UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Cancer Sciences Unit

Identification and characterisation of deregulated microRNAs during colorectal cancer progression – interplay between tumour stroma and malignant epithelium

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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IDENTIFICATION AND CHARACTERISATION OF DEREGULATED MICRORNAS DURING COLORECTAL CANCER PROGRESSION - INTERPLAY BETWEEN TUMOUR STROMA AND MALIGNANT EPITHELIUM

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MicroRNAs (miRNA; miR), which are key drivers of colorectal cancer (CRC) tumourogenesis, are increasingly implicated in metastatic progression. Equally relevant is the role of the tumour microenvironment and interactions between the malignant epithelium and cancer-associated stroma. Based on these observations the following hypothesis was formulated: *Stromal and epithelial miRNA expression patterns (individually and communally) have important consequences for CRC progression and metastasis.*

To identify miRNA candidates with putative roles in CRC progression, high throughput microarray profiling was used to compare miRNA expression in laser-microdissected CRC tissue and paired 'normal' colonic tissue; and in matched early stage CRC specimens with and without subsequent metastasis within 5 years. Subsequently, stromal miR-21 and miR-556, and epithelial miR-224, miR-153 and miR-106a were selected for further analysis.

MiR-21, a known oncogene was shown by in-situ hybridisation to be upregulated exclusively in tumour associated fibroblasts in CRC. In functional co-culture studies, ectopic miR-21 expression in fibroblasts protected CRC cells from chemotherapy induced apoptosis and increased their proliferative and invasive capacities. Furthermore, miR-21 significantly corrupted fibroblast secretory functions and supported fibroblast-to-myofibroblast transdifferentiation, a key pro-metastatic stromal event. Deregulated stromal miR-21 expression was also associated with an increasingly aggressive phenotype in a novel murine CRC-cell/fibroblast orthotopic co-implantation model and in 3-Dimensional synthetic organotypic tumour models purpose built for this study. These findings complemented mechanistic data which demonstrated stromal suppression of matrix-remodelling enzyme inhibitors RECK and TIMP3 and a reciprocal rise in Matrix Metalloproteinase 2 (MMP2) activity in response to ectopic miR-21 expression.

MiR-153 was also shown to play a functional role during disease progression by promoting CRC cell invasion directly. Furthermore, both stromal and epithelial miRNAs identified in this study predicted short disease-free survival (DFS) for patients with early stage CRC: stromal miR-21 (DFS: HR=2.68, p=0.015); stromal miR-556 (DFS:HR=2.60, p=0.018); epithelial miR-106a (DFS:HR 2.91, p=0.008); combined (All High vs. All Low. DFS: HR=5.83, p=0.008).

In summary, these data highlight the importance of miRNAs within the tumour microenvironment; they suggest a novel stroma mediated pro-metastatic mechanism in CRC and identify a potential application for stromal miRNAs as therapeutic targets and biomarkers of cancer progression.

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Publications arising from this thesis

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- Ling H, Pickard K, Ivan C, Isella C, Ikuo M, Mitter R, Spizzo R, **Bullock MD** et al., The Clinical and biological significance of miR-224 in colorectal cancer metastasis. *Gut* 2015 Mar 24 (Epub ahead of print).
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- **Bullock MD,** Pickard K, Schnack Nielsen B, Sayan AE, Jenei V, Mellone M, Primrose JN, Thomas G, Packham GK, Mirnezami AH. Deregulated stromal microRNA-21 and promotion of metastatic progression in colorectal cancer. *The Lancet* 2014: 383; S30 doi:10.1016/S0140-6736(14)60293-2
- **Bullock MD,** Mellone M, Pickard KM, Sayan AE, Mitter R, Primrose JN, Packham GK, Thomas G, Mirnezami AH. Molecular profiling of the invasive tumour microenvironment in a 3-dimensional model of colorectal cancer cells and ex vivo fibroblasts. *Journal of visualized experiments : JoVE* 2014(86); doi: 10.3791/51475
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- **Bullock MD,** Sayan AE, Packham GK, Mirnezami AH. MicroRNAs: critical regulators of epithelial to mesenchymal (EMT) and mesenchymal to epithelial transition (MET) in cancer progression. *Biology of the cell* 2012; **104**(1): 3-12.
- **Bullock M.D** and Mirnezami A.H. The Management of Rectal Cancer. In: Choudhury D (ed). General Surgical Operations. Jaypee Brothers Medical Publishers Ltd; 2nd edition (2013). ISBN: 81-8448-195-0. *In press.*

Presentations arising from this thesis

First author presentations at international and national conferences arising from this thesis are listed below

- SARS (Society of Academic and Research Surgery) Patey Prize session: ‘MicroRNA-21 expression in stromal fibroblasts supports metastasis in an orthotopic murine model of colorectal cancer by maintaining a pro-invasive tumour microenvironment.’ Durham, UK January 2015 (International).
- Tripartite Colorectal Meeting: Lunchtime Poster Prize Session: ‘Metastatic departure: understanding the origins of colorectal cancer metastasis by modelling the invasive tumour microenvironment in-vitro.’ Birmingham, UK July 2014 (International).
- Academy of Medical Science Clinician Scientist Meeting: Poster Prize Session: ‘Deregulated stromal microRNA-21 and promotion of metastatic progression in colorectal cancer.’ Royal College of Physicians of London. February 2014 (International).
- SARS (Society of Academic and Research Surgery) Oral presentation: ‘Deregulated stromal microRNA-21 promotes metastatic progression in colorectal cancer.’ at the University of Cambridge January 2014 (International).
- NCRI (National Cancer Research Institute) Poster Presentation: ‘Deregulated stromal microRNA-21 promotes metastatic progression in colorectal cancer.’ Liverpool, UK November 2013 (International).
- SOTA (Surgical Oncology Trainee Association) Oral presentation: ‘FOXO3 expression during colorectal cancer progression: potential utility as a biomarker reflects a tumour-suppressor role.’ Royal College of Surgeons of England. Nov 2013 (National).
- ACPGBI (Association of Coloproctology of Great Britain and Ireland) Oral presentation: ‘FOXO3 expression during colorectal cancer progression: potential utility as a biomarker reflects a tumour-suppressor role.’ Liverpool July 2013 (International).
- ASGBI (Association of Surgeons of Great Britain and Ireland) Oral presentation: ‘Pleiotropic actions of miR-21 highlight the critical role of

deregulated stromal microRNAs during colorectal cancer progression.' Glasgow May 2013 (International).

- AACR (American Association of Cancer Research) Poster Presentation: 'Pleiotropic actions of miR-21 highlight the critical role of deregulated stromal microRNAs during colorectal cancer progression.' Washington D.C March 2013 (International).
- BASO (British Association of Surgical Oncology) Oral Presentation Ronald Raven Prize session: 'Pleiotropic actions of miR-153 support colorectal cancer progression through enhanced invasion and platinum based chemotherapy resistance.' Royal College of Surgeons of England. Nov 2012 (National).
- NCRI (National Cancer Research Institute) Poster Presentation: 'Pleiotropic actions of miR-153 support colorectal cancer progression through enhanced invasion and platinum based chemotherapy resistance.' Liverpool, UK November 2012 (International).

Prizes and Awards arising from this thesis

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- 2015 Patey Prize shortlist - Society of Academic and Research Surgery
- 2014 Sir James Fraser Award - University of Southampton - Division of Surgery
- 2014 MRC supplementary training award
- 2014 **Fulbright-Royal College of Surgeons of England Research Award**
- 2014 Academy of Medical Sciences Spring Meeting - Poster Presentation Prize
- 2013 Nomination for the BACR/Gordon Hamilton - Fairley Young Investigator Award
- 2013 University of Southampton CSU conference - Prize for Best Oral Presentation
- 2013 Sir James Fraser Award - University of Southampton - Division of Surgery
- 2012 **Ronald Raven Prize at the British Association of Surgical Oncology**
- 2012 *Biology of the Cell* – Most Cited/Downloaded manuscript of 2012
- 2012 **Medical Research Council (MRC) Clinical Research Training Fellowship**
- 2012 *Biology of the Cell* Paper of the month award (May)

Declaration Of Authorship

I, Marc David Bullock

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as: [please list references below]:

Signed:
……

Date: ……

- Ling H, Pickard K, Ivan C, Isella C, Ikuo M, Mitter R, Spizzo R, **Bullock** MD et al., The Clinical and biological significance of miR-224 in colorectal cancer metastasis. *Gut* 2015 Mar 24 (Epub ahead of print).
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Abbreviations

5-FU	5-Fluorouracil
ACF	Aberrant crypt foci
AJCC	American Joint Committee on Cancer
AKT	Serine-threonine protein kinase
APC	Adenomatous polyposis coli
ATCC	American Type Culture Collections
AUC	Area under curve
AV	Annexin V
BM	Basement membrane
BMI	Body mass index
BMI1	B-lymphoma Mo-MLV
BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
CASP3	Caspase 3
CDH1	E-cadherin
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability pathway
CLL	Chronic lymphocytic leukaemia
CM	Conditioned media
CMV	Cytomegalovirus
COX	Cyclooxygenase enzyme
CRC	Colorectal cancer
CSS	Cancer-specific survival
CT	Computed tomography
CTGF	Connective tissue growth factor
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DCC	Deleted in colorectal cancer
DFS	Disease free survival
DIG	Digoxigenin
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
EGFR	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial-to- Mesenchymal transition
ENA-78	Epithelial-derived neutrophil-activating peptide 78
FACS	Fluorescence- activated cell sorting
FAP	Familial adenomatous polyposis
FAP	Fibroblast activation protein
FCS	Foetal Calf Serum

FDA	Food and drug administration
FDR	False discovery rate
FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FOXO	Forkhead box O
FSP1	Fibroblast specific protein 1
GFP	Green fluorescent protein
GTSP1	Glutathione S-transferase P1
HCV	Hepatitis C virus
HER2	Herceptin 2
HMGA2	High mobility group AT-hook 2
HNPCC	Hereditary non-polyposis colorectal cancer
HR	Hazard ratio
HRP	Horseradish peroxidase
ICAM	Intracellular adhesion molecules
ID	Identity
IGF	Insulin-like growth factor
ISH	In-situ hybridisation
Jag1	Jagged-1
KRAS	Kirsten rat sarcoma viral oncogene homolog
LMD	Laser microdissection
LN	Lymph node
LNA	Locked nucleic acid
LOH	Loss of heterozygosity
MIAME	Minimum information about a microarray experiment
Mam1	Mastermind-like co-factor
MAPK	Mitogen-activated protein kinase
miR	MicroRNA
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MMR	Mismatch-repair gene
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSI	Microsatellite instability
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-slfophenyl)-2H-tetrazolium
NK cell	Natural killer cell
NS	Not significant
OS	Overall survival
PBS	Phosphate buffered saline
PCF	Primary colon fibroblast
PCR	Polymerase chain reaction
PDCD4	Programmed cell death 4
PET	Positron emission tomography
PHLPP1/2	PH domain leucine-rich-repeats protein phosphatase 1/2

PI	Propidium iodide
PIK3	Phospatidylinositol-4, 5-bisphosphate 3-kinse
PJS	Peutz-Jegheres syndrome
PMS	Phenazine methosulphate
PTEN	Phosphate and tensin homolog
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs
REMARK	Reporting recommendations for tumour marker prognostic studies
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RT	Reverse transcription
SCC	Scrambled control and Squamous Cell Carcinoma
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDF	Stroma cell derived factor
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide
SF/HGF	Scatter factor/hepatocyte growth factor
siRNA	Short interfering RNA
SIRT1	Silent information regulator
SMA	Smooth muscle actin
SSC	Saline-Sodium citrate
stage II-NR	Stage II CRC without recurrence within 5 years
stage II-R	Stage II CRC with recurrence within 5 years
TBE	Tris-borate EDTA buffer
TBS	Tris-Buffered Saline
TF	Transferrin
TGFβ	Transforming growth factor beta
TIAM1	T-lymphoma invasion and metastasis 1
TIMP3	Tissue inhibitor of metalloproteinase 3
TMA	Tissue microarray
TNM	Tumour Node Metastasis
TRG	Tumour regression grade
TTP	Tristetraproline
UC	Ulcerative colitis
UK	United Kingdom
uPAR	Plasminogen activator urokinase receptor
US	United States (of America)
VEGF	Vascular endothelial growth factor
VIM	Vimentin
WHO	World health organisation
WT	Wild-type
ZEB	Zinc-finger E-box binding homeobox

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1 Introduction

Introduction

1.1 Colorectal cancer

Colorectal cancer (CRC) is a key public health issue, and accounts for the second highest cause of cancer related death in Europe.[1] Metastases and disease progression are the principal cause of death and occur in 30% of patients at presentation and subsequently in over 50% of patients after surgery with curative intent.[2, 3] The majority of patients with metastases remain incurable. Without treatment median survival is 8 months,[4] but the addition of chemotherapy and newer targeted therapies may enhance this figure to 12 and 16 months respectively.[4, 5] The emergence of targeted therapies has been made possible through a greater understanding of the molecular characteristics of CRC. Although results of clinical trials have proved disappointing, extending survival by only a few months,[6, 7] these studies offer a proof-of-principle that therapeutic strategies translated from a better understanding of the mechanisms of disease can be applied to CRC. They also highlight the need for a continued effort to unravel the biological mechanisms behind CRC progression.

1.1.1 Natural history of colorectal cancer

In 2011, CRC accounted for 15,659 deaths in the UK, and of the 41,581 new cases diagnosed, 56% were in men and 44% in women. The incidence of CRC is strongly related to age with peak risk occurring in the over 85 age group. In the 3 decades preceding 2011, CRC rates in Europe rose by 29% in men and 7% in women, meaning that in the UK currently, the lifetime risk of developing CRC is 1 in 14 for men and 1 in 19 for women.[8]

Approximately 25% of new cases of CRC arise in the rectum, and 75% in the colon. Although increasingly recognised as distinct biological entities, colon and rectal cancers share many clinical features and are often referred to as a single unified pathology. [9, 10]

Metastasis occurs through lymphatic, haematogenous or transcoelomic spread. Pathological staging, which is crucial for prognostication and therapy planning, is conducted using the Tumour Node Metastasis (TNM), Dukes' or American Joint Committee on Cancer (AJCC) classification systems.[11, 12] Although they differ subtly from one another, each system uses histopatholgical and radiological information to

describe the extent of local disease spread as well as the presence and pervasiveness of metastasis in regional lymph nodes and remote organ systems.

If an invasive cancer is confined to the wall of the colon (AJCC Stage I and II) surgical resection is often curative. In contrast, patients with disease spread to lymph nodes (Stage III CRC), are usually also offered chemotherapy, as this reduces relapse and mortality rates by 30% to 40% compared with surgery alone.[13] However, the majority of deaths from CRC are caused by metastatic (Stage IV) disease and not the primary tumour, and despite increasing use of metastectomy and chemotherapy over time, the proportion of patients in this group who survive beyond 2 years remains as low as 28%.[14]

1.1.2 Aetiology of colorectal cancer

The majority of CRCs arise spontaneously and are not associated with a pre-disposing genetic condition. However, much of our insight into the biological underpinnings of CRC was first achieved by studying inherited cancer syndromes in which colonic malignancies are a predominant feature.

Familial adenomatous polyposis (FAP) is an autosomal dominant disorder caused by a germline mutation in the adenomatous polyposis coli (*APC*) locus of chromosome 5q21.[15, 16] It is characterised by the development of multiple colonic adenomas and the inevitable onset of invasive carcinoma unless prophylactic colectomy is performed.[17]

Hereditary non-polyposis colorectal cancer (HNPCC) is more common than FAP, accounting for approximately 1-2% of all cases of CRC.[18] An autosomal dominant disorder resulting from mutation of one of 5 mismatch-repair (MMR) genes (primarily *MLH1* and *MSH2*) responsible for maintaining the fidelity of genomic DNA,[19] it is associated with an 80% lifetime risk of developing invasive colonic adenocarcinoma.[20, 21]

The third major familial cancer syndrome associated with CRC is Peutz-Jeghers syndrome (PJS), an autosomal dominant syndrome attributed in a proportion of patients to mutational inactivation of the gene *STK11* (*LKB1*) on chromosome 19p13.[22, 23] Characteristically associated with mucocutaneous pigmentation and hamartomatous

polyps of the small intestine, patients with PJS also have a lifetime risk of CRC of approximately 20%.[24]

Two other germline mutations relevant to CRC are associated with inherited polyposis syndromes, that of the *SMAD4* gene in Juvenile polyposis[25] and the *PTEN* gene in Cowden disease.[26]

Intriguingly however, these examples of high penetrance germ-line mutations are estimated to account for only 5% of the heritability of the disease.[27] This suggests other inheritance pathways must exist and that a plethora of low penetrance genetic variants play contributory roles during tumourogenesis.[27]

Against this heterogeneous and complex genetic backdrop, it has become increasingly clear that environmental factors also contribute significantly to the pathogenesis of CRC.

For example, a protective association with high fibre diets was identified as early as the 1970s.[28] Subsequent meta-analysis of relevant studies suggests the incidence of both CRC and precursor adenomatous lesions may be reduced by as much as 10% per 10g/day increase in dietary fibre intake.[29, 30]

Other dietary factors contribute to an increased risk of CRC, most notably red/processed meat and alcohol,[31] and on a related theme, which has attracted considerable attention recently, a significant association with obesity (defined by the WHO as a body mass index (BMI) $\geq 30 kg/m^2$) has also been identified.[32] Not only is the incidence of CRC significantly greater in obese people,[33, 34] but obesity is independently associated with impaired post-operative outcomes and response to therapy and decreased overall survival.[35]

This effect may be mediated in part through an associated metabolic syndrome; a state of corrupted physiological homeostasis, characterised by elevated levels of fasting glucose, insulin resistance, dyslipidaemia, high blood-pressure and pro-inflammatory and pro-thrombotic changes.[36, 37] Within this spectrum, the presence of type-II diabetes mellitus (Type-II DM) in particular, has been identified as an independent risk factor for developing CRC. Furthermore, Type-II DM affected patients have both poorer disease-free survival and cancer-specific mortality than their non-diabetic counterparts.[38, 39]

Other environmental agents associated with increased CRC risk include bile acids[40] and enteric bacteria.[41] Research into the nature of the gut microbiome has become especially prominent in recent times, as the apparent carcinogenic influence of certain bacteria, including *Helicobacter pylori* has been identified in colon.[41, 42]

1.1.3 The molecular basis of colorectal cancer development

As the complex interplay between genes and the environment, and the impact this has on tumourogenesis is increasingly well described, a number complimentary and interconnect systems have emerged to catalogue the molecular pathobiology of CRC:

Volgestein's hypothesis suggests the progressive accumulation of somatic mutations in genes involved in cell cycle regulation and apoptosis leads to a stepwise transition from normal colorectal mucosa, to adenoma and eventually to invasive adenocarcinoma.[43, 44]
Mutational inactivation of the tumour suppressor gene *APC* is a key initiating event, triggering the development of small adenoma through derestricted activity of the oncogene β-catenin in the wnt signalling cascade.[45, 46] Loss of p53 activity and TGFβ signalling are later events which coincide with progression from adenoma to carcinoma.[47, 48]
In addition, the inappropriate activation of oncogenes such as *KRAS* and *BRAF* play important roles. Mutations in either gene may trigger constitutive activation of the mitogen-activated protein kinase (MAPK) signalling cascade.[49, 50]
A second system used to describe the molecular basis of CRC is termed the genomic instability pathway. Loss of genomic stability may take several forms:
80-85% of sporadic CRCs follow the chromosomal instability pathway[45] (CIN) characterised by loss of heterozygosity (LOH) and altered chromosomal copy number and structure.[51, 52] It is commonly through the CIN pathway that the function of key tumour suppressors including p53, APC and SMAD4 is lost.[53]
Furthermore, inactivation of genes required for repair of base-base mismatches (MMR genes), leads to more frequent DNA replication errors and as a consequence, the mutation rate in colorectal epithelial cells may increase as much as 100-fold.[54] Loss of MMR function results in microsatellite instability (MSI). This is a feature characteristic of HNPCC which also occurs in 15-20% of sporadic colorectal tumours.[19, 55]
Microsatellites are repetitive DNA sequence elements identified throughout the genome

which change size in the absence of effective MMR 'proof-reading'. The CRC phenotype associated with MSI consists of poorly differentiated, proximal mucinous tumours in which lymphocytic infiltration is a prominent feature.[56]

Genomic instability results in the sequential loss-of-function of genes regulating the hallmark processes of carcinogenesis, and ultimately manifests as invasive carcinoma. Comprehensive analysis of the CRC genome revealed mutations in the coding sequence of 67 genes on average, of which 12 genes were proposed to be most relevant to tumour development, illustrating the complexity and potential heterogeneity of CRC pathogenesis.[57]

The most common somatic mutations in oncogenes and tumour suppressor genes associated with sporadic CRC include *APC* in 60% of cases,[58, 59] *p53* in 30-50% of cases,[60, 61] *KRAS* in 35-45% of cases,[49, 62] *SMAD4* in 9-35% of cases,[63, 64] *PIK3CA* in 15-18% of cases,[65-67] *BRAF* in 8-12% of cases [50, 68] and *PTEN* in approximately 5% of cases.[69]

In contemporary literature, it is commonplace for CRCs to be referred to as one of these two well characterised major tumour subtypes (CIN or MSI).[45] In addition to genomic instability, however cancer initiation and progression may occur through aberrant epigenetic gene regulation. In CRC, the dominant epigenetic mechanism is CpG island methylation. Frequently present in premalignant aberrant crypt foci (ACF), within colonic mucosa, the methylation of cytosine residues in CpG dinucleotides leads in-turn to the transcriptional silencing of tumour-suppressor genes and represents one of the earliest detectable changes in CRC.[19, 70]

In contrast to CIN, CpG island methylator phenotype (CIMP) tumours are more likely to develop in the proximal colon and display microsatellite instability and crucially, they define a distinctive patient group, in terms of prognosis and treatment outcome.[71, 72]

The fairly loose and overlapping system of tumour classification described above reflects how our understanding of CRC biology has evolved over time. However, the ability to more systematically categorise tumours based on molecular characteristics and phenotype despite the inherent heterogeneity of the disease is highly desirable as it could substantially improve prognostication and facilitate development of newer targeted therapies.

In CRC the closest we have come to a ubiquitous classification model, derives from a study in which unsupervised cluster analysis of 146-genes was performed for >1,100 tumour specimens, spread across 6 international patient cohorts, cross-referenced with MSI and CIMP status.[73] Crucially, 3 colon cancer subtypes (CCSs) were identified: CCS1 and CIN classifications were broadly aligned; MSI/CIMP+ tumours were largely absent from this group, which was more typically associated with *KRAS* and *P53* mutations; CCS2 tumours were associated with both MSI and CIMP +ve status; and CCS3 tumours were relatively heterogeneous with respect to MSI and CIMP status, but contained a large proportion of patients with *BRAF* and *KRAS* mutations. CCS1 tumours were mainly left sided, CSS2 tumours mainly right sided and CCS3 tumours evenly distributed throughout the colon and typically poorly differentiated.

CCS3 tumours were associated with significantly impaired clinical outcomes and resistance to cetuximab therapy. Furthermore, gene-set enrichment analysis comparing CCS3 and CCS1 tumours, highlighted biological themes particularly associated with aggressive disease, including EMT, matrix remodelling and TGFβ signalling.

