -1

MAGI -1 protein has significant clinical relevance, which makes it a very attractive area of research, but also has a potential for future diagnostic and therapeutic expression sites in cancer treatment. This is not only supported by our experimental work but also is in keeping with evidence from the literature. MAGI -1 is widely expressed at and have been found to be localised to the epithelial and endothelial cell to cell TJs, and acts as a scaffolding molecule to stabilise cadherin-mediated adhesions and to recruit molecules in TJ. (Laura et al. 2002). Roles attributed to MAGI include organisation of protein complexes at the cell and synaptic junctions, regulation of cell polarity, connecting trans-cellular proteins with the cytoskeleton in TJs. (Martin *et al.* 2011), and regulation of synapse formation and plasticity. (Emtage et. al.2009). Kimura *et al.* suggested that Endothelial cell-selective adhesion molecule (ESAM) regulates MAGI-1 recruitment to the cell contacts and subsequently promotes actin polymerisation and mature cell-to-cell adhesion (Kimura et al. 2010). Stetak *et al.* reported that the knockdown of MAGI protein

in *Caenorhabditis elegans* epithelia causes a loss of junctional compartmentalisation along the lateral membrane, and reduces the overall robustness of cell-to-cell adhesion. (Stetak et al. 2010). MAGI have also been reported to be involved in cancer cell signalling as a tumour suppressor regulator protein (Matsuda et al. 2013).

6.3. Effects of knocking down MAGI -1 in colorectal cancer cells

We chose three different human colorectal cancer cell lines for our experiments, RKO, HT-115 and HRT-18, because of their high invasive and metastatic potential, which is well known and also found in the literature. To start with, we created a different type of cell lines and checked the expression of MAGI -1 in them. After that, we created knockdowns of MAGI 1, which were confirmed on Q-PCR and agarose gel electrophoresis (figure 4.4.1). The experiments were repeated which showed consistency in the results. Once the KDs were confirmed, cell lines were generated, significant changes in morphology and growth pattern between the wild types colorectal cancer cell lines and their corresponding KDs were noted. To naked eye in the flasks (figure 4.4.2), WT cells were quicker to grow and would spread in a cluster pattern on the wall of the flask, and individual cells had rounded appearances, whereas KD cells would grow in a uniform pattern at a slower rate and individual cells had spiky appearances. This was noted across the board for all the three colorectal cancer cell lines (HT-115, RKO & HRT-18) used in our experiments. However, the differences were noted to be less apparent in the HRT-18 cell lines, which was possibly due to their slower to grow and more adhesive nature. Also, HRT-18 KD cells continued to have some expression of MAGI-1 on Q-PCR and agarose gel electrophoresis, which was again either due to their slow growing and more adhesive nature when compared to the other two cell lines or was due to the less successful

creation of KDs. Electronic microscopy following Immunofluorescence staining (figure 4.5.1 – 4.5.6) showed the presence of TJs in WT cells and their absence in HT115 and RKO MAGI -1 KD cells. This was noticed to a lesser degree in HRT18 MAGI -1 KD cell lines, which consistent with our findings on Q-PCR and agarose gel electrophoresis. ZO -1 -2 & -3 were used as controls, which had no or minimal loss of TJs by knocking down MAGI -1 gene.

In our experiments, we used TER to assess and compare the barrier function of reconstructed endothelial and epithelial of the grown WT and MAGI -1 KD cell lines. ECIS was used to assess cell behaviour based on growth, migration and adhesion by monitoring changes in electrical impedance of the cells. The TER experiments (figure 5.3.1, 5.3.1.2 & 5.3.1.1) were consistent with findings of reduced TJs function in the HT115 and RKO KDs colorectal cancer cell lines due to decreased MAGI -1 expression in comparison to PEFs. The TER findings were consistent with ECIS results (figure 5.3.2.1 – 5.3.2.3), which also showed a decrease in cell impedance in MAGI -1 KDs in comparison to the wild types in HT115 and RKO cell lines, which implied an increase in cell migration and invasion potential secondary to break down of the TJs. Again, the findings between TER and ECIS were similar in the HRT18 colorectal cancer cell lines in that there was a reduction to a lesser degree in cell resistance and cell impedance than HRT18 and HT115. As mentioned above, Zaric *et al.* also reported that knocking down MAGI -1 resulted in increased migration and invasion. (Zaric *et al.* 2012) Soler *et al.* also checked trans epithelial resistance (TER) in colonic cancer cell lines and compared it to normal colonic

cells. He found out that there was a decrease in the barrier function of cancer cell lines than that of normal colonic cell lines. (Soler *e. al.* 1999).