Finally, on this point, deregulated gene expression in sessile serrated adenomas showed a high degree of concordance with CCS3 tumours, suggesting this biologically and clinically distinct tumour sub-group may arise from a histologically distinct precursor lesion which does not feature in the classic Volgestein model.[73]

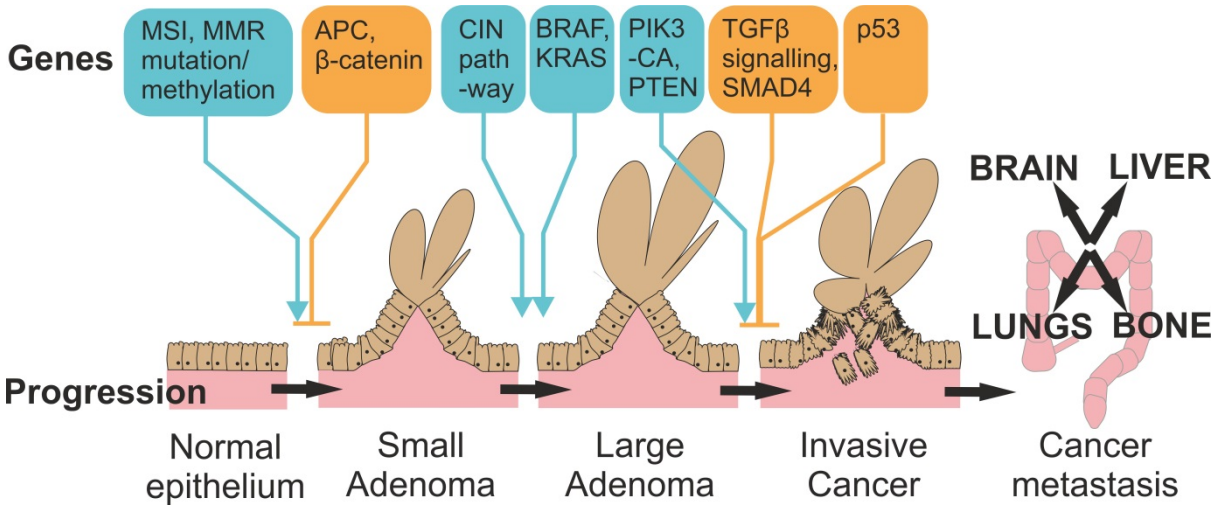


Figure 1.1 The molecular basis of colorectal cancer development

The sequential accumulation of mutations in tumour relevant genes produces an increasingly malignant phenotype characterised by progression from normal mucosa through benign and dysplastic adenomas to invasive adenocarcinomas. By contrast, the genes involved in promoting metastatic progression are relatively poorly understood. MSI, microsatellite instability; MMR, mismatch repair genes; APC, adenomatous polyposis coli; CIN, chromosomal instability; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PTEN, Phosphatase and tensin homolog; TGFβ, transforming growth factor β. Adapted from: Markowitz SD et al., 2009. [45]

1.1.4 The molecular basis of colorectal cancer development: clinical implications

Genetic and epigenetic molecular profiles are increasingly used in the clinical setting to sub-classify colorectal tumours:[74-76] Identification of molecular signatures with utility in the management of cancer promises to revolutionise our understanding of tumour biology and greatly enhance diagnostic and therapeutic strategies. Thus a molecular pathological approach to disease profiling in CRC, looking for individual and/or communal markers and profiles, when combined with modern imaging modalities, may provide a more accurate reflection of disease stage and prognosis and better inform the

multidisciplinary cancer team about the use of adjuvant therapy, heralding new opportunities for the delivery of personalised cancer care.

Improved molecular characterisation has already been translated into a more nuanced and targeted approach in CRC. Phase I and II clinical trials of the anti-epidermal growth factor (EGFR) monoclonal antibody agents cetuximab and panitumumab when used alone or in combination with chemotherapy, have been shown to be beneficial for patients with metastatic CRC. However, if *KRAS* mutation in codon 12 or 13 is detected, treatment is ineffective as *KRAS* remains constitutively active despite upstream inhibition of EGFR. In effect *KRAS* mutation status can be used to predict therapeutic response in this context.[77]

1.1.5 The metastatic cascade and colorectal cancer

Metastasis is a complex multi-step process during which certain cells acquire the capacity to break free from their sister cells, invade the extracellular matrix (ECM) and basement membrane (BM), intravasate into the circulation, evade immune detection and eventually extravasate and propagate at distant sites, establishing viable secondary tumour deposits in other organ systems.[78] This elaborate process is responsible for the vast burden of CRC-associated morbidity and mortality and recognition of this has provoked an increasing amount of research activity into the pathogenesis and treatment of metastasis, and a focus on the molecular mechanisms that underlie cancer progression. In CRC, this is particularly relevant as most deaths are not caused by the primary tumour, which is often resectable, but by metastatic disease, to which the most troublesome and intractable symptoms can be attributed and to which most patients eventually succumb.

The classic ‘late dissemination’ or ‘linear’ model of metastatic progression suggests that as a result of multiple mutations, transformed cells eventually achieve metastatic capability and break free from the primary tumour site.[79, 80] Metastatic cells then colonise distant organs provided the tumour micro-environment is receptive; the so-called ‘seed and soil hypothesis’.[81] The more contemporary ‘early dissemination model [82, 83] which recognises that metastasis is not necessarily a continuation of tumourogenesis, has two important implications; firstly that metastatic and primary tumour cells may be genetically divergent, and may therefore respond very differently

to treatment, and secondly; that the mechanics of metastatic progression are distinct from tumourogenesis and may be studied and targeted in isolation.

Biological evidence in support of early tumour dissemination is accumulating in the literature: For example, malignant epithelial cells have been found in lymph nodes, bone marrow and peripheral circulation of healthy patients with a history of early-stage cancer, but with no clinical evidence of local or metastatic recurrence.[84-86] Furthermore, analysis across a range of solid organ tumours suggests these disseminated cancer cells display highly divergent range of genetic aberrations compared with cells from the tumour of origin.[87, 88] Moreover, in a seminal study in breast, disseminated cancer cells actually displayed fewer chromosomal aberrations than the malignant tissue of origin, strongly suggesting that metastatic precursor cells are not necessarily the most genomically advanced clones within a primary malignancy.[89]

In CRC specifically, the picture is less clear. Several early studies reported marked heterogeneity in the mutational profiles of tumours compared with their corresponding lymph node or distant metastases[90, 91] however, these results contrast with more recent genomic screening data which points to high levels of mutational concordance in several key genes.[60] For *KRAS* for example, the concordance rate between primary CRC tissue and liver metastases may be as high as 94%.[92]

Recent evidence has uncovered a related biological theme, which complements the early dissemination model of tumour progression and also explains how metastatic recurrences may occur many years after successful treatment of a primary tumour. Tumour cell dormancy, a state of metabolic quiescence in which proliferation is suppressed, offers tumour cells protection from the cytotoxic effects of conventional adjuvant therapeutic agents.[93, 94] Furthermore, dormant tumour cells have been identified in secondary organs including lymph nodes and appear to exhibit relatively fewer genetic aberrations than other primary tumour cells.[88, 95, 96] Although the mechanisms underlying cellular dormancy and reactivation are somewhat lacking, this has become an important theme in cancer research, particularly in terms of metastatic progression and the optimisation of therapeutic strategies.[97]

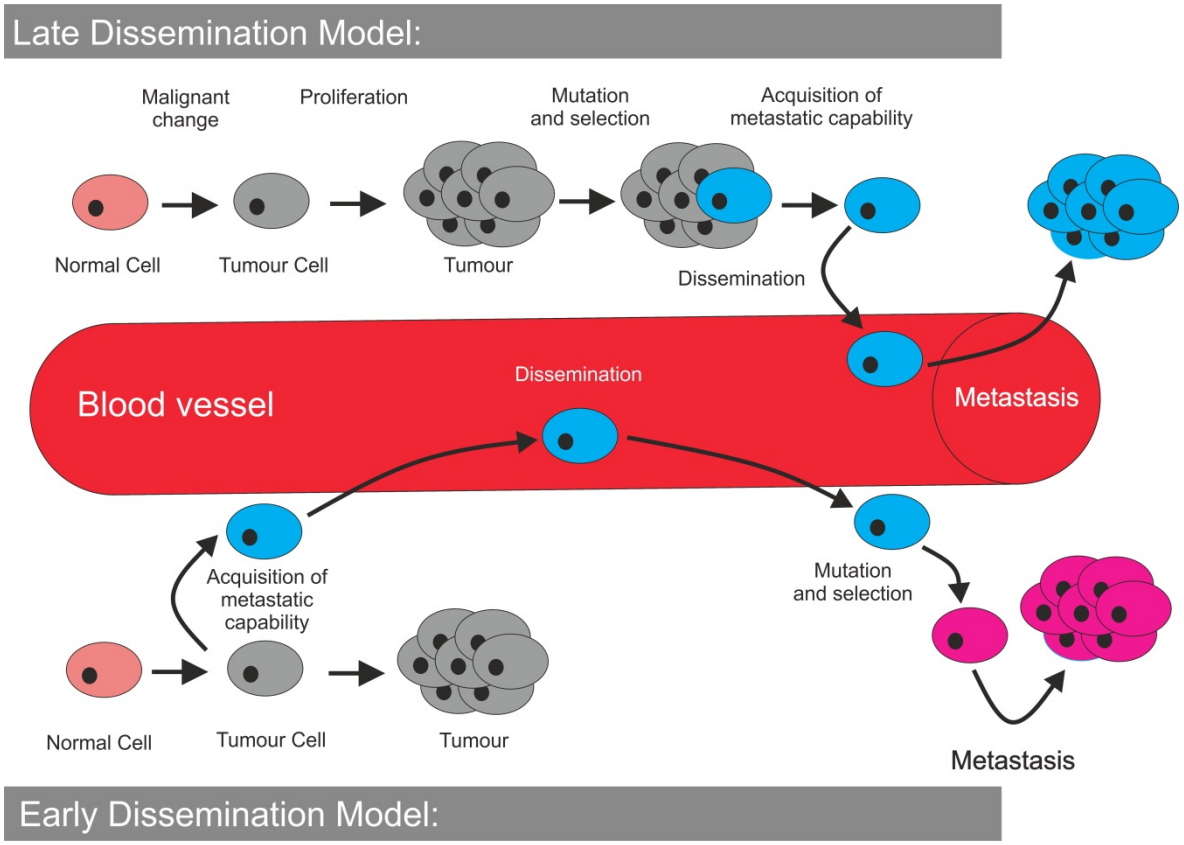


Figure 1.2 Early vs. late dissemination model of metastatic progression

The traditional late dissemination model suggests that metastasis occurs due to the progressive accumulation of genetic and epigenetic alterations within primary tumour. In which case, a high degree of genetic concordance would be expected between primary malignant tissue and associated metastases. By contrast, the early dissemination model is predicated on the concept that tumourogenesis and metastatic progression are biologically distinct processes. Thus, primary tumour and metastatic tissue, having diverged early and evolved separately, may be genetically dissimilar despite sharing the same origin. Adapted from: Klein et al., 2008.[83]

1.2 The tumour microenvironment

An emerging theme in recent years is the importance of the tumour microenvironment during tumour progression.

In the malignant state, the stroma surrounding a cancer stimulates a physiologically distinctive 'active' tumour microenvironment, maintained by various tumour-associated host cells including inflammatory cells, macrophages, immunocytes and cancer associated fibroblasts (CAFs).[98, 99] The dynamic and reciprocal interaction between these cells and the malignant epithelium promotes tumour growth/invasion, ECM remodelling and angiogenesis. [100-103]

There is mounting evidence to support the notion that chronic inflammation mediated through the stroma is also an important contributory factor in the development of cancer.[104] In colon specifically, the lifetime risk of developing invasive carcinoma is 2-4 times greater in patients with the chronic inflammatory condition Ulcerative Colitis (UC) compared with the population as a whole.[105, 106] This crucial pathophysiological link is emphasised by the fact that anti-inflammatory agents including Aspirin may reduce the overall risk of CRC development and the incidence of metastasis if introduced after CRC diagnosis.[107-111]

In addition, the stroma hosts an important immune response, balanced between the adaptive immune system (NK cells, CD8+ve and CD4+ve and other T cells) providing anti-tumour surveillance, and tumour promoting activity directed by innate immune cells, B cells and certain T cell subtypes, which suppress tumour-cytotoxicity and support malignant growth and angiogenesis.[112]

1.2.1 Cancer associated fibroblasts

The key cellular constituent of the transformed tumour microenvironment are CAFs.

CAFs are a heterogeneous population of cells which include myofibroblasts. The two terms are often used interchangeably because in effect, they represent phenotypically similar cell populations[99] however, myofibroblasts are defined specifically by the presence of mesenchymal markers including α-SMA, deposited in characteristic stress-fibre formations; the stress-fibre regulatory protein paladin 4Ig; the intermediate

filament vimentin; the transmembrane glycoprotein podoplanin; the Type I collagen maturation enzyme P4H and the absence of epithelial and endothelial markers such as cytokeratin and CD31 respectively.[113] In contrast, the term CAF is usually used to describe morphologically and functionally similar α-SMA expressing cells within the tumour micro-environment, which do not express the full complement of secondary markers.[98]

Myofibroblasts originate from a variety of stroma-resident progenitor cells which may include fibroblasts, smooth muscle cells, stellate cells, adipocytes and epithelial cancer cells,[114-116] and from migratory, bone marrow derived mesenchymal stem cells and CD34+ve fibrocytes[115] which in certain tumour contexts may contribute as much as 25% of the CAF population.[117]

TGF-β1, produced by the malignant epithelium is the dominant and best characterised fibroblast-to-myofibroblast transdifferentiation signal.[103, 118, 119] Mature myofibroblasts reciprocate by modulating the make-up of the tumour microenvironment to enhance invasion and metastatic progression. Myofibroblasts produce various secreted factors including: matrix metalloproteinase enzymes (MMPs) which stimulate ECM remodelling;[120, 121] COX products;[122] vascular endothelial growth factor (VEGF) which promotes angiogenesis; and cytokines and growth factors including IL-6, IGF-1, fibroblast growth factor (FGF), scatter factor/hepatocyte growth factor (SF/HGF), stromal cell derived factor (SDF) insulin like growth factor (IGF) and TGFβ1 which promote cancer cell proliferation and invasion.[100-102, 123-128]

To illustrate the importance of stromal myofibroblasts during *in vivo* tumour progression, Orimo and colleagues used mouse xenograft models to demonstrate that CAFs co-injected with MCF7 mammary tumour cells were associated with significantly increased tumour growth and angiogenesis compared with normal fibroblasts harvested from the same patients.[102] Furthermore, in human subjects, the increasing abundance of stromal CAFs in stage II and III CRC specimens, is associated with deteriorating prognosis and reduced disease free survival.[129]

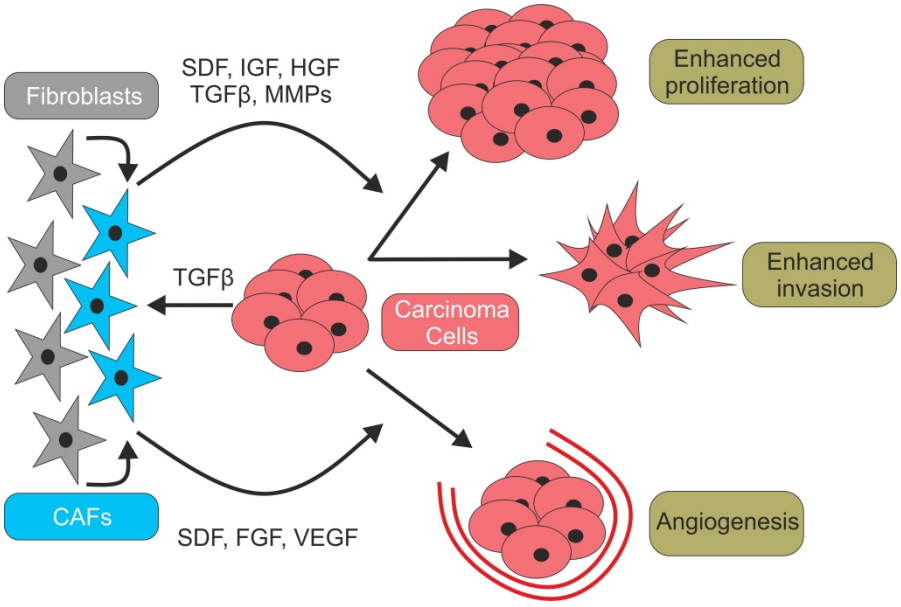


Figure 1.3 The biological impact of cancer associated fibroblasts during cancer progression

Paracrine signals such as TGFβ from transformed epithelial cells provoke profound biological and phenotypic change in fibroblasts. Cancer associated fibroblasts (CAFs) which include myofibroblasts, produce a host of secreted factors which contribute to a permissive tumour microenvironment which promotes angiogenesis, enhanced tumour invasion and increased tumour cell proliferation. Secreted factors include: Stroma cell derived factor (SDF;CXCL12); fibroblast growth factor (FGF); vascular endothelial growth factor (VEGF); insulin-like growth factor (IGF); TGFβ; hepatocyte growth factor (HGF) and matrix metalloproteinase enzymes (MMP). Adapted from Shimoda et al., 2010 [130]

This relationship between TGFβ signalling and the stromal response during CRC initiation and progression may in fact hold the key to an important paradox identified in the literature:

TGFβ has a protective influence in early colorectal carcinogenesis, and mutational inactivation of the TGFβ signalling is a crucial event during the transition from adenoma to invasive adenocarcinoma.[131] Yet high levels of TGFβ1 in the serum of patients with CRC is associated with poor clinical outcome, and in other epithelial

malignancies, where TGFβ signalling is generally preserved, TGFβ triggers a variety of prometastatic biological events.[132, 133]

One possible explanation for this observational inconsistency, points to a central role for TGFβ in the transformed tumour microenvironment rather than CRC cells during disease progression and the formation of metastasis:

In a seminal paper, Calon and colleagues demonstrated that indeed, high TGFβ expression in human CRC specimens was associated with a significantly greater risk of disease recurrence.[134] Furthermore, they showed that genes enriched in stromal cells, including fibroblasts, following exposure to TGFβ, also predicted short disease free survival in all pre-metastatic stages of CRC. However, perhaps more importantly, functional studies in mice, using CRC cells engineered to constitutively express TGFβ1 demonstrated not only that TGFβ increases the rate of tumour xenograft 'take', but also the incidence and size of liver and lung metastasis. Crucially, this predisposition to metastasise observed in CRC cells in the presence of TGFβ was mediated by the reactive production of IL11 from tumour associated fibroblasts in a manner which could potentially be inhibited with drugs.[134]

1.2.2 Genomic and epigenetic changes in tumour-associated stroma

It is accepted that the pro-invasive, pro-inflammatory phenotype adopted by tumour-associated stromal cells such as myofibroblasts is in response to paracrine signals from transformed epithelium; however it is not clear to what extent if any, the opposite holds true: that *de-novo* mutations or epigenetic changes in stromal cells initiate tumourogenesis in adjacent epithelial cells.

As discussed previously, evidence from everyday clinical practice supports a functional association between the stroma mediated inflammatory response and the onset of cancer. Although on the molecular level, this relationship remains largely obscure, the critical observation that cultured CAFs remain phenotypically stable without continued exposure to tumour cells, has focused attention on the potential contribution of genetic or epigenetic aberrations in cancer-associated stromal cells.[102, 135]

Bian and colleagues[136] examined the laser microdissected (LMD) stroma of formalin fixed paraffin embedded (FFPE) CRC specimens from 3 patients previously identified as carrying the TGFβ Receptor1*6A (TGFBR1*6A) tumour susceptibility allele; a sporadically acquired mutation which confers tumour cells with a selective growth advantage in the presence of TGFβ.[137] DNA was extracted, amplified by PCR and sequenced, revealing the mutation was present concomitantly in stromal cells in all 3 cases raising the intriguing possibility that somatic changes in cancer cells and CAFs might co-evolve.[136] Using similar methodology, Patocs et al.,[138] identified isolated *p53* mutations in 11/43 (25.6%) hereditary and 34/175 (19.4%) sporadic LMD stromal breast cancer specimens. Kurose and colleagues identified *p53* and *PTEN* (encoding phosphate and tensin homolog) mutations in 25/50 (50%) and 15/50 (30%) breast cancer specimens respectively,[139] however an alternative approach using fresh frozen tissue and cultured mammary CAFs produced contradictory results as un-mutated wildtype *p53* was identified in all samples examined (n=17).[140]

Other reports identifying loss of heterozygosity (LOH), MSI and gene copy number changes in stromal cells of various tumour types,[141-143] contrast with studies which suggest clonally selected somatic gene alterations are extremely rare:[144, 145] Having used single nucleotide polymorphism (SNP) arrays to examine fresh frozen LMD stromal tissue as well as cultured breast and ovarian cancer-associated CAFs, the authors of those studies suggested that given the technical limitations associated with allelic-imbalance profiling in FFPE tissue, the high frequency of genomic alterations previously described may have been artifactual.[140 144]

Despite conflicting data, it is likely that somatic mutations in stromal cells do occur however infrequently, but it remains uncertain whether this is sufficient to drive the acquisition and clonal expansion of the myofibroblast phenotype or trigger malignant change.

The epigenetic changes in reactive tumour-associated stroma are less controversial. Discrete areas of DNA hypermethylation in the context of global hypomethalation, has been identified in a number of tumour types.[144, 146] In prostate cancer for example, the glutathione S-transferate P1 (*GTSP1)* gene promoter is methylated in >90% of tumours. In a study of laser-microdissected tissue from multiple locations within the prostate, *GTSP1* promoter methylation was consistently identified in malignant epithelial cells.

Interestingly in cancer-associated stromal cells, methylation was also identified at some but not all equivalent locations within the gland, suggesting that epigenetically distinct clusters of otherwise histologically normal cells may exist within the tumour microenvironment.[146]

In breast cancer, comprehensive analysis of methylation profiles in epithelial and myoepithelial cells and stromal fibroblasts isolated from normal breast tissue, ductal carcinoma *in situ* and invasive breast carcinomas, demonstrated distinct epigenetic changes in all three cell types.[147] In a further analysis of 143 human breast cancer specimens, significant differences were identified in the methylation status of 5 genes in laser microdissected stroma from HER-2/neu hormone receptor positive vs. negative tumours. HER-2/neu receptor positive tumours have greater metastatic potential and are less responsive to standard chemotherapy, and by implication, the authors of the study suggested that altered methylation in stromal cells may contribute to this more aggressive phenotype. However, no mechanistic evidence to support this position was provided.[148]

These aberrant stromal methylation patterns may be driven by interaction with transformed cancer epithelial cells. Tyan and colleagues demonstrated that normal-tissue breast fibroblasts co-cultured with breast cancer cells acquired the ability to induce enhanced epithelial invasion through upregulated ADAM metallopeptidase with thrombospondin type 1 motif (ADAMTS1) activity, linked to the progressive methylation of the *ADAMTS1* promoter associated histone H3K27.[149]

Crucially however, there is also some evidence from animal models which suggests that intrinsic disruption of epigenetic regulation in stromal cells, although rare, may be sufficient to initiate *de-novo* tumourogenesis. Through expression of the gene High-mobility group AT-hook 2 (*Hmga2*) in mesenchymal stromal cells, Zong and colleagues were able to induce prostate intraepithelial neoplasia in adjacent normal prostate tissue and even frank neoplasia in a paracrine wnt-dependent manner when Hgma2 and androgen receptor (AR) were co-induced.[150] Hgma2 is potent epigenetic regulator which binds AT-rich DNA and interacts with histone modifying enzymes.[151]

In summary, it is clear that epigenetic changes are a feature of tumour-associated stromal cells; however the role this may play in the establishment of an abnormal

tumour microenvironment, and the potential functional consequences in terms of tumour initiation or progression, are yet to be uncovered.

1.2.3 Targeting the stroma: a promising therapeutic strategy

The tumour microenvironment has attracted particular attention in recent years as recognition of its importance during disease progression has made the development of stroma-targeted therapies an attractive prospect.[152, 153]

CAFs are particularly promising in this respect, as they powerfully promote tumour growth/invasion and angiogenesis through conserved intracellular signalling pathways in a manner which is not initiated by *de novo* mutations. This means they are likely to respond consistently to a targeted treatment and are unlikely to escape drug capture or develop resistance through genomic instability pathways. Furthermore, CAFs and normal fibroblasts are distinguishable on physiological, genetic and epigenetic grounds, which is a pre-requisite for selective therapeutic interventions; and because the tumour stroma impedes effective anti-tumour immune surveillance,[154] synergistic targeting of CAFs may prove an effective boost to both conventional treatment and newer immunotherapy regimes.

Broadly speaking, 2 stroma-targeted therapeutic strategies have been explored, firstly; inhibiting epithelial/stroma/ECM signalling, and secondly; modifying the stromal immune response.

The best characterised inhibitor of stroma/epithelial signalling is bevacizumab, a humanised recombinant IgG monoclonal antibody (mAb) that inhibits VEGF, produced in part by activated stromal cells, from binding the tyrosine kinase (VEGFR) receptor. Bevacizumab is the only agent which specifically targets the VEGF pathway, which is approved for the treatment of CRC by the US Food and Drug Administration (FDA).[155] Current use is restricted to metastatic disease, and as a first line therapy in conjunction with irinitecan, flurouracil and leucovorin, administration of bevacizumab was associated with 10.6 months progression-free survival compared with 6.2 months in bevacizumab naive patients ($p<0.001$).[7]

De Wever and colleagues demonstrated that myofibroblast derived conditioned medium (CM) stimulated c-Met/Rac mediated CRC cell invasion in a SF/HGF dependent

manner.[101] To achieve this they used NK4, a competitive antagonist for HGF-met association composed of NH_2-terminal hairpin domain and 4 kringle domains of the HGF α-chain. Subsequently, ectopic NK4 expression, induced in MC-38 CRC cells was shown to reduce liver metastases to 27% of the control value and reduce intracapsular invasion in nude mice models.[156] An alternative approach, using mice bearing intracranial glioma xenografts, demonstrated reduced angiogenesis and dramatic tumour regression associated with systemic administration of anti-HGF monoclonal antibodies (mAb).[157] However, rilotumumab, a fully human monoclonal antibody against HGF developed subsequently, was not associated with significant anti-tumour activity in human subjects with high grade recurrent glioblastoma.[158]

A number of targeted therapies have been developed predicated on the principle that malignant invasion can be prevented by inhibiting degradation of the ECM.

MMPs are a family of zinc dependent enzymes, produced by CAFs, which sit at the heart of a network of tumour promoting pathways and play a pivotal role in disease progression by promoting ECM remodelling, angiogenesis and altered cell-cell and cell-ECM interaction.[159] However, despite showing initial promise, the use of synthetic MMP inhibitors in human trials has proven disappointing.[160]

Urokinase plasminogen activator (uPA) is mainly expressed by tumour-associated stromal cells including CAFs, and converts plasminogen to plasmin, which degrades a number of ECM proteins, most notably collagen IV. Å6 is an 8 amino-acid inhibitory peptide which interferes with uPA activity and has anti-invasive and anti-angiogenic properties.[161] A limited phase II human trial which administered subcutaneous Å6 to women in remission from ovarian cancer in response to an asymptomatic rise in CA125, demonstrated a statistically significant delay in the time to clinical progression, however subject numbers were small (placebo, n=12; low-dose, n=8; high-dose, n=4)[162] and Å6 has not penetrated further into clinical practice.

Fibroblast activation protein (FAP) is an inducible cell surface glycoprotein, which supports ECM remodelling through type I collagenase activity, integrin engagement and induction of MMP2 activity.[163, 164] Inhibition of FAP by the mAb sibrotozumab showed limited efficacy in early trials and no overall benefit in phase II trials for metastatic CRC.[165, 166] However, FAP remains a potentially useful stromal target because it is

expressed in a highly selective manner by CAFs and there is evidence to suggest that specifically targeting the proteolytic activity of FAP may prove a more effective strategy.[167]

FAP+ve stromal cells may have a broader influence as they inappropriately suppress the immune response to tumour cells mediated by interferon-γ and tissue necrosis factor-α.[168] Furthermore, evidence suggests that the tumour-associated stroma may impede effective immune surveillance by acting as a barrier to antigen presentation and immune recognition by T-cells.[169] In a key experiment, cancer cells escaped destruction by adoptively transferred effector T-cells if antigen-loaded cells within the stroma were not also targeted and destroyed.[170] As the stroma significantly enhances tumourogenicity,[169] targeted destruction by immunotherapy, may also become an effective anti-cancer tool.

1.3 MicroRNA

The traditional model of carcinogenesis in which genetic and epigenetic transcriptional aberrations drive malignant transformation, has been revised following the discovery of novel microRNA (miRNA) dependent mechanisms acting at the level of oncogene/tumour-suppressor gene translation.

1.3.1 MicroRNA overview

MiRNAs are a class of highly conserved, non-coding 18-25 nucleotide RNA molecules which provide widespread expressional control through translational repression of mRNA.

Until the true nature of miRNAs was uncovered, these small but abundant molecules were dismissed as 'junk' RNA, however in 1993 the perception changed when Lee and colleagues[171] recognised that lin-4, a gene which did not encode protein but was essential for the normal development of the nematode *Caenorhabditis elegans*, negatively regulated LIN-14 protein expression instead. As lin-4 and the 3'untranslated region (3'UTR) of lin-14 mRNA contained elements of sequence complementarity, they suggested regulation occurred post-transcriptionally through antisense RNA-RNA interactions.

Since then, over 25,000 miRNAs in 193 species have been identified, experimentally validated and registered in the miRBase database.[172, 173] Each is assigned a unique name following a standard nomenclature consisting of 3 components: (1) A prefix which denotes species such as 'hsa-' for *Homo sapiens;* (2) mir or miR, denoting immature or mature miRNA status respectively and; (3) a numerical suffix indicating the order of discovery.[174]

MiRNA genes account for 2-5% of the human genome, and are commonly clustered within the introns of protein coding genes.[175] Bioinformatic studies estimate that each miRNA interacts with hundreds of mRNA targets and as a consequence, the miRNome may regulate more than 30% of all human genes.[176-178]

As well as regulating fundamental processes including cellular differentiation, proliferation and apoptosis, miRNAs have been implicated in the pathogenesis of numerous malignancies.[179] Growing numbers of oncogenes and tumour-suppressor

genes are found to be under miRNA control[180] and more than 50% of miRNA genes appear to be located at fragile sites or within genomic regions frequently altered in cancer cells.[181] Furthermore, miRNAs have promising clinical applications and form the basis of emerging therapeutic, diagnostic and prognostic tools in cancer.[182, 183]

1.3.2 MicroRNA biology and function

Primary miRNA transcripts (pri-miRNAs) are transcribed by RNA polymerase II. Many miRNAs are encoded within introns of protein-coding genes, but have distinct transcriptional start sites, whereas others share the promoter sequence of their hosts.[184, 185] MiRNA in close proximity to one another may be co-transcribed, with regulation of individual miRNAs within a cluster occurring at the post-transcriptional level.[186]

Pri-miRNAs which are typically >1kb in length are processed into 70-nucleotide stem-loop structures called pre-miRNAs by the ribonuclease Drosha, which is contained with multiple co-factors within a 'microprocessor' complex.[187] Pre-miRNAs are transported from the nucleus by the RAN GTP-dependent transporter exportin 5,[188] and undergo further processing by the cytoplasmic endonuclease enzyme Dicer to form mature, 18-25 nucleotide miRNAs.[189] One strand of mature miRNA associates with Argonaut 2 (Ago) and TNRC proteins, becomes incorporated into the RNA-induced silencing complex (RISC)[190] and through partial sequence complementarity interacts with the 3'UTR of target mRNAs to bring about translational repression or mRNA degradation, in a manner comparable to exogenous short interfering RNAs (siRNA):[191 192] MiRNAs bind their target mRNA through Watson-Crick base pairing between their 2nd and 8th nucleotides at the 5' end (the so called 'seed-sequence').[178] When it occurs, extensive pairing complementarity may induce heavy mRNA suppression via Ago dependent catalysis, however this may not be the dominant mechanism of action of miRNAs.[193] In fact, more profound biological consequences may arise through incomplete seed-binding, disrupting the initiation of translation of potentially hundreds of mRNAs simultaneously.[191, 194] As a consequence, miRNAs may reduce target protein expression without necessarily impacting significantly on the abundance of associated mRNA.

Interestingly, some studies have identified that miRNAs may also target the 5'UTR of protein coding genes to induce translational activation not repression, potentially

revealing a further layer of complexity with miRNA mediated regulatory apparatus.[195, 196]

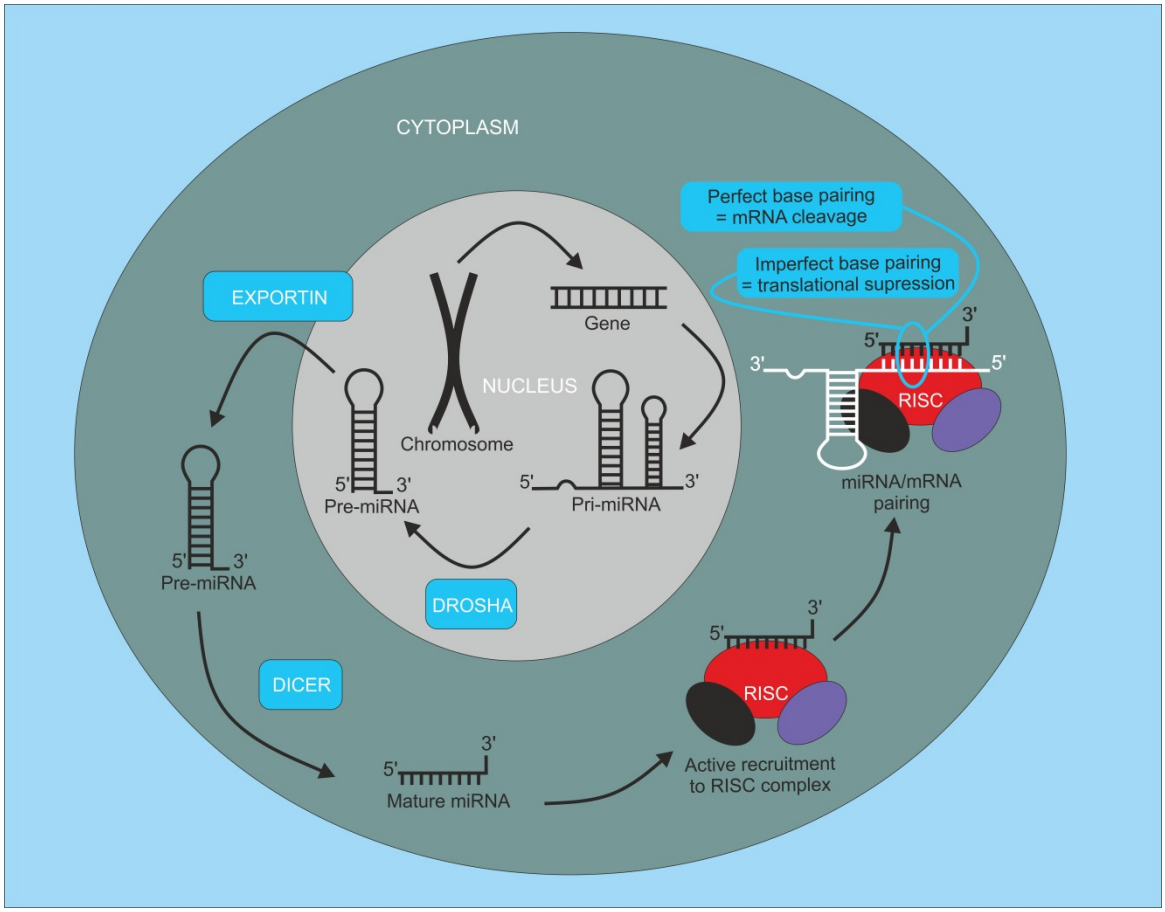


Figure 1.4 MicroRNA biogenesis and mechanism of action

Primary miRNA transcripts are converted to 70-nucleotide stem-loop structures called pre—miRNAs by the ribonuclease 'Drosha'. Pre-miRNAs are exported from the nucleus and further processed in the cytoplasm by the endonuclease enzyme 'Dicer' to form 18-22 nucleotide mature miRNAs. These mature sequences are incorporated into the RNA-induced silencing complex (RISC) to be presented to and bind with complementary mRNAs. Perfect miRNA-mRNA sequence complientarity induces mRNA cleavage, whereas imperfect binding inhibits mRNA translation. Adapted from: Aslam et al., 2009 [197]

1.3.3 MicroRNA and carcinogenesis

MiRNAs exert a pathogenic effect during carcinogenesis through targeted interactions with tumour-suppressor gene or oncogene encoding mRNA. Deregulated miRNA expression is a consistent feature of malignant transformation, however having

identified miRNAs with putative pathogenic roles in cancer, very few studies go onto characterise the genetic or epigenetic defects which may have set these events in motion and as a result the molecular mechanisms underlying miRNA deregulation in cancer are relatively poorly understood.

Notwithstanding, the question of whether mutations in miRNA encoding genes trigger malignant transformation remains an important one, not only because the key to developing targeted therapies lies in a deeper understanding of molecular pathogenesis, but also because distinguishing between a causal or supporting role for individually deregulated miRNAs helps to prioritise relevance within the context of complex synchronous tumour-promoting biological events.

In cancer, miRNAs may be aberrantly induced or supressed. MiRNA gene silencing through genomic instability pathways has been documented, but generally in solid organ tumours, miRNA expression is upregulated due to transcriptional activation or amplification of miRNA encoding genes.[181, 183, 198, 199, 200]

In one study, miRNA genes in human cancer specimens were analysed by array-based genomic hybridisation methods, revealing that a high proportion exhibit DNA copy number increases, ranging from 37.1% in ovarian cancer to 85.9% in malignant melanoma specimens.[201] Another study identified a high frequency of miRNA genes in cancer-associated genomic regions including fragile sites and common breakpoint regions.[202]

However direct evidence that mutations in miRNA encoding genes may translate into a malignant phenotype was lacking until 2005 when Carlo Croce and colleagues published a study in the *New England Journal of Medicine* in which the genomic sequence of 42 miRNAs in chronic lymphocytic leukaemia (CLL) specimens were profiled, revealing germ-line or somatic mutations in 11/75 CLL patients compared with 0/160 normal controls.[203] The group went on to look at the miR-15/16-1 cluster specifically because it resides at 13q14.3, a location frequently deleted in CLL.[204] MiR-15a and miR-16, downregulated in the majority of CLL specimens were shown to target the mRNA of anti-apoptotic protein BCL2, which is over-expressed in the malignant B cells of CLL. Furthermore, restoration of miR-15a/16-1 induced apoptosis in MEG-01 megakaryocytic leukaemia cells, data which when considered together, supports an inherited or somatically acquired oncogenic mechanism resulting from the loss of tumour suppressing miRNA genes.[205]

Epigenetic mechanisms including aberrant CpG islands hypermethylation and histone modifications have also emerged as important regulators of miRNA expression. In an integrated review of 45 articles describing epigenetically regulated miRNAs in cancer, human oncogenic miRNAs (oncomiRs) showed an order of magnitude higher methylation frequency (11.6%; 122/1048 known miRNAs) compared to protein coding genes. Nearly half (45%; 55/122) of epigenetically regulated miRNAs were associated with multiple cancer types. The other 55% (67/122 miRNAs) were epigenetically regulated in a single cancer type raising the possibility of using miRNA gene-associated methylation profiles as cancer-specific biomarkers.[206]

In recent years a third possible mechanism of miRNA deregulation in cancer has been described, which involves mutations within the genes encoding molecular components of the miRNA processing machinery.[207, 208] In an examination of high MSI CRC specimens, 27% harboured mutations in one or more of the genes encoding the RISC complex constituent proteins *AGO1, AGO2, TNRC6A, TARBP2* and *TNRC6C* and the nuclear exporter gene *EXPORTIN5*. Loss of Ago2 and TNRC6A expression was described in 35% and 54% of CRC specimens respectively; suggesting frameshift mutations in miRNA regulation-related genes may also contribute to miRNA deregulation and tumourogenesis in a subset of CRC patients.[209]

1.3.4 MicroRNA and colorectal cancer pathogenesis

In CRC, aberrantly expressed miRNAs derail a number of cellular signal transduction and cell survival pathways including the Wnt/β-catenin pathway, EGFR pathway, and *p53* function, tying miRNA biology to known mutational events in the classical adenoma-carcinoma sequence of malignant transformation and potentially to each of the hallmarks of cancer.[183]

Further evidence that miRNAs play roles in the sequential molecular events which lead to CRC comes from a microarray based profiling study of 866 miRNAs in 69 colorectal specimens, which revealed 2 overarching but distinct patterns of differential miRNA expression during the transition of normal mucosa to adenoma and through to adenocarcinoma. The largest miRNA grouping (n=108) were altered during the earliest step from normal mucosa to lesions displaying low grade dysplasia, of which 36 remained differentially expressed throughout the entire sequence to adenocarcinoma.

The second group consisted of 96 miRNAs which were altered only in highly dysplastic adenomas and adenocarcinoma or in adenocarcinoma alone.[210]

1.3.4.1 WNT pathway activation

A number of approaches have been employed to investigate the potential pathogenic roles of miRNAs during WNT signalling.

In an early study, a GFP sensory vector containing the 3'UTR of the *APC* gene was used to interrogate a miRNA library for potential regulatory interactions. MiR-135a+b both targeted APC and induced upregulated activity in the wnt/β-catenin cascade. Crucially, miR-135 was found to be increased in human CRC tissue compared with normal colon epithelium and *in vivo* expression correlated inversely with APC mRNA.[211]

A key activity downstream of APC regulatory suppression is the formation of bi-partite β-catenin/TCF complexes which recruit co-regulatory molecules to activate or supress the expression of numerous target genes. Schepeler et al.,[212] used an inducible CRC cell system to identify miRNAs deregulated in the context of disrupted TCF/β-catenin coupling. Their most important finding was that miR-30, miR-139, miR-145 and miR-126 were significantly induced, and this led to inhibited growth in functional cell assays and widespread changes in protein expression. Furthermore, elements responsive to TCF/β-catenin control were downregulated in CRC compared with paired normal tissue suggesting the pathogenic consequences of constitutive wnt/β-catenin activation *in vivo,* may in part be mediated by miRNAs. [212]

The transcription factor C-MYC also lies downstream of TGF/β-catenin control and positively regulates the oncogenic miR-17-92 miRNA cluster. Except for miR-18a all members of this miRNA family are upregulated during the transition from adenoma to CRC, through gene copy number increase and enhanced c-myc expression.[213]

In contrast YY1 (Yin Yang 1) regulates upstream elements of this cascade including wnt and β-catenin. Crucially, the 3'UTR of YY1 is targeted directly by miR-7, and over-expression of miR-7 leads to supressed proliferation and increased apoptosis in CRC cell lines. The role of miR-7 as a tumour suppressor was characterised by Zhang et al., who also identified downregulated expression of the gene in human CRC specimens compared with paired normal colonic tissue, and in various CRC cell lines.[214]

1.3.4.2 EGFR signalling activation

EGFR, consisting of an extracellular ligand-binging domain and an intracellular domain with tyrosine kinase activity, regulates two important intracellular signalling pathways (KRAS/BRAF/ERK and PI3K/AKT) responsible for cancer cell proliferation, survival, invasion and metastasis.[215]

EGFR activation suppresses expression of miR-143 and miR-145 in HCT-116 CRC cells.[216] MiR-143 and miR-145 act as tumour suppressor genes, and are generally downregulated in CRC. Along with let-7 they negatively regulate KRAS.[217, 218] In contrast, activation of KRAS in a CRC cell model induced a host of miRNAs with oncogenic potential, including miR-181, miR-200c and miR-210.[219]

BRAF, MEK and ERK are also induced by KRAS, and similarly co-regulated by the tumour suppressor miR-143.[217, 220] In addition, BRAF and MEK are targeted by miR-145, and ERK by miR-129 which are significantly downregulated in CRC. [217 221]

As discussed previously, aberrant PI3K signalling is a common feature of CRC. A specific mutation identified in a sub-set of patients in the 3'UTR of *PIK3CA* gene itself, is associated with decreased binding affinity for miR-520a and miR-525, resulting in amplified PI3K signalling and enhanced tumour growth *in vitro*.[222] Furthermore, the PIK3CD subunit of PI3K is a direct target of miR-30a, a miRNA downregulated in CRC specimens with lymph node metastasis compared with non-metastatic CRC specimens.[223] In a study by Zhong et al., CRC cell lines recruited from metastatic tissue (SW620s) demonstrated lower levels of miR-30a expression than cells originating from primary tumour tissue (SW480s). Furthermore, miR-30a upregulation significantly reduced the capacity of SW620 cells to migrate and invade in transwell assays, just as capacity was enhanced by miR-30a suppression in SW480 cells.[223]

AKT and mTOR activity is induced by canonical PI3K signalling, and both elements are co-regulated by miRNAs which are corrupted in CRC. The 3'UTR of mTOR for example, has 2 binding sites for miR-144, a tumour suppressor gene which when found in low abundance in primary CRC tissue, is associated with increased liver metastasis and significantly impaired prognosis.[224] Similarly, AKT2 is targeted by miR-203, a miRNA frequently suppressed in CRC, which correlates inversely with tumour size and TNM stage *in vivo*.[225, 226]

Other upstream regulators and downstream effectors of PI3K signalling are equally enmeshed in regulatory miRNA networks.

PTEN and PDCD4 are key tumour suppressors in CRC and they are regulated by perhaps the most thoroughly investigated oncomiR in the literature; miR-21. PTEN regulates cell-cell interactions and the migratory potential of CRC cells *in vitro* and their metastatic potential when injected into the tail-veins of mice.[227] MiR-21 induces cell proliferation and invasion by directly targeting the 3'UTR of PTEN mRNA, subjecting it to regulatory suppression and consequently activating AKT signalling.[228, 229] Similarly, the PDCD4 3'UTR has an evolutionary conserved target sequence for miR-21and the expression of miR-21 and PDCD4 is inversely correlated in CRC tissue *in vivo*.[230]

Recently, a further family of tumour suppressor genes have been identified as miRNA targets in CRC. Forkhead box O (FOXO) proteins are transcription factors characterised by the presence of 'Forkhead-box' DNA binding domains.[231] Phosphorylation by AKT inhibits FOXO transcriptional functions contributing to cell survival, proliferation and growth, and aberrant activation of the PI3K/AKT pathway of which FOXO sub-family member FOXO3 is an important component, drives CRC pathogenesis.[232-234]

MiR-153 is significantly upregulated in CRC specimens compared with paired 'normal' colonic epithelium, and expression further increases with progressive tumour stage. miR-153 mediates chemoresistance to cisplatin in CRC cells though a direct effect on FOXO3 expression and crucially, FOXO3 and miR-153 expression are inversely correlated *in vivo* in human CRC specimens.[235]

FOXO3 phosphorylation is also mediated through the tumour suppressors PHLPP1 and PHLPP2 (PH domain leucine-rich-repeats protein phosphatase 1 + 2) which antagonise PI3K/AKT signalling.[236] Another strongly upregulated miRNA in CRC compared with paired normal tissue, is miR-224 which directly targets PHLPP1+2, and drives CRC cell proliferation both in vitro and in sub-cutaneous xenograft mouse models.[237]

1.3.4.3 TGFβ response inactivation

MiR-224 is increasingly recognised as an important player during CRC pathogenesis. Identified in an early microarray profiling study amongst the most upregulated miRNAs in human CRC,[238] miR-224 is associated with impaired clinical outcomes and an increasingly malignant cellular phenotype.[239] An important target of miR-224 is

SMAD4, a central mediator of the TGFβ pathway, suggesting that miR-224 upregulation in CRC may attenuate the early protective transcriptional response of TGFβ signalling.[239, 240]

In a further attempt to dissect the pathogenic impact in CRC of miRNAs relevant to TGFβ signalling, Lui and colleagues examined the miR-130ac/301ab/454/721cluster of genes which share an identical SMAD4 3'UTR seed binding sequence. Their analysis identified upregulated expression of this miRNA family in primary CRC specimens and furthermore they demonstrated an enhanced proliferative capacity associated with ectopic expression in multiple CRC cell lines. They did not however, clearly establish that this effect was mediated through SMAD4 suppression.[241]

The TGFβ receptor 2 (TGFβR2) is directly targeted by miR-21, further emphasising the breadth of influence of this important oncomiR.[242] TGFβR2 is well known as a tumour suppressor and somatic mutations within this gene, present in around 30% of cases, are recognised to increase significantly the risk of CRC development.[131, 243] Intriguingly, Yu et al., suggested that miR-21 dependent downregulation of TGFβR2 may result in the expression of 'stem-like' cellular properties, which may be one mechanism through which CRC cells achieve their metastatic potential.[242] However, the importance of miRNAs during CRC progression, including the role of cancer stem-cells, forms the subject of a later section and will not be discussed in greater detail here.

1.3.4.4 p53 function

The *p53* gene sits at the centre of a complex signalling network involved in maintaining genomic stability and preventing tumour formation. Recently it has emerged that miRNAs are an integral component and during malignant transformation, deregulated miRNA expression may corrupt this essential tumour suppressive molecular apparatus.