Survival curves showed an increase in overall survival and disease-free survival in our cohort with consistent trends of higher survival rates related to increased expression of MAGI. Combined analysis of MAGI -1, -2 and 3 (Figure 3.8.1 and 3.8.2) showed higher survival rates in both, overall and disease-free survival, which were of statistical significance (Wilcoxon statistical significance of 0.015 and 0.045). Survival curves of individual MAGI -1, -2 and 3 were consistent with a similar pattern, and showed a higher rate of survival with increased expression of individual MAGI type (MAGI -1, -2 and 3) (Figures 3.8.3 – 3.8.8). Although individual MAGI type (MAGI -1, -2, -3) results did not reach statistical significance, the trends were consistent with increased overall survival and disease-free survival in tissues with increased expression. MAGI-1 overall survival was of note as it nearly reached statistical significance (0.074) (Figure 3.8.3). Our work is unique as it is the first of its type, considering the effect of MAGI protein expression (MAGI -1, -2, -3) on patients (human tissue) survival, as there is nothing published to date relating MAGI expression with human survival. However, Balbas *et al.* have published their work on MAGUK (MAGI -2) expression and survival function conducted on mice. (Balbas *et al.* 2014)

Following studying the expression of MAGI -1 in colorectal cancer cell lines, we established that reduced expression of MAGI -1 lead to not only reduced adhesion and barrier function, but also an increase in the migratory function. This translates into not only increased cancer metastasis in the patient's cohort but also reduced survival. This makes MAGI proteins of significant clinical relevance.

6.4. Future work

If time had allowed, we would have done the following:

- We would have carried out Western Blotting (WB) experiments to confirm expression levels of MAGI in colorectal cancer cell lines at the protein level.
- Further time is permitting we would have also studied phosphorylation status and regulation pathways, which were areas of our future interest.

In future, further work is needed on the functions and roles of this essential protein, especially in cancer metastasis, which was out of the scope of this study mainly due to mainly time constraints.

6.5. Conclusion

From our analysis of MAGI-1 protein expression in human colorectal cancer cell lines, we have compelling evidence that MAGI-1 protein plays an important role in the invasiveness and metastasis of human colorectal cancer, mainly by their role in the maintenance of TJ structure and function.

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Appendix-1

Ethical approval

NHS WALES GIG CYMRU

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From: Professor MF Scanlon
Trust R&D Director
Radnor House

20 December 2005

Professor WG Jiang
Department Of Surgery
Wales College Of Medicine
Heath Park
Cardiff

Dear Professor Jiang

Project ID : 05/DMD/3562 : Tumour-Associated Lymphangiogenesis In The Development And Metastasis Of Human Colorectal Carcinoma

Thank you for your recent communication with the Trust R&D Office regarding the above study. The project was reviewed by the Joint Trust/University Peer/Risk Review Committee on 16 December 2005. The committee approved the project and Cardiff University will sponsor the study under the Research Governance Framework for Health and Social Care.

The Trust is therefore happy for the project to begin subject to it receiving ethical approval from the appropriate MREC and/or LREC, and honorary contracts, where required, being in place. Please forward a copy of this letter to the ethics committee. Once you have ethical approval, please forward a copy of the letter to the Research and Development Office at the above address.

The committee would like the following comments to be taken into consideration.

1. The Principal Investigator intends to study 48 stored carcinoma specimens plus 40 from fresh patients, and 34 specimens of stored normal mucosa. Some rationale for the sample size is needed in terms of the size of difference that this would give a high power to detect.
2. The information sheet states that the tissue sample may be used in more than one research project. This should be consented to on the consent form.

May I take this opportunity to wish you success with the project and remind you that as Principal Investigator you are required to:

a) inform the Trust R & D Office if any external funding is awarded for this project in the future
b) maintain a record of the number of patients /samples in this study
c) complete any questionnaires sent to you by the Trust R & D Office regarding this project