For example in CRC cells, direct negative regulation is achieved by miR-504 at 2 sites within the 3'UTR of p53. Furthermore, ectopic miR-504 expression has been shown to both reduce *p53* mediated cell cycle arrest in HCT116 cells *in vitro* and promote tumour proliferation *in vivo*. However, the authors of this study failed to quantify miR-504 expression in human CRC tissue and thus, the pathological relevance of their findings is not clear.[244]

Perhaps the best characterised miRNA-p53 interaction is with the conserved miR-34a-c family, which was identified by comparing miRNA expression in *p53* wild-type and mutated HCT116 CRC cells following exposure to DNA damaging agents. Upregulation of miR-34a in particular promoted cellular apoptosis and impacted powerfully on the expression of a host of genes involved in cell-cycle regulation. [245] Thus, miR-34a appears to cooperate with and be induced by the tumour suppressor p53, leading to the downstream suppression of numerous target genes include CDK4/6, BCL2 and SIRT1.[245-248] Crucially, downregulated expression of miR-34a is frequently identified in human CRC specimens.[249]

Recently in CRC a number of other miRNAs have been implicated in p53 dependent cell cycle arrest *in vitro* including miR-16 and miR-192.[250, 251] Their proposed involvement during Vogelstein's adenoma-carcinoma sequence is summarised in figure 1.5.

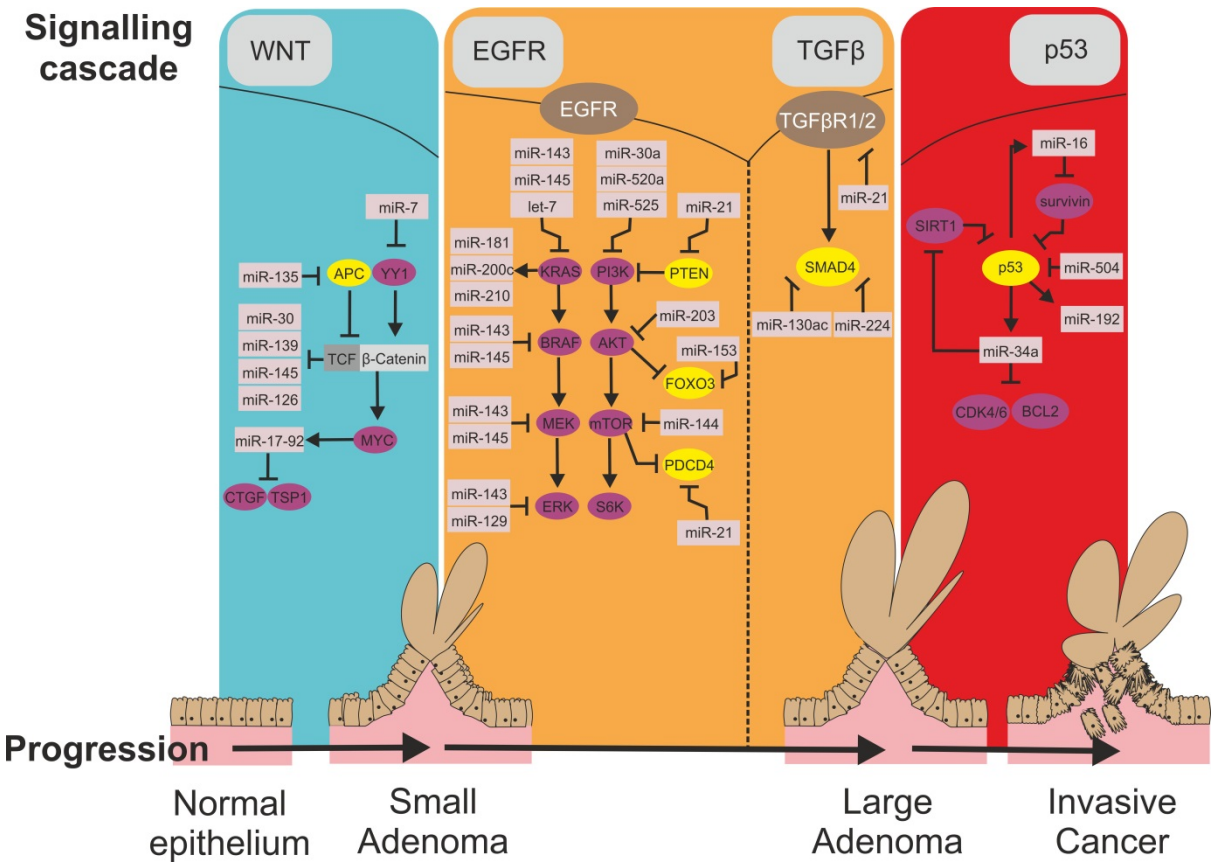


Figure 1.5 Proposed involvement of microRNAs during Volgenstein's adenoma-to-carcinoma sequence

MiRNAs are implicated at each stage of Volgenstein's adenoma-to-carcinoma sequence. CTGF, connective tissue growth factor; TSP1, thrombospondin; YY1, Yin Yang 1; MEK, Mitogen activated protein kinase; mTOR, mammalian target of rapamycin; PTEN, Phosphate and tensin homolog; FOXO3, Forkhead box 03; PDCD4, programmed cell death 4; SIRT1, sirtuin 1; CDK4/6 cyclin-dependent kinase 4/6; BCL2, B-cell lymphoma 2.

Although an increasingly detailed impression is emerging of the complex web of interaction between deregulated miRNAs and the intracellular signalling cascades which trigger malignant transformation in CRC, comparatively little is known about the role of miRNAs during disease progression. Relevant areas of research regarding the impact of miRNAs during the metastatic cascade, with an emphasis on CRC, are outlined in the following section.

1.3.5 MicroRNA and cancer progression

MiRNAs have established roles in cancer pathogenesis. Deregulation of miRNAs in malignancy has profound consequences as individual miRNAs target multiple genes and are capable of inducing broad downstream and feedback effects simultaneously in numerous fundamental genetic programmes.

MiRNA regulation also extends to some of the key processes in metastasis development. A fuller dissection of these processes will undoubtedly lead to a deeper understanding of the nature of disease progression and the requirements for successful metastasis, and in doing so, trigger innovations in the treatment of metastatic disease.

In this section, the focus is on miRNA families with critical roles in the initiation of the metastatic cascade.

1.3.5.1 Epithelial to Mesenchymal transition

Epithelial to mesenchymal transition (EMT) is a programme of gene expression responsible for gastrulation and neural crest cell migration during embryogenesis. Recently, this paradigm has been extended to include cancer progression as this critical phenotypic change may explain how during the course of malignant transformation, polarised epithelial cells, bound firmly to the basement membrane and to one other, assume mesenchymal characteristics such as enhanced motility, invasiveness, and resistance to apoptosis.[252]

EMT is activated by various signalling pathways including TGFβ, Notch, and the wnt cascade which converge at the level of key transcription factors ZEB, SNAIL and TWIST, repressing the expression of epithelium-specific genes such as *E-cadherin* and upregulating expression of mesenchymal genes such as vimentin.[253] Several miRNAs are crucial regulators in this process, actively influencing the balance between EMT and the reverse process termed mesenchymal to epithelial transition (MET).[254]

One such miRNA is miR-9, which promotes breast cancer progression by sensitising malignant cells to EMT inducing signals. Over-expressed in primary breast cancer tissue compared with normal breast epithelium, mir-9 targets CDH-1 mRNA, which encodes E-cadherin, and is itself a transcriptional target of the MYC/MYCN oncogenes.[255] As a further consequence of E-cadherin downregulation, miR-9 may

enhance pro-metastatic intracellular signalling cascades by releasing β-catenin, sequestered at the cytoplasmic membrane, making it available for nuclear translocation.[256] E-cadherin expression is reduced by 70% in response to ectopic miR-9 expression, and this corresponds to a reciprocal increase in the expression of vimentin. The functional result of this is a 3-5 fold increase in cellular invasiveness and motility *in vitro*, and enhanced angiogenesis and micrometastasis formation *in vivo*.[255]

MiR-155 is overexpressed in numerous malignancies and promotes EMT by disrupting tight junction formation and enhancing cell migration and invasion. Mir-155 is under direct transcriptional control by the TGFβ signalling pathway,[257] which has emerged as the key regulatory mechanism of EMT.[253] MiR-155 induces EMT by targeting RhoA GTPase and restoration of RhoA using an expression vector cloned without its 3'UTR eliminates this transformation.[257]

Perhaps the most powerful downstream mediators of TGFβ dependent EMT activation are the zinc finger E-box-binding homeobox transcription factors ZEB 1 and 2.[258] ZEB has been shown to promote metastases in mouse xenograft models and has been implicated in the progression of several human cancers including breast and colon.[259, 260]

Studies of EMT activation have demonstrated a crucial functional link between ZEB 1 and 2 and the miR-200 family of miRNAs.[261] The miR-200 family consists of 5 members arranged as 2 clusters, 200a/200b/429 and 200c/141.[262] Forced overexpression of miR-200c in *in vitro* cell models promotes an epithelial phenotype and upregulation of E-cadherin, and is sufficient to prevent TGF-β dependent EMT induction.[262, 263] Furthermore, overexpression in mesenchymal cells can induce MET.[264] MiR-200 specifically targets and downregulates ZEB expression.[262-264] Importantly however, the common miR-200 family promoter region also contains highly conserved ZEB binding sites through which ZEB 1 and 2 exert reciprocal control on miR-200 transcription.[265, 266] Based on this evidence, it has been postulated that miR-200 and the ZEB family of transcription factors are involved in a double negative feedback loop which acts to stabilise cellular differentiation in response to prevailing extracellular cues.[261]

The implications of this are potentially profound and may help explain a central dilemma in our understanding of the metastatic cascade: miR-200 expression is lost in invasive breast cancer cell lines which have a mesenchymal phenotype[264] and yet miR-

200 expression is also associated with increased metastatic potential and suppression of Sec23a, a regulator of metastasis-suppressive proteins.[267, 268] This apparent paradox helps to illustrate that mechanistically, in order for malignant cells to metastasise, they must be capable of expressing both mesenchymal and epithelial characteristics; at times the capacity for enhanced motility, invasiveness and resistance to apoptosis required for dissemination and at other times the capacity for colonisation and proliferation in order to establish histologically identifiable metastases at distant locations. The ZEB/miR-200 feedback loop may be one mechanism through which this reversible phenotypic switch is achieved, implying that on a molecular level the metastatic programme is dynamic and that tumour cell plasticity in the face of changing environmental cues is a prerequisite for tumour progression.[261]

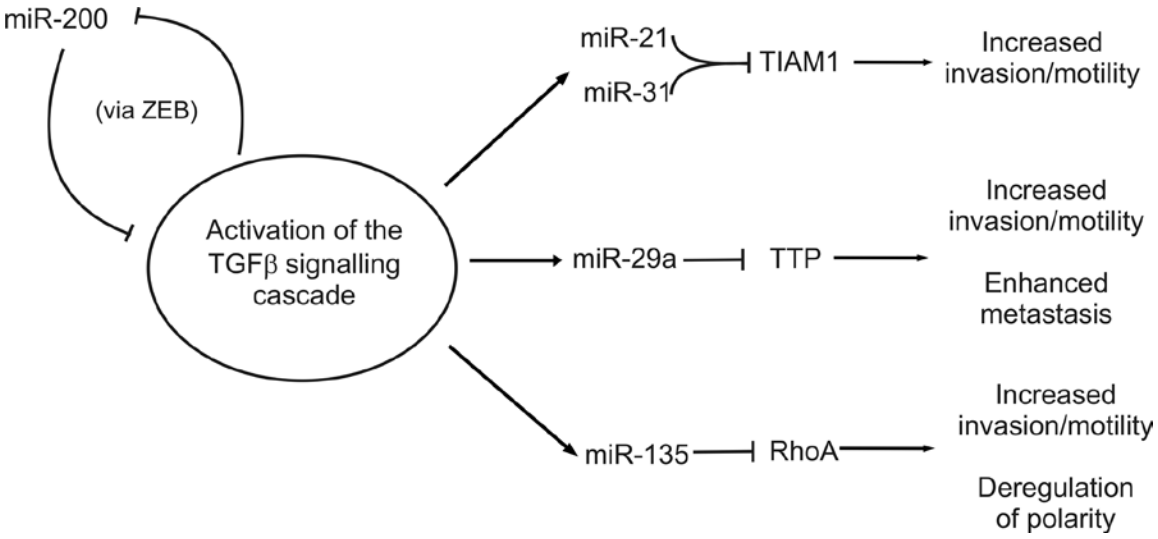


Figure 1.6 MicroRNAs implicated in the TGF βEMT pathway

TGFβ derived from tumour-associated stroma is the dominant EMT trigger. Profiling studies have uncovered a number of miRNAs, implicated in tumour progression, with important roles in TGFβ dependent EMT. TIAM1, T-lymphoma invasion and metastasis 1; TTP, tristetraproline; RhoA, RhoA GTPase. From: Bullock et al., 2012.[269]

1.3.5.2 Stemness

Another important concept, linked to EMT is that of cancer stem cells; de-differentiated cells derived from primary tumours that have acquired the capacity for self-renewal and are capable of dissemination and micrometastasis formation. Mani et al.,[270] using mammary epithelium, demonstrated that EMT activation by ectopic SNAIL or TWIST transcription factor expression produces cells with stem-like antigenic signatures (specifically high CD44 and low CD24 surface expression) and behavioural properties, and furthermore, that naturally occurring neoplastic human mammary stem-like cells co-express markers of EMT.[270] This important observation supports the view that early in the metastatic programme, a subpopulation of malignant epithelial cells undertake a de-differentiation step which confers the capacity for both self-renewal, and disassociation from their sister cells. MiRNA profiling of hepatoblastomas, embryonic neoplasms derived from liver progenitor cells, revealed a pattern of miRNA expression resembling that of embryonic stem cells, including over-expression of the miR-371-3 cluster, which has been shown to play a critical role in stem cells renewal.[271, 272]

There is also evidence to suggest that the ZEB/miR-200 feedback loop plays a role in promoting stemness: Stem-like cells from both normal and malignant mammary tissue under-express miR-200, resulting in the upregulation of the Stem-cell factor BMI-1, (a negative regulator of apoptosis, senescence and differentiation pathways). Furthermore, overexpression of miR-200c reduces both clonogenic and tumour initiation capacity.[273]

A deeper understanding of the mechanistic link between EMT and stemness remains elusive. However, recent miRNA profiling studies have revealed a number of important insights: The let-7 family of miRNAs control the expression of multiple stem cell characteristics and are markedly downregulated in breast cancer stem cells.[274] Furthermore, the maturation of primary Let-7 miRNA transcripts is blocked by the stem cell marker Lin28B.[275] Recently, in cultured prostate cancer cells, which have undergone EMT in response to PDGF treatment, miR-200b and c have been shown to target Lin28B such that down-regulation of miR-200 during EMT is matched by a reciprocal rise in Lin28B expression.

In contrast, Lin28B knock-down using siRNAs leads to strong let-7 induction which in turn, inhibits the capacity for self-renewal of Prostate cancer cells.

Together, these data provide evidence for the first time, of a possible molecular mechanism through which EMT and the stem cell phenotype may be co-induced.[276]

An upstream role for p53 in this process has also been described. Loss of p53 function has profound oncogenic consequences reflecting its pleotropic regulatory activity in fundamental cellular programs including cell cycle progression, apoptosis and DNA repair.[277] Several groups have shown that ectopic p53 expression leads to upregulation of numerous miRNAs including miR-200 and miR-192 family members.[278, 279] Chang et al.,[278] further demonstrated that miR-200c is a direct transcriptional target of p53 and that in mammary epithelial tissue p53 dependent miR-200c induction is mirrored by suppression of important EMT and stem-cell markers including BMI-1 and ZEB. Inhibition of Wild-Type (WT) p53 expression in MCF12A, 'normal' epithelial breast cells using siRNA or TGF-β treatment promotes EMT and a stem-like phenotype, a process which can be reversed by ectopic expression of miR-200c.

A crucial relationship has therefore been established between the loss of p53 function, one of the most important genetic alterations in human cancer, EMT, and the concept of cancer stem cells.[280]

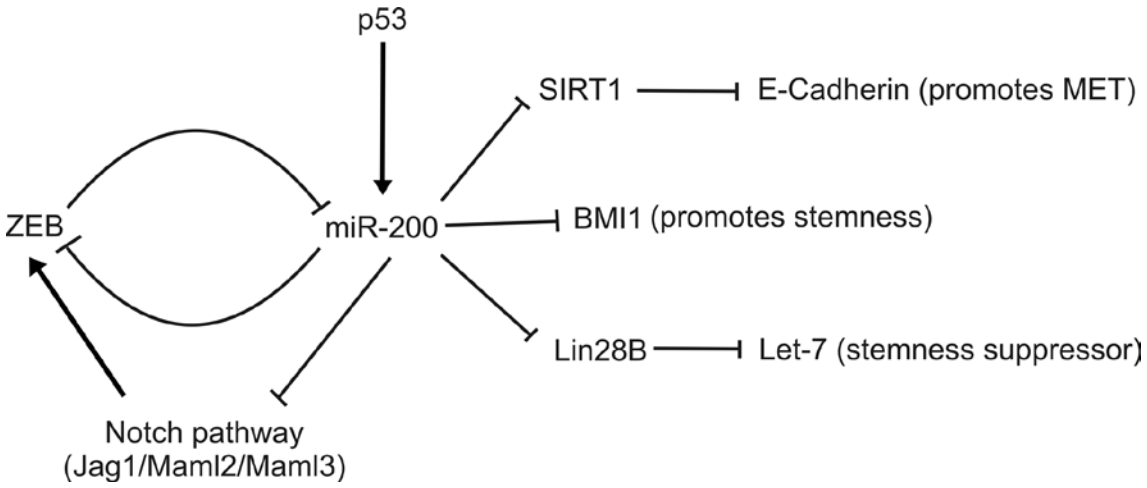


Figure 1.7 The ZEB/microRNA-200 feedback loop

The ZEB/microRNA-200 feedback loop regulates EMT and stemness. The ZEB family of transcription factors are the most powerful downstream mediators of TGFβ dependent EMT activation. ZEB and the miR-200 family are involved in a double feedback loop that stabilises differentiation along the EMT-MET axis in response to extracellular cues. This key regulatory mechanism allows cancer cells to transit between epithelial and mesenchymal differentiation states and links EMT with the concept of 'stemness'. Jag1, Jagged 1; Maml, Mastermind-like co-factor; SIRT1, silent information regulator 1; BMI1, B-lymphoma Mo-MLV insertion region 1 homolog. From: Bullock et al., 2012.[269]

1.3.5.3 Extracellular matrix remodelling

In order for malignant cells to invade the stroma in the earliest stages of the metastatic programme, remodelling of the ECM is required. Broadly speaking three enzymatic groups, involved in ECM remodelling are known to be under miRNA control.

MMPs

Matrix-metalloproteinases (MMPs) are an important family of enzymes, active at the cancer-stroma interface, and involved in ECM remodelling and degradation of the basement membrane (BM). Several miRNAs target MMPs directly, including miR-146b

which inhibits breast cancer metastasis by silencing MMP16, and miR-222 which exerts an anti-metastatic influence by directly targeting MMP1 in oral squamous cell carcinomas (SCCs).[281]

In CRC specifically, overexpression of miR-153 led to increased invasiveness *in vitro* in various cell lines, by indirectly inducing MMP9 activity.[235]

MMP inhibitors

RECK (reversion-inducing cysteine rich protein with Kazal motifs) is a membrane anchored glycoprotein and TIMP3 (tissue inhibitor of metalloproteinase) a secreted inhibitor of MMPs, both of which act as tumour suppressors in solid organ malignancies.[282, 283]

In Glioma models, miR-21 activates MMPs by suppressing TIMP3 and RECK expression. Conversely, the experimental down-regulation of miR-21 results in elevated RECK and TIMP3 mRNA and protein levels, which in turn decreases MMP activity *in vivo* and *in vitro*, leading to reduced cellular motility and invasiveness. In addition, the 3'UTRs of both RECK and TIMP3 mRNA contain putative miR-21 binding sites, although it is possible that only RECK is targeted directly.[284] Recently, the interaction between TIMP3 and miR-21 has been shown to be clinically relevant in other malignancies including cholangiocarcinoma and hepatocellular carcinoma.[285]

Mir-21 negatively regulates RECK in cooperation with at least two other families of miRNA: miR-15b/16 and miR-372/373. MiR-373 expression correlates with increased metastasis and invasion in breast cancer, and in metastatic SW620 colon cancer cells, miR-372/373 is upregulated by the EMT inducing transcription factor TWIST.[286]

Urokinase plasminogen activator

u-PA has emerged as an important regulator of breast cancer metastasis. uPA is a serine protease which catalyses the conversion of inactive zymogen plasminogen into the broad acting enzyme Plasmin. Plasmin, in turn, activates various enzymes involved in ECM remodelling including MMPs.

MiR-193b directly targets uPA mRNA.[287] Clinically its expression inversely correlates with both metastasis-free survival and overall survival in breast cancer patients and the

effect of ectopic miR-193b expression is to decrease the migratory capacity and invasiveness of breast cancer cells *in vitro*. MiR-193b is under-expressed in the highly metastatic MDA-MB-231-HM breast cancer cell line in comparison with parental cells, consistent with xenograft metastasis models in which forced over-expression of miR-193b inhibits the development of pulmonary metastases.[281] uPA is also targeted by miR-23b which *in vitro*, suppresses the migratory capacity of hepatocellular carcinoma cells.[288]

1.3.5.4 Chemotherapy resistance

Anti-cancer drug resistance is a major clinical challenge which impedes treatment and worsens prognosis. Increasingly, as drug resistance pathways are described, it is apparent that miRNA deregulation plays a significant role in this process.[289]

Multidrug resistance genes encode proteins which are characterised by similar trans-membrane domains capable of blocking harmful drugs or expelling them from the cell.[290]

In breast cancer, miR-451 and miR-298 regulate the multidrug resistance gene 1 (mdr-1) which is likely to be a crucial factor in doxorubicin resistance.[291]

In CRC, upregulation of miR-153 in cell lines promotes chemoresistance to platinum-based agents through downregulated expression of the transcription factor FOXO3, an effect which is abrogated in the presence of ectopic FOXO3 induction.[235]

A similar regulatory pattern has emerging across a spectrum of other malignancies including glioblastoma, ovarian, prostate and intestinal cancers, reaffirming the importance of miRNAs in malignant progression, and highlighting a pro-metastatic mechanism potentially targetable by drugs.[292-295]

1.3.6 MicroRNA, the cancer-associated stroma, and tumour progression

As discussed previously, an important feature of metastatic progression is the role played by the tumour microenvironment and the promotion of tumour growth, invasion and angiogenesis by cancer-associated fibroblasts.[100, 103] This dynamic and reciprocal interaction between stromal and malignant epithelial cells has a profound impact on tumour progression *in vivo* and as stromal cells are less likely to acquire *de-novo* mutations, evade capture or develop drug resistance, the development of targeted therapies based on improved molecular characterisation of CRC stroma is an attractive prospect.[101, 152, 153] MiRNAs are strongly implicated in the pathogenesis of numerous malignancies and hold promise as therapeutic targets as they regulate fundamental intracellular processes and are deregulated, often in a tissue and tumour specific manner, in all cancer types examined to date.[179, 182]

Although the role of miRNAs in promoting progression in malignant epithelial cells is becoming increasingly clear, very little is understood about their potential roles within cancer-associated stroma.

Nishida et al.,[296] compared miRNA expression patterns between LMD CRC stroma and normal colonic stroma and revealed that 2 important oncogenic clusters (miR-17-92a and miR-106b-25) were upregulated in cancer. Bioinformatic analysis highlighted a number of putative mRNA targets, but crucially, these data were not supported by detailed mechanistic or functional studies necessary to assign pathogenic significance to individual miRNAs in this context. However, one notable miRNA; miR-21, overexpressed 3.9 fold in CRC stroma compared with normal stroma, has been validated to some extent in other studies.[297-299] Nielsen and colleagues suggested that miR-21 overexpression is exclusively a stromal phenomenon in CRC and breast cancer and that stromal miR-21 expression has prognostic relevance in stage II CRC;[298] and Yao *et al*., demonstrated that miR-21 is upregulated during TGFβ dependent fibroblast-to-myofibroblast transdifferentiation and that downregulation of miR-21 with specific anti-sense inhibitors prevents transdifferentiation of fibroblasts in response to TGFβ.[299]

Other data also supports a role for miRNAs in the control of fibroblast differentiation and phenotype:[299, 300] For example, Bronisz and colleagues powerfully demonstrated

that downregulation of miR-320 in mammary stromal fibroblasts activates a pro-oncogenic secretome capable of reprograming the tumour microenvironment to support tumour invasion and angiogenesis.[300]

Furthermore, comparative miRNA analysis of CAFs isolated from endometrial and ovarian tumours and matched normal fibroblasts identified consistent miR-31 downregulation in cancer.[301, 302] Subsequently, ectopic miR-31 expression was shown to impair significantly the ability of CAFs to stimulate tumour cell migration and invasion by targeting and downregulating homeobox-gene SATB expression.[301]

In a similar study in breast cancer, miR-31 and miR-221 upregulation in CAFs was accompanied by downregulated expression of eight further miRNA candidates including miR-200 family members and let-7, involved in cell differentiation, migration and secretory functions.[303]

These findings again emphasise the significance of tumour-stroma interactions and suggest miRNAs which regulate CAF phenotype, are important regulators of pro-invasive tumour cell functions. They also reflect a growing interest in cell-cell communication roles in cancer mediated by miRNAs. Exosomes for example, which are 30-100nm secreted vesicles which form from cell membrane invaginations, are capable of delivering proteins, lipids and nucleic acids between cells or into the extracellular space, in a targeted manner.[304] Accumulated evidence suggests that miRNAs in cancer exosomes are an important component of tumour-stroma cross talk promoting tumour-potentiating effects both in malignant epithelial cells and cancer associated stromal cells.[305] Exosome derived RNA molecules also provide valuable prognostic and diagnostic information in cancer and it appears that exosome driven tumour growth is potentially targetable by drugs.[306]

Furthermore, it has also emerged that exosomal miRNAs originating from the stroma mediate tumour-potentiating activities[307, 308] however, the role of key stromal cell types such as fibroblasts has not yet been explored in detail.

1.3.7 Clinical applications of microRNAs

The importance of the miRNome during tumourogenesis is reflected in the abundance of research seeking to translate novel insight in miRNA pathobiology into clinically useful applications.

Early emphasis was placed on the diagnostic and prognostic potential of miRNA profiling but, more recently hopes have been raised that miRNA based approaches might also be adapted for therapeutic purposes, either through manipulation of tumour-relevant miRNAs or the use of synthetic miRNAs to silence oncogenes *in vivo.*

1.3.7.1 MicroRNAs as diagnostic tools

The unique pattern of deregulated miRNA expression in individual tumour types may be utilised to improve diagnostic accuracy in challenging clinical scenarios for example, distinguishing malignant from chronic inflammatory change in the pancreas:

In an analysis of 65 pancreatic duct adenocarcinomas and 42 cases of chronic pancreatitis, deregulated expression of 22 miRNAs successfully separated benign and malignant disease with 93% accuracy.[309] A similar study reported accuracy of 90% in classifying over 400 tumour samples of 22 tissue types based on the expression of 48 miRNAs.[310]

MiRNA profiling has also proven useful in identifying, in the absence of recognisable histological features, the tissue of origin of poorly differentiated tumours. In one study, a miRNA expression signature consisting of 217 miRNAs successfully classified 12/17 poorly differentiated tumours compared with only 1/17 cases using an equivalent mRNA profile.[182] The superior performance of miRNAs in this context may be because miRNAs, which control whole programs of gene expression, are fewer in number and less heterogeneously expressed in cancer than mRNA or; that miRNA transcripts are less prone to degradation because they are considerably shorter and fold into stable stem-loop structures.

In CRC, profiling has been used to compare miRNA expression in primary tumours and normal tissue; tumours of progressive pathological stage; primary tumours and paired

liver metastases; CRC-associated stroma and malignant epithelium; and the serum, plasma, lymph nodes and even stool of CRC patients and matched, healthy control subjects.[183, 235, 311-314]

Bandres and colleagues were amongst the first to compare miRNA in CRC and adjacent non-neoplastic tissue using high sensitivity real-time PCR techniques. They examined the expression of 156 mature miRNAs and identified 13 that were significantly deregulated in cancer, including miR-31, the expression of which also correlated positively with disease stage.[315]

Subsequently, it has emerged that high levels of miRNA also circulate in the plasma of cancer patients and that miRNA profiles generated from serum are reproducible and reliable.[316] In one study, 69 miRNAs were differentially expressed in the serum of CRC patients compared with normal control subjects; however all but 14 miRNAs were similarly corrupted in lung cancer, raising doubts as to the signature-uniqueness of serum miRNA expression profiles in different cancer types.[317] Furthermore, as individual biomarkers in early studies, serum miRNAs achieved diagnostic sensitivity and specificity values of only 65%-84%, and 41%-89% respectively, which is insufficient for the purpose of tumour screening.[318-320]

However, two recent developments have re-ignited interest in blood-based miRNA-predicated diagnostic tools. In a study of 282 patients, a group at Baylor University Medical Centre examining serum miR-21 expression in isolation, were in-fact able to distinguish very effectively between 'normal' healthy subjects and matched patients with CRC (ROC AUC=0.919; 95%CI=0.867-0.958) or colonic adenomatous disease (ROC AUC=0.813; 95%CI=0.691-0.910).[314] Furthermore, it has emerged that blood-borne miRNAs are transported predominantly in exosomes rather than circulating freely in serum, and that isolating the exosome fraction prior to profiling, may significantly enhance sensitivity and specificity of miRNA detection.[321]

The molecular profiling of faeces in CRC is another recent development, but once again, the instability of mRNAs in this context means they are of limited clinical use.[322] In contrast, miRNAs extracted from cells which have exfoliated into the gastrointestinal tract can be isolated from stool and profiled in a robust and reproducible manner.[323] Notwithstanding, miR-21 and miR-92a, which are both overexpressed in CRC and

abundantly present in stool, are also inadequate as stand-alone tests for the purpose of cancer screening.[312]

However, when considered together, these promising preliminary data show that stool and plasma based molecular markers have significant diagnostic potential in CRC. In future, they may even form the basis of a non-invasive adjuvant screening tool, however reservations regarding sensitivity and specificity suggest they are most likely to be used in combination with other clinical tools as part of a broader, integrated screening platform.

1.3.7.2 MicroRNAs as prognostic tools

Surgery is the standard treatment for the majority of patients with CRC. The discretionary use of other treatment modalities such as adjuvant or neo-adjuvant chemotherapy and radiotherapy depends on the perceived risk of tumour recurrence based on the analysis of histopathological and radiological parameters including tumour stage and differentiation status; tumour margin and lymphovascular involvement; and clinical parameters including tumour perforation, and patient performance status.[324] However, there is increasing evidence that a more refined approach tailored to the individual and based on the distinct molecular profile of each tumour may improve prognostic accuracy and outcome.[325]

A number of miRNAs have shown promise as prognostic biomarkers in CRC, predicting with varying accuracy, disease stage, response to therapy, overall survival, disease free survival, progression free survival and the presence or absence of lymph node (LN) metastases (summarised in Table 1.1).

The ability to detect occult LN metastases would be particularly valuable as it presents the opportunity of identifying high risk patients early and of optimising their treatment.[326, 327] In a limited study of just 6 CRC patients with (n=3) and without (n=3) LN metastases, miRNA expression profiling was conducted on uninvolved tissue close to the tumour site. Two miRNAs (miR-129 and miR-137) were significantly differentially expressed in the LN positive group compared with the LN negative group. Although low patient numbers and sub-optimal study design limit the impact of the

data, the study supports the use of miRNA based molecular profiling to assist identification of high risk patients.[328]

Molecular profiling may also be useful to deliver adjuvant therapies in a more tailored and specific manner. As discussed previously, the *KRAS* mutation strongly predicts resistance to cetuximab mAb therapy in metastatic CRC.[329] MiRNAs let-7b, let-7e and miR-17 expression may be similarly used to identify patients who are likely to be responders or non-responders during the treatment planning phase.[330, 331]

Thus a deeper understanding of the molecular characteristics of cancer may also enable more accurate prediction of a patient's response to treatment. Svoboda and colleagues biopsied rectal cancers *in situ* prior to and during combined pre-operative capecitabine and radiation therapy, and found that miR-137 expression, upregulated in 26/31 cases correlated positively with increasing tumour regression grade (TRG); a measure of positive response to treatment in rectal cancer.[332]

On a related theme, treatment of CRC cells with 5 fluorouracil (5-FU), part of most CRC chemotherapy regimens produced significant alterations in miRNA expression *in vitro*, including upregulated miR-21 expression.[333]

MiR-21 has increasingly well validated prognostic value in CRC. For example, in one study, stromal miR-21 expression, quantified using a highly sensitive ISH technique, predicted reduced disease free survival and overall survival independently of other clinical parameters in stage II (LN negative) disease.[298]

In this study, the authors were addressing an area of urgent clinical need as disease recurrence remains a substantial problem in stage I/II CRC. Currently, 20-25% of patients following surgery with curative intent develop metastases within 5 years.[2] There is strong evidence to support the routine use of adjuvant chemotherapy in stage III (LN positive) disease however, in stage II CRC there is no evidence of benefit for the group as a whole and treatment is recommended only for those judged to be at high risk based on loosely defined clinical and pathological parameters.[326] Hence, a significant minority of patients with stage II CRC are sub-optimally managed. A molecular staging approach enabling early identification of these patients would create the opportunity to offer some patients targeted chemotherapy as appropriate, whilst avoiding overtreatment in other cases.

Table 1.1					
Study	**Technique**	**Comparison**	**Samples (n)**	**miRNAs deregulated**	**Prognostic implications**
Michael et al. 2003[334]	Cloning	Tumour vs. normal	25	MiR-143, 145 downregulated	
Xi et al. 2006[335]	Real time qRT-PCR	Tumour vs. normal	48	miR-15b, 181b, 191, 200c upregulated	Elevated miR-200c associated with short survival
Bandres et al. 2006[315]	Real time qRT-PCR	Tumour vs. normal	24	miR-133b, 145 downregulated. miR- 31, 96, 135b, 183 upregulated	Elevated miR-31 correlates with increasing tumour stage
Volinia et al. 2006[200]	Microarray	Tumour vs. normal	55	miR-9-3 downregulated. miR-10a, 17-5p, 20a, 21, 24-1, miR-24-2, 29b, 30c, 32, 106a, 107, 126, 128b, 150, 155, 191, 203, 213, miR-221, 223 upregulated	
Nakajima et al. 2006[336]	Real time qRT-PCR	Tumour vs. normal	42	Let-7g, miR-181b, m200c upregulated	
Slaby et al. 2007[337]	Real time qRT-PCR	Tumour vs. normal	35	miR-143, 145 downregulated. MiR-21, 31 upregulated	Elevated miR-21 correlates with stage, LN status and distant metastasis
Lanza et al. 2007[338]	Microarray	MSS vs. MSI-H	39	miR-17-5-p, 20, 25, 92, 93-1, 106aupregulated	
Schetter et al. 2008[311]	Microarray	Tumour vs. normal	Test: 84 Validation: 113	miR-20a, 21, 106a, 181b, 203 upregulated	Elevated miR-21 correlates with increasing tumour stage and poor survival

Monzo et al. 2008[339]	Real time qRT-PCR	Tumour vs. normal	46		28 miRNAs deregulated in stage I disease; 64 miRNAs deregulated in stage II disease
Schepeler et al. 2008[340]	Microarray	Tumor vs. normal; MSS vs. MSI	59	miR-20a, 92, 510 downregulated. miR- 26b, 30b, 101, 145, 455, 484 upregulated.	Elevated expression of miR-320 and miR-498 correlates with longer DFS in stage II MSS
Diaz et al. 2008[341]	Real time qRT-PCR	Tumour vs. normal	220	miR-17-5p, 106a, 126 downregulated	Supressed expression of miR-106a predicts shorter DFS and OS
Wang et al. 2009[342]	Real-time qRT-PCR	Tumour vs. normal	196	miR-145, 143 downregulated. miR-31 upregulated	Elevated miR-31 correlates with advanced stage and deeper invasion
Motoyama et al. 2009[238]	Microarray and qRT-PCR	Tumour vs. normal	Test: 8 Validation: 138	miR-143, 145 downregulated. miR-17-5p, 18a, 20a, 31, 92, 183 upregulated	
Huang et al. 2009[328]	Microarray	Stage II vs. Stage III CRC	6 (all normal tissue samples around CRC	miR-129, 137 upregulated in stage III	
Baffa et al. 2009[343]	Microarray	Tumour vs. paired LN metastasis	20	miR-17, 20, 25, 106a, 204 downregulated in LN. let-7e, miR-21, 99b, 106b, 125b, 127-3p, 132, 138, 199b, 342, 409-3p, 449a, 491-3p, 518-3p, 671 upregulated.	
Ng et al. 2009[318]	Real-time qRT-PCR	CRC plasma vs. normal	Test: 10 Validation:	miR-17-3p, 92 upregulated	miR-92 elevated in plasma of CRC

		plasma	90		patients. First potential blood bourn marker identified.
Arndt et al. 2009[344]	Microarray	Tumour vs. normal	49	miR-1, 10b, 30a, 30c, 125a, 133a, 139, 143, 145, 195, 387, 422, 497 downregulated. miR-17-5p, 18a, 19, 20a, 21, 25, 29a, 31, 34a, 93, 95, 96, 106a, 130b, 105b, 181b, 182, 183, 203, 224 upregulated	miR-31 and miR-7 upregulated and miR-99b, 378, 133a and 125a downregulated in late stage (III/IC) vs. early stage (I/II) disease.
Sarver et al. 2009[345]	Microarray	Tumour vs. normal; MSS vs. MSI	108	miR-1, 9, 10b, 20b, 30a, 133a, 137, 138, 139, 147, 328, 363. 375, 378, 486, 497, 511, 551b, 642, 650 downregulated. miR-17-3p, 29, 31, 32, 33, 96, 135b, 182, 183, 188, 224, 503, 552, 542, 584 upregulated	
Shibuya et al. 2010[346]	Real-time qRT-PCR	Tumour vs. normal	156 paired samples	miR-21, 155 upregulated	Elevated miR-21 expression associated with increasing T-stage, venous invasion, liver metastasis, decreased DFS and OS
Wang et al. 2010[347]	Microarray	Tumour (LN – ve) vs. normal	6 paired samples	miR-378 downregulated. miR-18, 19a, 20a, 106b, 135b, 196b,	

				224, 301b, 335, 374, 424 upregulated	
Wang et al 2010[348]	Microarray	LMD tumour epithelium vs. adenoma and normal	18	miR-20a, 21, 106a, 181b upregulated	First documented use of LMD paired with miRNA screening
Earle et al. 2010[349]	Real-time qRT-PCR	Tumour vs. normal	55 paired samples	Let-7a, miR-16, 26b, 143, 145, 192, 191, 196a, 215 downregulated. miR-17, 31, 20, 25, 92, 93, 133b, 135a, 183, 203, 223 upregulated	
Huang et al. 2010[319]	Real-time qRT-PCR	CRC plasma vs. normal plasma	Test: 40 Validation: 119	miR-29a, 92 upregualted	
Kulda et al. 2010[350]	Real-time qRT-PCR	Tumour and liver metastasis vs. normal tissue	46 CRC vs. 30 liver mets	miR-143 downregulated. miR-21 upregulated in liver metastasis and normal tissue	Elevated miR-21 and miR-143 (paradoxically) associated with short DFS
Pu et al. 2010[320]	Real-time qRT-PCR	CRC plasma vs. normal plasma	140	miR-221 upregulated	Elevated miR-221 associated with poor OS
Akcakaya P et al. 2011[351]	Real-time qRT-PCR	Short vs. Long survivors	50	miR-133b downregulated. miR-185 upregulated	Elevated miR-185 and supressed miR-133b correlate with poor survival and increased metastasis
Cheng et al. 2011[352]	Real-time qRT-PCR	CRC plasma vs. normal plasma	Test: 102 Validation: 16	miR-21, 92, 141 upregulated	Elevated miR-141 correlates with advanced stage and predicts poor survival

Karaayvaz et al. 2011[353]	Real-time qRT-PCR	Tumour vs. normal	34 pairs	miR-192, 215 downregulated	Elevated miR-215 (paradoxically) associated with poor survival
Nielsen et al. 2011[298]	In-Situ Hybridisation	Stage II CRC	197	Stromal miR-21 upregulated	Elevated stromal miR-21 associated with poor DFS
Ma et al. 2012[354]	Real-time qRT-PCR and In-situ hybridisation	Tumour vs. adenoma and normal	Test: 239 Validation: 185	miR-150 downregulated during adenoma to carcinoma sequence	Supressed miR-150 associated with poor survival and response to therapy
Pichler et al. 2012[355]	Real-time qRT-PCR	Tumour vs. normal	77 pairs	miR-143 downregulated	Supressed miR-143 expression associated with poor CSS
Vickers et al. 2012[356]	Real-time qRT-PCR	Early stage CRC with and without subsequent metastasis	34	miR-206 downregulated. miR-21, 135a, 355 upregulated	First published use of a multi-candidate miRNA prognostic platform
Wang et al. 2012[357]	Reak-time qRT-PCR	Tumour vs. normal	85 pairs	miR-15, 16, 195 downregulated. miR-424 upregulated	miR-195 predicts LN status and is associated with poor overall survival
Weissmann-Brenner et al. 2012[358]	Microarrays	Early stage CRC with and without subsequent metastasis	110	miR-29a downregulated in metastatic group	Elevated miR-29a expression associated with longer DFS
Yu et al. 2012[359]	Real-time qRT-PCR	Tumour vs. normal	48 pairs	miR-17-92 cluster upregulated	Elevated miR-17 associated with poor survival
Bovell et al. 2013[360]	Real-time qRT-PCR	Tumour vs. normal	345 sub-divided by ethnicity	miR-20a, 21, 106a, 181b, 203 upregulated	miR-21, miR-181b and miR-203 associated with poor outcome

Liu et al. 2013[361]	Real-time qRT-PCR	Tumour vs. normal	148 pairs	miR-182 upregulated	Elevated miR-182 associated with presence of LN and distant metastasis and poor survival
Liu et al. 2013[362]	Real-time qRT-PCR	CRC serum vs. adenoma and normal serum	330	miR-21, 92a upregulated	Elevated serum miR-92a associated with poor survival
Lou et al. 2013[363]	Real-time qRT-PCR	Tumour vs. normal	96 pairs	miR-625 downregulated	Supressed miR-625 expression correlates with LN and distant metastasis and poor survival
Toiyama et al. 2013[314]	Real-time qRT-PCR	CRC plasma vs. normal plasma	Test: 24 Validation: 239	miR-21 upregulated	Elevated serum miR-21 correlates with tumour size, metastatic status and poor survival
Wang et al. 2013[364]	Real-time qRT-PCR	Tumour vs. normal	96 pairs	miR-124 downregulated	Supressed miR-124 expression associated with poor DFS and OS
Zhang et al. 2013[235]	Real-time qRT-PCR	Tumour vs. normal	100	miR-153 upregulated	Elevated miR-153 expression associated with poor DFS
Zhang et al. 2013[365]	Microarray	Stage II CRC vs. normal	Test: 40 Validation: 136 and 460	miR-21, 20a, 103a, 106b, 143 and 215 deregulated	Utility of multi-candidate miRNA prognostic platform in CRC
Zhou et al. 2013[366]	Real-time qRT-PCR	Tumour vs. normal	82 pairs	miR-92a upregulated	Elevated miR-92a associated with poor overall survival

Oue et al. 2014[367]	Real-time qRT PCR	Early vs. late stage CRC	Test: 156 Validation: 145	miR-21 upregulated	Elevated miR-21 associated with poor prognostic and therapeutic outcomes. Robust miR-21 expression from FFPE tissue
Toiyama et al. 2014[368]	Real-time qRT-PCR	CRC plasma vs. normal plasma	Test: 12 Validation: 206	miR-200c upregulated	Elevated serum miR-200c associated with LN and distant metastasis and poor prognosis. miR-200c predictive of LN metastasis and recurrent disease

Table 1.1 Summary of miRNA expression profiling studies and their prognostic significance in CRC

1.3.7.3 MicroRNAs and therapy

The development of miRNA based therapies follows on from a decade of research exploring the therapeutic potential of short interfering RNA (siRNA) molecules. The advantage of miRNA over siRNA-based therapeutic agents is two-fold, firstly; miRNAs target putative disease effectors and as such directly influence pathobiology and secondly; miRNAs regulate whole programs of gene expression and may impact powerfully on multiple upstream and downstream target genes simultaneously.[369, 370]

In fact, the first study to demonstrate that mutations in miRNA encoding genes are sufficient to initiate carcinogenesis also introduced the concept of miRNA based therapy, as Croce and colleagues induced apoptosis in leukaemic MEG01cells, by correcting deficient miR-15a/16-1 expression *in vitro*.[205, 371] Conversely, miR-21 was identified in early studies as a candidate miRNA which when antagonised in cancer cells *in vivo*, produced pro-apoptotic, anti-proliferative effects.[372]

However, despite the innate potential of miRNA based therapies, they are yet to emerge in the clinical setting because achieving stable and safe drug delivery is proving a major challenge.[373] Unconjugated therapeutic miRNAs which are around 7-20kDa, are rapidly

filtered by the kidney and excreted. Furthermore, circulating phagocytes effectively isolate and remove exogenous RNA from the bloodstream.[374]

Two potential solutions to this problem are being examined. The first involves chemically modifying oligonucleotides to provide resistance from endonuclease degradation making them stable enough to be administered systemically.[375, 376] The second involves conjugation with delivery proteins or complexing therapeutic miRNAs with lipids.[377-379]

The most advanced miRNA-based therapy developed to date, anti-miR-122, is a synthetic RNA analogue with a fructose ring backbone known as a locked nucleic acid (LNA).

Anti-miR-122 LNA, was designed specifically to produce anti-sense inhibition of miR-122, which is expressed in the liver and is essential for hepatitis C virus (HCV) accumulation in liver cells. In the first *in vivo* demonstration of the power of miRNA based therapy in primates, intravenous administration of anti-miR-122 LNA in chronically infected Chimpanzees, resulted in sustained reductions in HCV viremia and significant improvements in HCV-induced liver pathology.[380]

In this case, drug delivery was tissue-specific because the pathogen itself (HCV) targets the liver. Similar techniques may not therefore work as effectively in cancer.

In 2010 Davis and colleagues at the California Institute of Technology tried to address this issue by examining an alternative drug delivery mechanism. Their group undertook human clinical trials of a targeted nanoparticle delivery system administered intravenously to patients with malignant melanoma. The nanoparticle consisted of a linear cyclodextrin-based polymer, a human transferrin (TF) protein to engage with TF receptors on the surface of cancer cells, a hydrophilic polymer to promote stability and a miRNA designed to reduce the expression of ribonucleotide reductase (RRM2), an established anti-cancer target. Following treatment, biopsied melanoma tissue was analysed, revealing intra-cellularly localised nanoparticles in dose dependent quantities; a reduction of RRM2 protein and mRNA compared to pre-treatment levels; and the presence of specific mRNA cleavage products.[381] These data suggest that tissue-specific, targeted gene silencing can be achieved in humans using a systemically

administered synthetic miRNA based agent; and that drug stability and targeted action can be achieved through parallel developments in nano-particle and miRNA technology.

1.4 The principles of experimental miRNA analysis

Interest in miRNA pathobiology has increased exponentially with the realisation that deregulated miRNAs play important gene regulatory roles in cancer and that miRNA expression profiles have valuable clinical applications.

The aim of the following section is to describe the principles of miRNA expression analysis and how data generated may be applied to enhance understanding of tumour biology and uncover novel miRNA based biomarkers in cancer.

1.4.1 MicroRNA expression profiling

MiRNAs represent just 0.01% of total RNA content; miRNAs within a family may differ from one-another by as little as one nucleotide and the sequence length of individual mature miRNA molecules may vary due to post-transcriptional 3'UTR sequence modifications;[382] all of which presents a considerable challenge for the accurate identification and quantitation of miRNAs within biological samples. Nevertheless, three major methodologies, used alone or in combination, have evolved. These are: high-throughput miRNA-hybridisation microarrays; next generation RNA sequencing techniques and quantitative reverse transcription PCR (qRT-PCR).

MicroRNA micro-arrays are an established high-throughput platform capable of screening many thousands of miRNAs simultaneously and at low cost. Typically, miRNAs are enzymatically conjugated with a fluorophore and hybridised to an array of captured DNA-based probes which is then washed, removing unbound miRNAs and analysed. Typically, miRNA microarrays are useful for biomarker profile studies but not for absolute miRNA quantitation. Furthermore, relatively low specificity requires expression of individual miRNAs to be confirmed using more accurate quantitative techniques before proceeding with functional or mechanistic studies.

Next generation RNA sequencing is the second major approach for miRNA expression profiling and involves the simultaneous sequencing of a small cDNA library created from the total RNA of the sample of interest, and a parallel library consisting of millions of individual cDNA molecules. The advantage of RNA sequencing techniques is their capacity to identify novel miRNAs not previously sequenced, and distinguish between

very similar miRNAs. However, they cannot be used for absolute miRNA quantitation. qRT-PCR, consists of a reverse transcription step which converts miRNA to cDNA, followed by a PCR step in which the accumulation of reaction product is quantified in real time. Highly sensitive qRT-PCR assays (eg Taqman®) which use specific miRNA primers in both forward and reverse PCR reactions are frequently used to validate expression of individual miRNAs identified by high-throughput methodologies. However, qRT-PCR can be adapted to screen many hundreds of miRNAs simultaneously on customisable medium through-put plates or microfluidic cards, using cDNA generated by reverse transcription from a universal miRNA 3' poly(A) tag sequence.

1.4.2 MicroRNA expression profiling – technical considerations

Conventional miRNA expression profiling studies using whole tumour samples do not differentiate between miRNAs deregulated in malignant epithelium and cancer-associated stroma and ignore potentially crucial biological distinctions between a tumour and its microenvironment.

Laser microdissection (LMD) enables the separation of tumour tissue into component sections which subsequently undergo analysis in order to produce distinct stromal and epithelial miRNA profiles. This approach has been used in a wide variety of solid organ tumours.[296, 383, 384] Nishida and colleagues were the first to publish stromal miRNA profiles in CRC however, their study design was suboptimal. In their 2011 study, the group identified deregulated miRNAs in 13 LMD CRC specimens by conducting comparative analysis with 4 normal colorectal tissue samples, rather than using paired normal tissue which may have reduced error from inter-patient variability. Furthermore, they only commented on miRNAs upregulated in the malignant state and ignored downregulated miRNAs which are potentially of equal importance and; having used miRNA microarrays in the first instance, the group failed to validate their data using more sensitive and specific techniques such as Taqman® qRT-PCR.[296] As a

consequence, the identity and biological significance of deregulated stromal miRNAs during CRC pathogenesis and progression remains largely unknown.

Historically, a second important technical consideration concerns the method of preservation of tumour specimens, and whether this affects the quality of RNA, or the reliability of miRNA profiles extracted. In particular it was felt that in contrast to cell lines and fresh frozen tumour tissues which yield high quality RNA, miRNAs would degrade rapidly in formalin in a similar fashion to mRNAs. However, it is now clear that reliable miRNA profiles can also be generated from archived FFPE specimens: For example, a comparison between snap frozen and formalin fixed tumour cell lines revealed 66% of miRNAs (n=154) examined were less than 1-fold differentially expressed between groups; and 95% were less than 2-fold differentially expressed.[385] Other studies highlight very little degradation in the quality of miRNA profiles extracted from archived FFPE tumour tissue over 10 years and that miRNA stability is independent of formalin fixation time.[386, 387]

1.4.3 MicroRNA expression profiling – picking 'winners'

Profiling studies which use high-throughput methodology may identify hundreds or even thousands of candidate miRNAs deregulated in the malignant state. In order to prioritise the most promising miRNAs it is necessary to identify which are likely to mediate significant biological functions before detailed and time consuming mechanistic studies are considered.

This challenge has traditionally been met using computer-based bioinformatic algorithms such as PicTar and TargetScan which correlate binding domains within miRNA 3'UTRs with putative mRNA target binding domains.[388] However, as functional mRNA regulation requires as few as 6 nucleotides of miRNA/mRNA seed sequence complementarity[389] this approach has 2 significant drawbacks. Firstly; bioinformatic programs are likely to predict very large numbers of putative mRNA targets without providing any sense of priority or context and secondly; different algorithms produce divergent results and high false positive rates.[388]

DAVID (Database for Annotation, Visualisation and Integrated Discovery) functional annotation bioinformatics was devised to address the first issue; it is a method of

contextualising the potential pathogenic impact of diverse mRNAs by assigning function to clusters of related genes, highlighting enriched biological themes and facilitating identification of putative cancer-relevant target pathways.[390] Despite this, the shortcomings of bioinformatics more generally have invited new innovations which enable identification of miRNA-mRNA interactions in a more specific manner.

HITS-CLIP uses ultra-violet radiation to covalently crosslink RNA molecules with proteins, which after purification by immunoprecipitation and partial RNA digestion permits high-throughput sequencing of miRNA-mRNA couplets *in vivo,* at their point of interaction with Ago proteins within the RISC complex.[391]

Although this technique has not completely supplanted *in silico* methodologies, identifying only mRNAs which are directly bound to miRNA has the potential to significantly reduce false-positive predictions and enhance the speed and efficiency with which miRNA profiling data is prioritised.

A further innovation has been the development of a quantitative proteomic approach to miRNA target prediction,[300, 392] which has the advantage of detecting miRNA mediated changes in gene expression at the protein rather than mRNA level. A recent study, in which miR-320 was shown to be co-supressed with PTEN in murine mammary fibroblasts, used proteomic mass-spectrometry to identify 51 proteins differentially expressed in conditioned media from *pten*-null fibroblasts compared with *pten*-null fibroblasts in which miR-320 had been ectopically expressed. Reintroduction of miR-320 inhibited the secretion of a cluster of pro-oncogenic factors including ECM remodelling enzymes MMP2 and MMP9, establishing a PTEN-miR-320 dependent pro-oncognenic axis in murine mammary tumour stroma.[300]

This approach further reduces the rate of false positive prediction however; the disadvantage is that indirect downstream molecular events are highlighted as well as alterations in directly targeted genes. This illustrates a common point relevant to all miRNA target prediction techniques; that although they streamline research by prioritising miRNAs with presumed biological relevance, more detailed mechanistic and functional studies are always required to develop true understanding of the pathogenic relevance of individual miRNAs.

1.5 Summary and Hypothesis

MiRNAs are a class of small highly conserved non-coding RNAs that provide widespread expressional control through translational repression of mRNA. MiRNAs have fundamental roles in regulation of intracellular processes and are increasingly recognised to play a key role during malignant transformation. Deregulated miRNAs are strongly implicated in the initiation of human carcinogenesis, and a growing number of oncogenes and tumour suppressor genes are subject to miRNA regulation.[180]

The vast majority of deaths from cancer are not, however, caused by primary tumours, which are often resectable, but by metastatic disease, to which the most troublesome and intractable symptoms can be attributed and to which most patients eventually succumb. Metastasis is a complex multi-step process during which certain cells acquire the capacity to break free from their sister cells, invade the extracellular matrix and basement membrane, intravasate into the circulation, evade immune detection and eventually extravasate and propagate at distant sites, establishing viable secondary tumour deposits in other organ systems.[78] The biological make-up of these malignant cells has been informed recently by advances in our understanding of miRNAs, and has altered the conventional narrative of metastasis development.

Furthermore, miRNA profiling is able to distinguish tumours of different developmental origins and has potentially powerful applications as a prognostic and diagnostic tool in CRC.[177, 393]

A further theme in recent years has been the critical role of the cancer microenvironment and the dynamic interactions between cancer and stromal cells in promoting invasion and progression. In the malignant state, the stroma is phenotypically distinctive and termed ‘activated stroma’ to reflect this. Key cellular components of this stroma are myofibroblasts, a heterogeneous population of cells derived from fibroblast progenitors which provide permissive signals to the malignant epithelium.[98] Although recent studies have shown that deregulated miRNA expression in myofibroblasts provides clinically relevant prognostic information in stage II CRC, little is known about the biology of stromal miRNAs during cancer progression.

Based on the observations detailed above, the following hypothesis was formulated:

- *Stromal and epithelial miRNA expression patterns (individually or communally) have important consequences for CRC progression and metastasis*

1.6 Objectives

To test my hypothesis, my project will address the following objectives:

1. Identify and validate candidate miRNAs differentially expressed in the stroma and epithelium of different stage clinically relevant CRC samples, with emphasis placed on the comparison of stage II CRC specimens with and without subsequent metastatic progression.

2. Determine biological consequences of deregulated miRNAs using *in vitro* functional cell assays for disease progression and a 3-D CRC organotypic model to facilitate better understanding of the complex tumour-stroma cross-talk.

3. Validate the functional impact of candidate miRNAs using a novel *in vivo* model of CRC progression

4. Identify potential gene targets of candidate miRNAs in order to map the molecular pathways which mediate their biological effects.

5. Establish the utility of miRNA expression profiling as a clinical prognostication tool in CRC.

1.7 Preliminary data from the group

Preliminary data from the host group provided an exceptional platform on which to base this research.

Colleagues identified a panel of miRNAs deregulated in epithelial and stromal tumour compartments of 10 CRC specimens compared with paired normal colonic tissue using QuantimiR™ qRT-PCR arrays comprising 95 human miRNAs.[235] Specimens consisted of 5 stage I/II (non-metastatic) and 5 stage III/IV (metastatic) CRCs as well as paired sections of unaffected proximal colon >5cm from tumour (figure 1.8).

A

	Fold Change*
miR-224	+11.48
miR-106a	+7.61
miR-20a	+6.89
miR-18a	+5.44
miR-29a+b+c	+5.40
miR-19a+b	+3.79
miR-95	+3.49
miR-23a	+3.27
miR-200b	+3.06
miR-24	+2.73
miR-92	+2.71
miR-106b	+2.42
miR191	+2.25
miR-181c	-3.30
miR-132	-3.00
miR-181a	-2.87
miR-134	-2.57
miR-135b	-2.51

B

	Fold Change*
miR-19 a + b	+3.4
miR-21	+2.6
miR-101-1	+2.4
miR-17-5p	+1.8
miR-183	+1.8
miR-30a-3p	-1.73
miR-26a	-1.76
miR-215a	-1.84
miR-132	-1.96
miR-204	-1.97
miR-195	-2.15
miR-16	-2.20
miR-218	-2.26
miR-194	-2.30
miR-192	-2.56
miR-150	-2.57
miR-9-1	-5.97
miR-215	-8.65

Figure 1.8 Preliminary miRNA profiling analysis (I)

*Differentially expressed miRNAs in (A) epithelium and (B) stroma of CRC specimens compared with paired normal colonic tissue by QuantimiR™ miRNA microarray analysis. *P<0.05.*

Using a complementary approach, colleagues also identified miRNAs differentially expressed between paired non-metastatic (SW480) and metastatic (SW620) CRC cell lines (figure 1.9).

A

	Fold Change*
miR-375	153.88
miR-192	55.39
miR-200a	53.12
miR-213	45.70
miR-194	34.27
miR-429	24.32
miR-200b	21.01
miR-181a	15.50
miR-153	12.75
miR-181b	9.50
miR-181d	9.08
miR-308-3p	8.05
miR-215	7.82
miR-148b	6.39
miR-7	6.35
miR-542-3p	5.45
miR-17-3-p	5.38
miR-92	5.30
miR-142-5p	5.27
miR-485-3p	4.76
miR-142-3p	4.52
miR-186	4.40
miR-452	4.24

B

	Fold Change*
miR-518b	4.14
miR-519a	4.34
miR-199a	4.53
miR-520h	4.97
miR-187	5.61
miR-330	5.84
miR-520d	6.68
miR-527	6.97
miR-498	7.40
miR-526c	9.55
miR-371	9.95
miR-193a	10.27
miR-150	11.58
miR-515-3p	13.51
miR-146a	14.14
miR-512-5p	15.09
miR-145	19.05
miR-516-5p	20.24
miR-512-3p	27.12
miR-520g	30.84
miR-372	41.97
miR-373	43.10
miR-139	63.96
miR-517a	107.72
miR-517b	155.95

Figure 1.9 Preliminary miRNA profiling analysis (II)

*Significantly (A) upregulated and (B) downregulated miRNAs in metastatic SW620 CRC cells compared with the paired non-metastatic SW480 CRC cells *P<0.05.*

Profiling data was subsequently used in conjunction with bioinformatic analysis to select 2 epithelial miRNA candidates for further study: miR-224, overexpressed in tumour epithelium compared with normal tissue and miR-153; overexpressed in metastatic CRC cells compared with paired non-metastatic cells.[235]

Expression of both epithelial miRNAs was confirmed in human CRC tissue using high-sensitivity Taqman® qRT-PCR (figure 1.10).

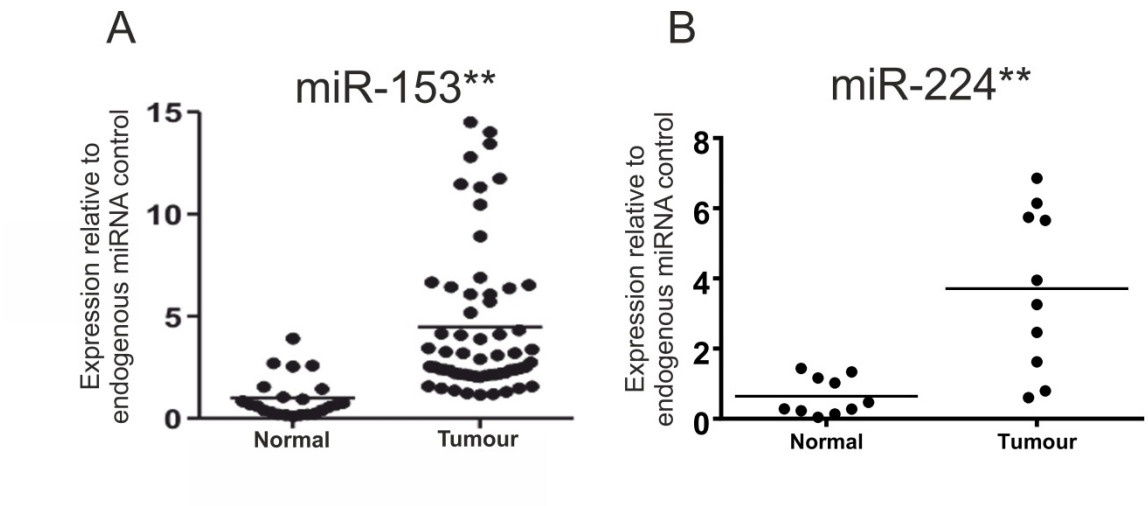


Figure 1.10 Preliminary miRNA profiling analysis (III)

(A) MiR-153 expression in 23 normal colonic epithelial specimens compared with 60 CRC epithelial specimens by Taqman® qPCR from Zhang et al., 2013.[235] *(B) MiR-224 expression in 10 CRC epithelial specimens and paired normal colonic epithelial specimens by Taqman® qPCR. Mean expression for the group is represented by a horizontal bar. **P<0.005.*

2 Materials and Methods

2.1 Tissue culture

2.1.1 General principles

All tissue culture work was conducted in a laminar flow hood. Cells were grown in a humidified environment at 37^{o}C with 10% CO_2 (Heraeus HeraCell incubator, Thermo Scientific). Reagents were stored at 4^{o}C, except phosphate buffered saline (PBS), which was stored at room temperature.

2.1.2 Cell lines

The following section contains details of all cell lines used in this study. Unless otherwise stated, cell lines were purchased from American Type Culture Collections (ATCC; Manasses, VA, USA).

Cell lines were regularly tested for mycoplasma contamination and only used if they were mycoplasma free. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Lonza Group Ldt, Basel, Switzerland) supplemented with 10% foetal calf serum v/v (FCS), 2mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). DMEM supplemented in this way is referred to as 'complete' in the remainder of the text.

SW480

SW480 is an immortalised human colon adenocarcinoma cell line derived from a grade (III/IV) tumour in a 51 year old Caucasian male.[394]

SW620

SW620 is an immortalised human colon adenocarcinoma cell line derived from a lymph node metastasis of a 51 year old Caucasian male.[394] SW620s and SW480s are paired metastatic and non-metastatic CRC cell lines isolated from the same patient.

DLD1

DLD1 is an immortalised colon adenocarcinoma cell line with a strongly epithelial morphology, recruited from a human adult male. DLD1 cells were a kind gift from Dr A. Emre Sayan at the University of Southampton.[395]

HCT116

HCT116 is an immortalised human colon adenocarcinoma cell line deficient for p300, a transcriptional cofactor involved in multiple cellular processes.[235, 396]

AAC1/82

AAC1/82 human colon adenocarcinoma cells were a kind gift from Professor C. Paraskeva at the University of Bristol.[235]

HT29

HT29 human colonic adenocarcinoma cells were isolated from a low grade (I) primary tumour specimen from a 44 year old Caucasian male.[397]

HFFF2

Human Foetal Foreskin Fibroblasts were derived from a 14-18 week old human foetus.[398]

MRC5

MRC5 Fibroblasts were originally established from normal lung tissue of a 14 week old male foetus. MRC5 fibroblasts had been immortalised by ectopic expression of the telomerase reverse transcriptase gene, in a manner which affects neither cell morphology or growth characteristics.[399] MRC5 fibroblasts were a kind gift from Dr Jeremy Blaydes at the University of Southampton.

2.1.3 Primary colon fibroblasts

Primary colon fibroblast (PCF) cells were isolated from normal uninvolved human colon tissue within resection specimens of patients undergoing surgery for CRC at

University Hospital Southampton. Ethics committee and Trust R&D approval were obtained and patient's written informed consent was sought in each case.

Tissue specimens, transported from the operating theatre in 10ml PBS supplemented with 100u/ml Penicillin (Lonza Biowhittaker™, Walkersville, MD, USA), 100µg/ml Streptomycin (Lonza Biowhittaker™, Walkersville, MD, USA, and 0.25µg/ml Fungizone® (Gibco®, Invitrogen, Carlsbad, CA, USA), were divided into 2 mm sections, placed in the centre of a 10cm dish and washed 3 times with PBS/Pen-Strep/Fungizone. With a sterile scalpel, a cross was drawn in the centre of the dish, to encourage adherence by the tumour specimen. Specimens were cultured in 20% DMEM supplemented with 20% FCS, 100u/ml Penicillin, 100µg/ml Streptomycin and 292µg/ml L-Glutamine. Over the next 5 days the plates were not disturbed except to re-feed every 2 days. After 5-7 days, PCFs began to grow out from the edge of the tissue sections and by 3-4 weeks they were present in sufficient numbers to transfer to a 25cm^2 tissue culture flask in complete DMEM.

Cell cultures were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2.

2.1.4 Cell culture technique

Cells were grown as adherent monolayers in sterile culture flasks (Greiner Bio-One Ltd., Gloucertershire, UK) in 5-10% CO_2 at 37^{o}C. Confluent cells were sub-cultured once, twice or three times per week at a ratio of 1:2-1:10. Briefly, media was removed from the culture flask; cells were washed with PBS to remove residual serum; 4ml Trypsin-Versene® (Lonza Biowhittaker™) was added and cells were returned to the incubator until they had detached. Once cells had detached, trypsin was inactivated by the addition of medium containing serum (complete DMEM) and appropriate aliquots of cell suspensions were transferred to a new flask or were used in experimental analysis. Complete DMEM was added to the remaining cells to achive a final volume of 20ml per 175cm^2 flask. Medium was changed twice each week.

2.1.5 Freezing cells for long term storage

Freezing media was prepared by adding 10% dimethyl sulphoxide (DMSO; Sigma-Aldrich) to 90% FCS. Cells in a 175 cm^2 culture flask were trypsinised in the standard

fashion (section 2.1.4). Half (v/v) were transferred to a 15ml sterile Falcon® tube (Corning®, Tewksbury, MA, USA), counted and centrifuged at 1300 rpm (360g) for 3 minutes in a Sorvall® Legend RT (Thermo Scientific) bench-top centrifuge. Pelleted cells were suspended in freezing mixture on ice at a concentration of $1x10^6$ per ml for fibroblasts and $3\text{-}6x10^6$ per ml for CRC cell lines. 1ml aliquots were transferred to labelled cryovials (Grenier Bio-One Ltd) and frozen in NALGENE® Mr Frosty containers at -80^{o}C overnight before being transferred to liquid nitrogen for long term storage thereafter.

2.1.6 Thawing cells for culture

Cells were retrieved from storage in liquid nitrogen and transported on dry ice to the tissue culture room. 5ml complete DMEM was pre-warmed in labelled 25cm^2 culture flasks. Cells were thawed by hand and resuspended in 5ml complete DMEM. Cells were then centrifuged at 1300 rpm (360g) for 3 minutes in a Sorvall® Legend RT bench-top centrifuge and the supernatant (containing DMSO) discarded. The cell pellet was subsequently resuspended in 5ml fresh complete DMEM and transferred directly to the appropriate culture flask. Cells were subsequently allowed to recover at 37^{o}C/10% CO_2.

2.2 Protein expression analysis

2.2.1 Cell harvest

Cellular assays involving protein harvest for western blot, were conducted in a 6 well format for both cultured fibroblast and CRC cell lines.

Protein lysates were prepared on ice. Growth media was decanted and the cells washed twice in ice cold PBS. Cells were subsequently lysed in 1x radioimmunoprecipitation buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris.Cl pH7.5) plus 1% complete protease inhibitor (ROCHE) applied directly to the cells. Cell detachment was encouraged using a plastic cell scraper (Greiner Bio-One Ltd.). After 15 minutes the entire volume was transferred to a fresh 1.5ml microcentrifuge tube.

Lysates were subsequently clarified by centrifugation at 13,000 rpm (16100g) for 5 minutes at 4°C in a Heraeus® Biofuge® fresco microcentrifuge. The resulting lysate supernatant was transferred to a fresh microcentrifuge tube and stored on ice. The total protein content of lystate supernatant was calculated using the BioRad Protein Assay (BioRad, Hercules, CA, USA) as described in the next section.

2.2.2 Calculating protein concentration and Gel Electrophoresis

BioRad Protein Assay concentrate was diluted 1:5 in distilled H_2O. 250µl aliquots of this stock solution were transferred to the appropriate number of wells of a 96 well plate and supplemented with 5µl of the protein sample of interest.

In addition, BSA standards were made up in the following concentrations from 1mg/ml bovine serum albumin (BSA; Promega) stock in distilled H_2O (Table 2.1):

Protein Standard mg/ml	1mg/ml BSA (µl)	PBS dilutant (µl)	BioRad Assay concentrate (µl)
0	0	5	250
0.2	1	4	250
0.4	2	3	250
0.6	3	2	250
0.8	4	1	250
1	5	0	250

Table 2.1 BSA standards for protein quantitation

Wells were mixed thoroughly and allowed to stand for 5 minutes at room temperature before light absorbance at 595nm was read using a Varioskan Flash plate spectophotometer (ThermoScientific). The data acquired was used to calculate a standard curve from which the protein concentration of each sample was calculated.

Sodium Dodecyl Sulphate-Polyacrylamide (SDS-PAGE) gels were made in accordance with the protocol described in *'Molecular Cloning a Laboratory Manual.'*[400] 10 or 12% acrylamide gels were allowed to polymerise between glass plates, before a stacking gel was poured and moulded into wells using a 10 or 15 well comb.

Protein samples were transferred to an eppendorf tube and supplemented with loading dye containing Dithiothreitol (DTT). The samples, along with a protein marker (New England Biolabs, Ipswich, USA) were heated to 95^{o}C for 2 minutes to denature protein. In the first well of the polyacrylamide gel, 5-15µl protein marker was loaded. In subsequent wells, equal amounts of the sample proteins of interest were loaded. Electrophosesis was carried out in 1X running buffer (Table 2.2) at 200V for 1 hour or until the dye marker reached the base of the gel.

Fractionated proteins were subsequently transferred onto nitrocellulose membrane (Whatman Protran, GE healthcare) by wet transfer. This was achieved by 'sandwiching'

the gel and nitrocellulose membrane between 2 pieces of filter paper and 2 sponges within a transfer cassette (BioRad Laboratories).

The cassette was then immersed in transfer buffer (Table 2.2) such that an electrical current (100V for 90 minutes) could pass through it and drive proteins from the gel onto the membrane where they would be captured.

Reagent	Composition
Running Buffer (X10)	250mM Tris-base; 1.9M Glycine and; 35 mM SDS
Transfer Buffer	200ml X10 Running buffer; 500ml 100% EtOH; 1300ml dH_2O
Tris-Buffered Saline-Tween (TBS-Tween)	20mM Tris pH7.6; 137mM NaCl in 1000ml dH_2O suppliemted with 1ml Tween-20 (0.1%)

Table 2.2 Reagents used for SDS-PAGE electrophoresis, antibody incubation and visualisation

2.2.3 Antibody incubation and visualisation

After blotting, membranes were blocked for 1 hour at room temperature in 3% (w/v) non-fat dry milk dissolved in 0.1% TBS-Tween. Blots were subsequently incubated with primary antibody in 1% milk in 0.1% TBS-Tween over night at 4^oC, then washed 3 times for 10 minutes (0.1% TBS-Tween) and incubated with horseradish peroxidase-conjugated secondary antibody (Dako; Diluted 1:5000) in 0.1% milk in 0.1% TBS-Tween for a further 1 hour at room temperature. After a further 3 washing steps, immunoblots were visualised using Supersignal® West Pico Chemiluminescent detection kit (ThermoScientific). Band intensity was quantified by densitometry using Image-J image processing software. A range of primary antibodies used in this work and their corresponding dilutions are summarised in Table 2.3.

Primary antibody	Manufacturer	Dilution
Rabbit α-RECK	Abgent	1:500
Rabbit α-TIMP3	Abgent	1:100
Rabbit α-E-cadherin	Abcam	1:1000
Rabbit α-N-cadherin	Abcam	1:1000
Rabbit α-β-actin	Santa Cruz	1:2000
Mouse α-HSC70	Santa Cruz	1:10000
Mouse α-SMA	Sigma	1:1000

Table 2.3 Antibodies used in Western blotting

2.3 mRNA and microRNA expression analysis

2.3.1 mRNA and microRNA expression in cell lines

2.3.1.1 Total RNA extraction

Total RNA was extracted from cells using the miRVana™ RNA extraction kit (Life Technologies) in accordance with manufacturer's instructions. Cells were trypsinied, transferred to a 15ml sterile Falcon® tube (Corning®) in complete DMEM and centrifuged at 1300 rpm (300 x g) for 3 minutes in a Sorvall® Legend RT (Thermo Scientific) bench-top centrifuge. Pelleted cells were lysed in 300µl miRVana™ lysis/binding buffer and transferred to a microtube, to which $1/10^{th}$ volume of Homogenate Additive was added and left for 10 minutes on ice. 300µl Acid-Phenol:Chloroform was then added and vortexed for 30-60 seconds. Samples were centrifuged at 10000 x g in a Heraeus® Biofuge® fresco microfuge at room temperature for 5 minutes to separate aqueous and organic phases. The upper aqueous phase was then removed, measured by volume and transferred to a fresh microcentrifuge tube. To each sample, 1.25 volumes of 100% ethanol was added. The lysate/ethanol mix was then transferred 700µl at a time to a filter cartridge placed in a fresh tube and centrifuged for 15 seconds at 10000 x g to pass the mixture through the filter. The filter was then washed in 3 successive steps using 2 separate wash solutions from the kit. Flow-through was discarded between each wash step. Total RNA was eluted by applying 50µl nuclease-free water (Severn Biotech Ltd.) pre-heated to $95^{o}C$ to the centre of the filter (place in a fresh microcentrifuge tube) and spinning in a bench-top centrifuge for 30 seconds at 10000x g.

The concentration of total RNA extracted was quantified using a Nanodrop 1000 Specrophotometer (Thermo Scientific). RNA purity assessed using the A_{260}/A_{280} ratio method with a ratio >2 deemed acceptable.

2.3.1.2 Taqman® qRT-PCR quantitation

To analyse miRNA and mRNA transcript levels for the purpose of measuring transfection efficiency, Taqman® (Applied Biosystems) gene expression assays were used.

miRNA quantitation

The Taqman® qRT-PCR assay consists of reverse transcription (RT) and forward PCR steps, both of which require specific primers for each miRNA analysed. Primers used are summarised in Table 2.4.

5µl of total RNA diluted to 10µg/µl in nuclease-free water was converted into cDNA using a miRNA-specific RT step in accordance with manufacturer's instructions: The RT reagents (10 × RT buffer, dNTPs, RNase Inhibitor, RNase-free dH_2O and MultiScribe^{TM} Reverse transcriptase (50 u/µl)) were combined with total RNA and the appropriate RT primer was added to the tube. The following RT reaction conditions were applied; 16^0C for 30 min; 42^0C for 30 min, 85^0C for 5 min and 4^0C hold.

PCR reactions were set-up in triplicate using miRNA specific primers and probes. For the 20µl PCR reaction; 10 µl Taqman® 2 × Universal PCR Master Mix (Applied Biosystems) was added to 1.33µl of cDNA, 7.67µl RNase-free dH_2O and 1µl of Taqman® microRNA Assay primer and probe mix. The PCR step was performed using the ABI 7500 qPCR instrument and the following the cycling parameters: 95^oC for 10 mins and 40 cycles of 95^oC for 15 sec and 60^oC for 60 sec.

MiRNA expression levels were normalised to U6 miRNA and calculated from the triplicate of C_T values using the $\Delta\Delta C_T$ method.

mRNA quantitation

The Taqman® qRT-PCR assay consists of a reverse transcription (RT) step which uses a universal primer, and a forward PCR step which requires specific primers for each mRNA analysed. Primers used are summarised in Table 2.4.

100ng-200ng of total RNA diluted in 7.7µl of nuclease-free water was combined for 5 minutes at 70^oC with lµl universal RT primer (GoScript™ kit from Promega) and

returned to ice. RNA was subsequently converted to cDNA in accordance with manufacturer's instructions using a master-mix comprised of 4µl GoScript™ 5X reaction buffer; 4.8µl $MgCl_2$; 1µl PCR nucleotide mix; 0.5µl Ribonuclease inhibitor and 1µl GoScript™ Reverse Transcriptase to make a total reaction volume of 20µl. The following RT reaction conditions were applied; 16^0C for 30 min; 42^0C for 30 min, 85^0C for 5 min and 4^0C hold. cDNA was subsequently diluted by adding 80µl nuclease-free water.

For the 20 µl PCR reaction; 10µl Taqman® 2 × Universal PCR Master Mix (Applied Biosystems) was added to 5µl of cDNA, 4µl RNase-free dH_2O and 1µl of Taqman® miRNA Assay primer and probe mix. PCR reactions were set up in triplicate using specific primers and probes and 2x Taqman® Universal PCR mastermix and performed using the ABI 7500 qPCR instrument and the following cycling parameters: 95^oC for 10 mins and 40 cycles of 95^oC for 15 sec and 60^oC for 60 sec.

mRNA expression levels were normalised to β-actin and calculated from the triplicate of C_T values using the $\Delta\Delta C_T$ method.

Gene	Assay ID
RECK mRNA	Hs0101917_ml
TIMP3 mRNA	Hs00165949_ml
PDCD4 mRNA	Hs00377253_ml
CDH1 mRNA	Hs01023894_ml
Vimentin mRNA	Hs00185584_ml
FSP1;S100A4 mRNA	Hs00243202_ml
β-actin mRNA	Hs99999903_ml
hsa-miR-224	000599
hsa-miR-215	000518
hsa-miR-21	00397
hsa-miR-19a	002424
hsa-miR-556	002344
hsa-miR-106a	000578
U6B miRNA	001093

Table 2.4 Applied Biosystems gene ID for probes used in Taqman® qRT-PCR assays

2.3.2 microRNA expression in primary colorectal cancer tissue

2.3.2.1 Patients and samples

Consecutive pairs of primary colonic tumour and adjacent non-tumourous colonic tissue (located >5cm from tumour) were obtained during surgery at University Hospital Southampton as part of a prospective National Institute of Health Research study (UKCRN ID 6067). Half of each specimen was frozen in liquid nitrogen and stored at -80^0C, and half fixed in formalin and embedded in paraffin. From this archive 50 consecutive and formalin fixed paraffin embedded (FFPE) CRC specimens were obtained. This cohort comprised 25 patients with stage I/II disease who developed metastasis within 5 years (stage II-R) and 25 carefully matched patients who remained metastasis free in that time (stage II-NR) (Table 2.5). Pathological verification of diagnosis and staging was in accordance with the Association of Coloproctology of Great Britain and Ireland guidelines on the management of CRC.[401]

All specimens were stored in a designated UK Human Tissue Act approved tumour bank. Comprehensive demographic, clinical and pathological information was collated by the author and hereditary tumours, mucinous tumours, and tumours with histologically identified necrosis were excluded. All patients provided written informed consent in accordance with the Helsinki protocol and the study was approved by the regional research ethics committee.

Characteristics	Stage II-R (n=25)	Stage II-NR (n=25)	p value
Age, years at diagnosis (mean+sd)	73.92 (11.45)	71.36 (11.57)	0.435[a]
Gender (m:f) absolute numbers	21:4	17:8	0.321[b]
Tumour site			0.482[b]
Right Colon	6	9	
Left Colon	11	12	
Rectum	7	4	
Stoma	1	0	
Surgical setting			0.762[c]
Elective	18	16	
Emergency	7	9	
Histopathological data			
Maximum tumour diameter (mm) Mean (SD)	50.22 (26.00)	49.54 (18.70)	0.919[a]
T stage			0.347[b]
T2	0	2	
T3	18	16	
T4	7	7	
N stage: N0 status	25	25	
M stage: M0 status	25	25	
AJCC staging 7th ed			0.347[b]
Stage I	0	2	
Stage IIA	18	16	
Stage IIB	7	7	
R0 resection margin	22	23	1.000[c]
Differentiation status			0.177[b]
Poor	2	2	
Moderate-Poor	4	1	
Moderate	7	14	
Well-Moderate	12	8	
Well	0	0	
Extramural vascular invasion	4	1	0.349[c]
Tumour perforation	1	2	1.000[c]
Adjuvant therapy			
Neoadjuvant Chemotherapy	3	2	1.000[c]
Neoadjuvant Radiotherapy	4	2	0.667[c]
Adjuvant Chemotherapy	6	2	0.247[c]
Adjuvant Radiotherapy	1	2	1.000[c]
Mean follow up, years (s.d)	2.89 (1.62)	5.40 (2.02)	0.000[a]

Table 2.5 Demographic data and tumour characteristics for all patients with stage II CRC with (stage II-R) and without (stage II-NR) recurrence at 5years *[a] = Independent t-test, [b] = Chi squared test, [c] = Fisher's exact test*

2.3.2.2 Slide preparation, Laser microdissection and RNA extraction

FFPE specimens were sectioned at 8-10µm using a microtome and subjected to deparaffinisation in Xylene for 1 minute. Tissue sections were then fixed in 75% ethanol for 30s and stained with Cresyl Violet (Sigma-Aldrich) for 1 min before undergoing a further dehydration step in 100% ethanol. Once air dried, LMD was performed using the Leica AS LMD microdissection platform (Leica Microsystems, UK) and cut tissue was collected directly into 50 µl of cell lysis buffer (RecoverAll™; Ambion®). Approximately 1×10^6 μm^2 was dissected from each slide. Malignant colonic epithelium and tumour associated stroma were each microdissected separately.

Total RNA extraction was conducted immediately using the RecoverAll™ kit for FFPE tissue in accordance with the manufacturer's instructions, except that RecoverAll™ filters were replaced by RNAqueous®-Micro Filters (Ambion) designed specifically for use with LMD tissue.

A further 50 µl of cell lysis buffer was added to each microcentrifuge tube containing LMD tissue to make a total volume of 100µl. To this, 1µl protease was added and the sample incubated for 15 minutes at 50^{o}C and then for 15 minutes at 80^{o}C in heat blocks.

During this time 120µl isolation additive from the kit was combined with 275µl 100% ethanol. After 30 minutes total incubation, the isolation additive/ethanol mix was added to each sample on ice. After thorough mixing, the full volume of each sample was pipetted through a filter contained within a fresh microcentrifuge tube and centrifuged at 10000 x g in a Heraeus® Biofuge® fresco microfuge at room temperature for 30 seconds. The flow-through was subsequently discarded and the filter washed in 3 successive steps using 2 separate wash solutions from the kit. Flow-through was discarded between each wash step.

After the final wash, the filter was transferred to a fresh microcentrifuge tube. RNA was eluted by applying 15µl nuclease-free water (Severn Biotech) at room temperature to the centre of the filter and spinning in a centrifuge for 1 further minute at 10000x g.

RNA concentration was determined using the Nanodrop™ 1000 spectrophotometer (Thermo Scientific). RNA quality was also assessed using this method: a ratio of absorbance at 260nm and 280nm ≈ 2 was considered 'pure'. If RNA yield <2ng/ml or

purity <1.8 (260nm:280nm ration) was achieved, LMD and RNA extraction steps were repeated.

2.3.2.3 MiRnome wide gene expression analysis

From the cohort of 50 patients with stage II CRC, 20 carefully matched specimens with (stage II-R) and without (stage II-NR) recurrence at 5 years were selected for differential gene expression analysis (Table 2.6). LMD and RNA extraction from stromal and epithelial tumour layers were performed as previously described (2.3.2.2).

Microarray data acquisition and presentation was conducted in accordance with MIAME guidelines.[402] The biomarker potential of data acquired was assessed in accordance with REMARK guidelines. [403]

For each LMD specimen, 100ng total RNA was dried down by vacuum centrifugation and resuspended in 3µl of nuclease-free water. Hy3™ fluorescence labelled RNA was prepared using the miRCURY LNA™ microRNA Array Hi-Power labelling kit (Exiqon, Vedbæk, Denmark) in accordance with the manufacturer's instructions:

For each hybridisation reaction, 3µl total RNA was combined with 1µl 'Spike-in' miRNA control, 0.5µl CIP buffer and 0.5µl CIP enzyme. The 'Spike-in' control contains 52 different synthetic unlabelled miRNAs corresponding to capture probes on a miRCURY LNA™ microarray slide at various concentrations.

This reaction mix was incubated for 30 minutes at 37^{o}C using a PCR cycler with heated lid. After 30 minutes, the enzyme reaction was stopped and the RNA denatured by incubation at 90^{o}C followed by snap cooling on ice for 5-15 minutes. Subsequently, this 5µl reaction mix was combined with 1.5µl Hy3™ fluorescent label, 3µl Hi-Power Labelling buffer, 2µl DMSO and 1µl of Hi-Power Labelling enzyme and incubated in darkness for 2 hours at 16^{o}C.

To stop the Hi-Power labelling reaction after 2 hours the sample was heated to 65^{o}C for 15 minutes and then placed immediately on ice.

Labelled RNA was subsequently hybridised to miRCURY LNA™ 7th generation microRNA microarray slides using a commercially available kit from Exiqon. These pre-printed arrays consist of control probes and 3100 capture probes, complimentary to

most human, mouse and rat sequences from the v19.0 release of miRbase (http://www.mirbase.org/blog/2012/08/mirbase-19-released/).

The fluorescent labelled RNA sample from the previous step was adjusted to 200µl by the addition of 178.5µl nuclease free water. To this, 200µl 2X hybridisation buffer was added and the mixture vortexed for 1 minute. RNA was then denatured in darkness at 95^{o}C for 2 minutes using a heat block, before being transferred directly to ice for a further 5-15 minutes.

In advance, microarray hybridisation cassettes (CapitalBio corp.) were prepared by cleaning once with RNaseZAP™ (Sigma-Aldrich) and once with nuclease free water. 400µl of the target sample mix was then added to the cassette reservoir before an array slide was placed onto it face down. The hybridisation cassette was then assembled making sure to avoid bubble formation, and clamped shut before the entire assembly was wrapped in aluminium foil to exclude light. Hybridisation was subsequently conducted for 16 hours at 56^{0}C in a Techne HB hybridisation incubator oven.

The following day, the cassettes were disassembled and the hybridisation slides subjected to serial washes in 3 different salt buffer/detergent solutions, culminating in a final wash in 99% ethanol, each step having lasting for 1 minute.

Finally, the hybridisation slides were placed into individual 50ml falcon tubes (Corning®) and spun-dried for 5 minutes at 1000 rpm in a Sorvall® Legend RT (Thermo Scientific) bench top centrifuge.

Once dry, array slides were scanned using a GenePix 4000B scanner (Molecular Devices) to detect Hy3™ at a wavelength of 532nm. Image analysis was performed using GenePixPro 3.0.5 software, which permitted a miRbase v19 updated gal file (www.exiqon.com/Gal-downloads) containing a mapping grid to be superimposed and accurately aligned onto the array image in TIFF format.

Mean fluorescence intensities minus background fluorescence were subsequently normalised and expression ratios calculated between LMD stroma/epithelium from recurrence and non-recurrence groups.

Characteristics	Stage II-R (n=10)	Stage II-NR (n=10)	p value
Age, years at diagnosis (mean+sd)	73.90 (11.31)	76.20 (8.43)	0.612[a]
Gender (m:f) absolute numbers	8:2	6:4	0.329[b]
Tumour site			0.132[b]
Right Colon	2	5	
Left Colon	2	4	
Rectum	5	1	
Stoma	1	0	
Surgical setting			0.370[c]
Elective	4	7	
Emergency	6	3	
Histopathological data			
Maximum tumour diameter (mm) Mean (SD)	50.50 (26.71)	49.50 (10.12)	0.913[a]
T stage			0.171[b]
T2	0	2	
T3	10	7	
T4	0	1	
N stage: N0 status	10	10	
M stage: M0 status			
AJCC staging 7th ed			0.171[b]
Stage I	0	2	
Stage IIA	10	7	
Stage IIB	0	1	
R0 resection margin	10	9	1.000[c]
Differentiation status			0.306[b]
Poor	0	1	
Moderate-Poor	2	1	
Moderate	3	6	
Well-Moderate	5	2	
Well	0	0	
Extramural vascular invasion	1	0	1.000[c]
Tumour perforation	0	0	
Adjuvant therapy			
Neoadjuvant Chemotherapy	0	0	
Neoadjuvant Radiotherapy	0	0	
Adjuvant Chemotherapy	0	0	
Adjuvant Radiotherapy	0	0	
Mean follow up, years (s.d)	3.37 (1.41)	5.90 (2.23)	0.007[a]

Table 2.6 Demographic data and tumour characteristics for stage II CRC specimens used in differential gene expression analysis

[a] = Independent t-test, [b] = Chi squared test, [c] = Fisher's exact test.

2.3.2.4 MiRNA validation in primary CRC tissue by Taqman® qRT-PCR

Singleplex Taqman® miRNA assay reactions were performed to quantitate candidate miRNA expression in all 50 tumour samples (Table 2.5) to validate data acquired through high-throughput screening (2.3.2.3).

For each qPCR assay 2.5 ng total RNA from LMD samples (0.5 ng/µl) was converted into cDNA using a miRNA-specific RT step. The Taqman® microRNA Reverse Transcription kit (Applied Biosystems) was used according to the manufacturer's instructions in conjunction with a miRNA-specific looped reverse primer (Table 2.4). The RT reagents (10 × RT buffer, dNTPs, RNase Inhibitor, RNase-free dH_2O and MultiScribe^{TM} Reverse transcriptase (50 u/µl)) were combined with total RNA and the appropriate RT primer was added to the tube. The following RT reaction conditions were applied; 16^0C for 30 min; 42^0C for 30 min, 85^0C for 5 min and 4^0C hold. For the 20 µl PCR reaction; 10 µl Taqman® 2 × Universal PCR Master Mix (Applied Biosystems) was added to 1.33 µl of cDNA, 7.67 µl RNase-free dH_2O and 1 µl of Taqman® MicroRNA Assay primer and probe mix.

Reactions were performed in triplicate in 96 well plates covered with optical adhesive seal. The ABI-HT7500 qPCR instrument (Applied Biosystems) was used with the following cycling parameters: 95^0C for 10 min and 40 cycles of 95^0C for 15 sec and 60^0C for 60 sec. Expression levels, normalised with U6 qPCR assay were calculated using the $\Delta\Delta C_T$ method and expressed relative to one of the specimens that was assigned the value 1.

2.3.3 microRNA expression in LMD tissue from CRC and Pancreatic organotypic models

CRC organotypics were created in triplicate as described in section 2.6.3 using SW480 and SW620 CRC epithelial cells and PCF8 or MRC5 stromal fibroblast cell lines. After 14 days incubation, organotypics were fixed in formalin for 24 hours, washed in 70% ethanol for 24 hours and subsequently embedded in paraffin blocks.

In addition, FFPE pancreatic cancer organotypics containing CAPAN1 and BXPC3 epithelial cancer cell lines and primary stellate stromal cells were aquired as a kind gift from Dr Jo Tod at the University of Southampton.

As for primary CRC tissue, 8-10μm organotypic sections were mounted on membrane coated slides, de-paraffinated and stained with cresyl violet solution as described in section 2.3.2.2.

LMD was used to isolate organotypic stroma from malignant epithelial cells at the invasive tumour margin and non-invading malignant cells in well organised stratified epithelial layers. Total RNA was subsequently extracted from LMD tissue using the RecoverAll™ kit (Ambion) for FFPE tissue.

As before miRnome gene expression analysis was performed using the miRCURY LNA™ 7th generation microRNA microarray from Exiqon™. 75ng total RNA was utilised for each hybridisation reaction. In total, 3 hybridisation reactions were performed per organotypic, corresponding to RNA extracted from stroma, malignant cells at the invasive tumour margin and non-invasive malignant epithelial cells.

Singleplex Taqman® miRNA assay reactions were performed to quantitate candidate miR-4454 expression in invasive vs. non-invasive malignant epithelial cells in all 4 organotypic models. This protocol is described in detail in section 2.3.2.4.

2.4 Generating stably transfected cell lines

Stable miR-21 and scrambled control miR-SCC expression in MRC5 fibroblasts (referred to as MRC5^{21} and MRC5^{SCC} cells), and stable miR-224, miR-153 and miR-SCC expression in CRC cell lines was achieved using precursor miRNA expression clones from GeneCopoeia™ (Rockville, Maryland, USA) (Figure 2.1).

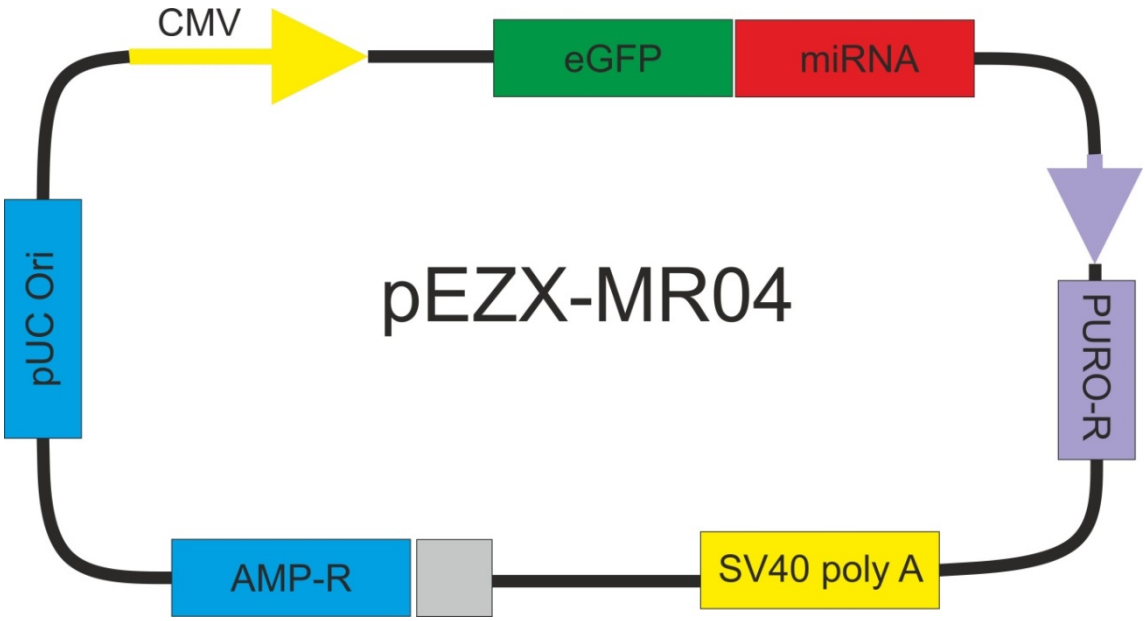


Figure 2.1 The pEZX-MR04 gene construct from GeneCopoeia™

Plasmid contains a pUC Ori (plasmid origin of replication) sequence, an AMP-R (ampicillin resistance) and a PURO-R (Puromycin resistance) gene, is driven from a Cytomegalovirus (CMV) promoter sequence and contains a GFP (Green fluorescent protein) reporter as well as the gene of interest (miR-21; miR-224; miR-153 or miR-SCC).

In contrast, stable miR-21 and miR-SCC expression in HFFF2 fibroblasts was achieved using Lentifect™ purified lentiviral particles also from GeneCopoeia™.

2.4.1 Preparing plasmid DNA stocks

2.4.1.1 Transforming competent JM109 bacteria

A 50µl vial containing competent JM109 bacteria to which 1µl of the precursor miRNA expression plasmid had been added was subjected to heat-shock treatment by incubation on ice for 20 minutes followed by incubation at 42^{o}C for 45 seconds followed by immediate cooling on ice for a further 2 minutes. 50µl of the DNA/bacteria mix was then added to 450µl of sterile LB broth and agitated in an Innova 4000 incubator at 37^{o}C. After 1 hour, 100µl of broth was seeded and spread on pre-warmed LB agar plates (containing 50µg/ml ampicillin from Sigma-Aldrich) which were then incubated overnight at 37^{o}C. The following day, individual bacterial colonies were transferred to separate falcons containing LB broth laced with ampicillin (50µg/ml) which were placed in an Innova 4000 incubator-shaker overnight at 37^{o}C. The following day, falcons were spun down at 3000rpm for 10 minutes to produce a pellet of bacterial cells.

2.4.1.2 Extracting plasmid DNA

Plasmid DNA was extracted from the bacterial pellet using the QIAprep® Miniprep kit from Quiagen as per the manufacturer's instructions. DNA was eluted in 50µl of nuclease-free water, quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and stored at +4^{o}C.

2.4.1.3 Restriction digest and Electrophoresis

To confirm successful purification of plasmid DNA, 1µl of stock solution digested using the restriction enzyme EcoR1 (16.3µl dH_2O; 2µl EcoR1 buffer; 0.2µl Acetylated BSA; 5µl EcoR1 enzyme incubated at 37^{o}C for 4 hours) was electrophoresed using an 0.8% Agarose gel made up in TBE and containing the Nucleic acid stain SafeView (NBS Biologicals).

2.4.2 Transfection and selection of cells

2.4.2.1 Epithelial CRC cell lines

Adherent cells were plated one day prior to transfection so that they were 80% confluent on the day of transfection. 2µg plasmid DNA was diluted in 100µl Opti-MEM I (Invitrogen), to which 6 µl of FuGENE® HD (Promega) transfection reagent was added. The FuGENE® HD/DNA mixture was incubated for 20 minutes at room temperature before being added dropwise to cells. Cells were subsequently incubated at 37^{o}C/10% CO_2. Assessment of transfection efficiency at 48 hours was conducted by visual examination under fluorescence as plasmid DNA co-expresses a GFP-reporter gene.

Once confluent, cells were trypsinised, and cell suspensions transferred to a fresh 6 well plate at the following dilutions in complete DMEM: 1:2; 1:4; 1:8; 1:16. Cells were allowed to adhere for a further 24 hours before the culture media was aspirated and replaced with 2ml of Puromycin at a concentration of 4µg/ml in complete DMEM. After 48 hours, selection media was removed, the cells washed in PBS and surviving cells expanded as per standard laboratory protocol in complete growth media. Under fluorescence, it was judged that a 1:8 dilution provided the most effective selection of GFP positive cells for both SW480 and HCT116 under these conditions. At greater dilution (1:16), only few cells survived and those that did failed to recover once selective media had been removed.

96 hours later, cells were trypsinised and suspended in serum free DMEM and the most fluorescent 5% of cells were selected using a FACSAria II (BD Bioscience) Cell sorter. $1x10^5$ cells selected in this manner were returned to 6 well plates and expanded in culture without further Puromycin selection, for use in functional and mechanistic studies.

Ectopic miRNA expression was subsequently confirmed using Taqman®qRT PCR.

2.4.2.2 Fibroblast cell lines

One day prior to transfection, MRC5 cells were plated in 1ml complete DMEM in 6 well plates, so that they would be 50% confluent the following day. Transfection with plasmid DNA was carried out using Xfect™ transfection reagent in accordance with the manufacturer's instructions (Clontech):

8µg plasmid DNA was combined in a microcentrifuge tube with Xfect reaction buffer to a final volume of 100µl. In a second microcentrifuge tube, 0.3µl of Xfect polymer per 1µg DNA was also diluted to a final volume of 100µl in Xfect reaction buffer. After thorough vortexing, the polymer solution and plasmid solution were combined and incubated at room temperature to enable nanocomplexes to form. After 10 minutes, the entire volume was added dropwise to the cell culture medium. Cells were incubated at $37^{o}C/10\%$ CO_2 for 4 hours, before transfection medium was decanted and replaced with fresh, complete DMEM. Transfection efficiency was assessed visually under fluorescence after 48 hours.

Under identical conditions, the transfection of HFFF2 fibroblasts was not successful. Instead, stable ectopic expression of miRNA candidates was achieved in HFFF2 cells using Lentifect™ purified lentiviral particles from GeneCopoeia™ (Rockville, Maryland, USA):

HFFF2 cells were plated in complete DMEM in 24 well plates to achieve 80% confluence at the time of infection with lentiviral particles. 10mg/ml Polybrene® (Sigma-Aldrich) stock made up in 150mM NaCl was diluted 1:2000 in complete DMEM. For each well, 5µl or 30µl of pseudoviral stock was added to 0.5ml of diluted Polybrene. To achieve ectopic miR-21 or control miR-SCC expression, target cells were subsequently infected by removing old culture medium and replacing it with the appropriate viral supernatant. Plates were then incubated at $4^{o}C$ for 2 hours and then overnight at $37^{o}C/10\%$ CO_2. The following day, viral supernatant was removed and replaced with fresh DMEM and after 48 hours, transfection efficiency was assessed visually under fluorescence. When confluent, cells were trypsinised, transferred to a 6 well plate and subsequently expanded as per standard laboratory procedure.

Fibroblasts stably expressing miRNA/GFP co-expression plasmid were subsequently enriched by Puromycin selection: To determine the minimum cytotoxic Puromycin dose

for MRC5 and HFFF2 fibroblasts, triplicate wells of a 24 well plate were seeded with $1x10^4$ cells in 500µl complete growth medium. The following day old media was decanted and replaced with 1ml Puromycin (Sigma-Aldrich) solution diluted in complete DMEM to the following final concentrations: 0µg/ml; 0.1 µg/ml; 0.5 µg/ml; 1 µg/ml; 2 µg/ml; 5 µg/ml; and 10 µg/ml.

At 24, 48, 72 and 96 hours post exposure to Puromycin, old media was discarded and the wells washed once with PBS. Viable cell numbers were quantified using the CellTiter 96® AQ_{ueous} Assay from Promega. Briefly, 2ml MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-slfophenyl)-2H-tetrazolium) reagent was combined with 100µl PMS (Phenazine methosulphate) and 10ml serum free DMEM, to produce a stock, of which 400µl was added to each well. After 90 minutes incubation at room temperature, light absorbance at 490nm was measured using used a Varioskan Flash plate reader spectrophotometer. Results represent a proxy measure of viable cell numbers.

2.5 Transient ectopic microRNA expression or suppression

2.5.1 Transient CRC cell line transfection

CRC cell lines were transiently transfected with miRNA mimic or control miR-SCC from Ambion® (Table 2.7) in accordance with a protocol pre-optimised by the group. Briefly, cells were plated in 500µl complete growth medium, in a 24 well format such that they were 80% confluent at the time of transfection.

In a microcentrifuge tube, 20pmol miRNA mimic or control miR-SCC oligomer was diluted in 50µl Opti-MEM® I Reduced Serum Medium. In a second microcentrifuge tube, Lipofectamine 2000™ was diluted 1µl in 50µl Opti-MEM® I. After 5 minutes incubation at room temperature, diluted oligomer was added to dilute Lipofectamine 2000™ and incubated for a further 20 minutes at room temperature. Once oligomer-Lipofectamine 2000™ complexes had formed, the mix was added drop-wise to cells to produce a final RNA concentration of 33nM.

Cells were incubated for a further 24-48 hours at $37^{o}C/10\%CO_2$ before harvest for use in functional cell assays and for assessment of transfection efficiency by Taqman® qPCR.

2.5.2 Transient fibroblast transfection

$5x10^4$ PCF, HFFF2 or MRC5 fibroblasts were plated in 6 well plates 16 hours prior to transfection. Fibroblasts were transfected using MiRIDIAN™ miRNA mimics and stem-loop inhibitors from Dharmacon® at a final concentration of 100nM (Table 2.7).

For one 6 well plate, a bijoux containing 1188µl Opti-MEM® I Reduced Serum Medium was supplemented with 33µl RNA at a concentration of 20µM. In a separate bijoux, 79.2µl Opti-MEM® I was supplemented with 19.8µl Oligofectamine™. Solutions were incubated separately for 10 minutes at room temperature, before being combined and incubated at room temperature for a further 20 minutes.

During this time, old culture medium was decanted and the cells washed once in Opti-MEM® I Reduced Serum Medium. Wash was subsequently discarded and replaced with 800µl fresh Opti-MEM® I per well. After 20 minutes, 200µl transfection mixture was added dropwise to each well and the cells were returned to the incubator at 37^{o}C/10% CO_2. After 4 hours, each well was supplemented with 500µl complete growth medium. 24-48 hours later, cells were used in functional assays or harvested for assessment of transfection efficiency by Taqman® qPCR.

Product	Manufacturer	Assay ID
Hsa-miR-224 mimic	Ambion®	AM17000
miR-SCC negative control	Ambion®	AM17110
Hsa-miR-21 mimic	Dharmacon®	C-300492-03
Hsa-miR-21 hairpin inhibitor	Dharmacon®	IH-300492-05
miR-SCC negative control	Dharmacon®	CN-001000-01
miR-153 Plasmid vector	GeneCopoeia™	HmiR0039
miR-224 Plasmid vector	GeneCopoeia™	HmiR0406
miR-21 Plasmid vector	GeneCopoeia™	HmiR0284
miR-SCC control vector	GeneCopoeia™	CmiR0001
miR-21 Lentipac™ Lentiviral particles	GeneCopoeia™	LP-HmiR0284
miR-SCC Lentipac™ control Lentiviral particles	GeneCopoeia™	LP-CmiR0001

Table 2.7 MiRNA mimic, hairpin inhibitors and scrambled (SCC) controls

Oligonucleotides used for stable and transient ectopic miRNA expression or suppression in CRC cell lines and fibroblasts.

2.6 Deregulated epithelial microRNAs – Functional studies

2.6.1 Transwell invasion assay

Cell invasion assays were performed over 72 hours using polycarbonate invasion chambers with 8µm pore sizes (Corning, Sigma) coated with Matrigel™ (BD) (diluted 1:2 in serum free DMEM). Transiently transfected DLD1, HCT116 or SW480 CRC cells were seeded in 200µl serum free DMEM at a density of $5x10^4$ per invasion chamber. Invasion chambers were then fitted into a 24-well plate containing 500µl of complete DMEM containing 10% FCS. Cells invading the lower chamber were trypsinised without washing and counted using a Casy cell counter (Roche Innovatis, Bielefeld, Germany).

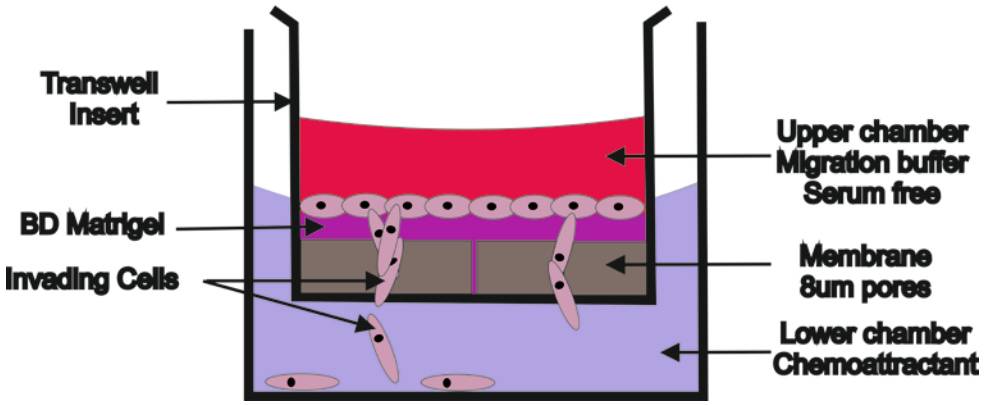


Figure 2.2 Illustration of Transwell invasion assays

CRC cells seeded in the upper chamber invade through a synthetic basement membrance composed of Matrigel™ along a chemoattractant gradient in to the trough below.

2.6.2 Cell proliferation assay

Cell proliferation was measured at various time points using the CellTiter 96® AQ_{ueous} Assay from Promega according to manufactures' instructions. 5000 HCT116 CRC cells

stably transfected with miR-224 or control miR-SCC were plated in triplicate, in complete DMEM in a 96 well plate and incubated overnight at 37^{o}C/10% CO_2.

On each subsequent day, 1ml MTS reagent was combined with 50µl PMS and 5ml serum free media to produce a stock reagent. At 24, 48 and 96 hour time points, old media was decanted and cells washed once in PBS. Cells were then incubated for 90 minutes in 120µl diluted MTS/PMS stock. Light absorbance at 490nm using a spectrophotometric plate reader (Thermo Scientific, Waltham, MA, USA) was used as a proxy measure of viable cell numbers.

2.6.3 Organotypic culture

Organotypics are synthetic tumour constructs which mimic *in vivo* conditions through the 3-D juxtaposition of an epithelial tumour cell layer with a stromal layer containing fibroblasts and essential ECM components.[404]

Day 1: 3.5 volumes of type I rat tail collagen (Millipore), 3.5 volumes of Matrigel™ (BD), 1 volume of filtered 10x DMEM, 1 volume of FCS and 1 volume of 10% DMEM containing 5x10^{5}/ml fibroblasts (MRC5/HFFF2 or PCF depending on the purpose of the study) were mixed on ice. 1ml of gel mixture was pipetted into each well of a 24 well plate and allowed to polymerise at 37^{o}C. After 1 hour, 1ml of 10% DMEM was added to the top of each gel and they were returned to the incubator for a further 24 hours at 37^{o}C.

Day 2: Medium was removed from the gels and replaced with 1 ml serum free DMEM containing 5x10^{5} cancer epithelial cells. Gels were then returned to the incubator for a further 24 hours at 37^{o}C.

Concurrently on day 2, 250µl of a collagen mix (containing 7 volumes collagen; 1 volume filtered 10x DMEM; 1 volume FCS and; 1 volume 10% DMEM) was added to sterile nylon sheets and allowed to polymerise at 37^{o}C for 30 minutes. Nylon sheet were subsequently fixed with 1% Glutaraldehyde (Sigma-Aldrich) for 1 hour at 4^{o}C, washed 3 times in PBS and once in 10% DMEM and then left in 10% DMEM over night at 4^{o}C.

Day 3: 2.5cm^{2} sterile stainless steel grids were place in 6 well plates. One collagen coated nylon sheet was placed collagen side up onto each grid. Gels were then carefully

lifted from the 24 well plates and transferred using a sterile spatula, onto the nylon sheets. Complete DMEM was then carefully added to each 6 well plate in sufficient volume to reach the under-surface of the nylon sheet without spilling onto it.

Media was changed every 3 days and after 14 days, gels were bisected, fixed in formal-saline and embedded in paraffin blocks.

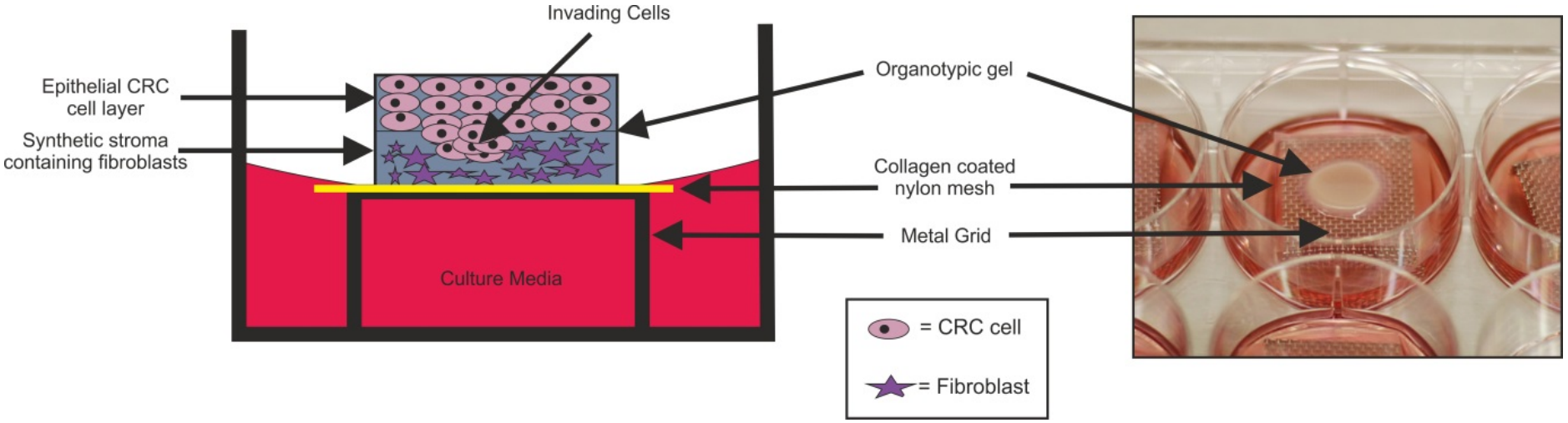


Figure 2.3 Illustration of organotypic co-culture

Organotypic models contain epithelial cancer cells and stromal cells such as fibroblasts, juxtaposed in 3-dimensions in a gel consisting of collagen and Matrigel™. Organotypics are incubated at an air-fluid interface, achieved by plating gels onto a collagen coated membrane suspended on the surface of chemoattractant solution on a wire plinth.

2.7 Deregulated stromal microRNAs – Functional and mechanistic studies

2.7.1 In-situ hybridisation (ISH)

ISH was performed as described by our collaborative colleagues (Bioneer; Horsholm: Denmark) except for minor modifications.[298] 6µm FFPE CRC tissue sections were mounted on slides and placed in a Tecan Freedom Evo automated hybridisation instrument (Tecan, Männedorf, Switzerland) in which the following steps were performed: proteinase-K treatment using 15µg/mL for 8 minutes at 37^{o}C, pre-hybridisation for 15 minutes at 57^{o}C, hybridisation for 60 minutes at 57^{o}C with double DIG (digoxigenin)-labelled miRCURY LNA™ probes (Exiqon, Vedbæk, Denmark) for miR-21 and a scramble sequence (both at 40nM), stringent washes with saline-sodium citrate (SSC) buffers, DIG blocking reagent (Roche, Mannheim, Germany), alkaline phosphatase-conjugated anti-DIG (Roche), enzymatic development using NBT-BCIP substrate (Roche) for 60 minutes, and finally nuclear fast red counterstain (Vector Laboratories, Burlingname, CA).

2.7.2 Transwell invasion assay

Transwell invasion assays were performed as described in 2.6.1 except for minor modifications.

MiR-21 and miR-SCC control transfected fibroblasts were plated separately at a density of 1x10^{5} cells per well in 6 well plates. After 24 hours culture medium was removed from cells and following 3 washes in PBS, replaced with serum free DMEM or DMEM supplemented with 1% Bovine Serum Albumin (BSA). Between 24 and 72 hours later conditioned media (CM; CM^{21} and CM^{SCC}) was removed, clarified by centrifugation at 13,000 xg for 10 minutes and the supernatant transferred in 500µl aliquots to a 24 well plate. 24 hours was identified as the optimal incubation time for fibroblasts in serum free conditions in order to minimise cellular stress, although in similar studies using various cultured fibroblast cell lines incubation time varied between 16 hours and 2 days. [405, 406]

SW480 or DLD1 cells were seeded at a density of 5 $x10^4$ per well in 200 µl of serum free DMEM. Invasion chambers were fitted into a 24-well plate containing 500 µl of CM^{21} or CM^{SCC} and allowed to invade for 72 hours at 37°C. Invaded cells were trypsinsed and counted using a Casy Cell Counter (Roche Innovatis, Bielefeld, Germany).

In addition, specific MMP2 inhibitor I (Merck, UK) was prepared at a stock concentration of 10mM in DMSO and added to CM^{21} and CM^{SCC} at a final concentration of 25µM. As little as 2.5 µM of MMP2 inhibitor was capable of producing a significant reduction in invasion. MMP2 inhibitor at 50 µM concentration induced some apoptosis but at 200 µM concentration significant cell death occurred which is likely to reflect the high final concentration of DMSO in which the MMP2 inhibitor had been dissolved. At a final concentration of 25 µM maximal inhibition of invasion was achieved with no cytotoxic effects. These optimisation steps will be discussed in greater detail in section 3.3.4.

2.7.3 Cell proliferation assay

Cell proliferation assays were performed using the CellTiter 96® AQ_{ueous} proliferation assay as described in section 2.6.2 except for minor modification.

5000 DLD1 or SW480 CRC cells were plated in triplicate in complete DMEM in 96 well plates. 24 hours later culture media was removed from cells and following 3 washes in PBS replaced with CM^{21} or CM^{SCC} from MRC5 fibroblasts. After 24 or 48 hours CM was decanted and replaced with 120µl of diluted MTS/PMS stock solution. After 90 minutes incubation at room temperature, absorbance readings at 490nm were taken in triplicates using a spectrophotometric plate reader (Thermo Scientific, Waltham, MA, USA).

2.7.4 Apoptosis assay

$6.5x10^5$ $MRC5^{21}$ or $MRC5^{SCC}$ cells were cultured in $75cm^2$ flasks containing 10mls of complete DMEM. After 24 hours, media was decanted and replaced with 10ml serum free media supplemented with 1% BSA. Fibroblasts were then returned to the incubator for 48 hours at $37^oC/10\%$ CO_2.

The next day, $1x10^5$ SW480 CRC cells were plated in triplicate wells of a 24 well plate in complete DMEM.

24 hours later, CM^{21} and CM^{SCC} was decanted from fibroblasts and clarified by centrifugation for 5 minutes at 13,000xg. Concurrently, culture medium was decanted from SW480 CRC cells, and the cells washed 3x in PBS.

After the final wash step, SW480 cells were treated with 1ml of clarified supernatant from miR-21 or miR-SCC control transfected MRC5 fibroblasts (CM^{21} and CM^{SCC}). A further 24 hours later, the SW480 cells were again washed 3x in PBS and treated either with 1ml complete DMEM containing 30µg/ml Oxaliplatin (Sigma, UK) or unmodified complete DMEM.

Cell viability was examined using the Annexin V/Propidium iodide (PI) double staining method (BD Bioscience) after 24 hours: Oxaliplatin or mock treated SW480 cells were trypsinised without washing, resuspended in 300µl 1X Binding buffer (10mM Hepes-pH7.4; 140mM NaCl; 2.5 mM $CaCl_2$), pooled and double stained with 2.5µl Annexin V-FITC (BD) and 2.5µl PI (50µg/ml). 10,000 cells were analysed per sample using a FACS Canto Flow Cytometer (BD). The assay was repeated 3 times. Results are presented as the percentage of total cells that are viable (Ann-/PI-), early apoptotic (Ann+/PI-) or late apoptotic (Ann+/PI+).

2.7.5 Gelatin Zymography

$MRC5^{21}$ and $MRC5^{SCC}$ cells were plated in triplicate at a density of $2x10^4$ in 96 well plates. 24 hours later, culture media was decanted; the cells washed 3 times in PBS, and media replaced with 200µl serum free DMEM. 24 hours later CM^{21} and CM^{SCC} were sampled, clarified by centrifugation at 13,000 xg for 5 minutes and used immediately. The volume of CM supernatant was adjusted to cell number and prepared in 2X SDS buffer without DTT and loaded without boiling to a 10% polyacrylamide gel copolymerised with 1 mg/ml of gelatin (Sigma-Aldrich). Gels were washed twice for 30 minutes in 2.5% Triton X-100 (Sigma-Aldrich) to remove SDS and incubated in developing buffer (50mM Tris-HCl; 0.2M NaCl; 5mM CaCl2; and 0.02% Triton X-100) at 37°C overnight. Gels were then stained for 30 minutes (0.5% Coomassie blue; 30% MeOH; 10% Glacial Acetic Acid) before de-staining (30% MeOH; 10% Glacial

Acetic Acid). Gelatin degrading enzymes were identified as clear bands against the dark background of the gel. To validate the chosen method of MMP2 inhibition, MMP2 inhibitor I (Merck. UK) was either added at a final concentration of 25µM, to CM^{21}/CM^{SCC} for 1 hour prior to fractionation or to developing buffer following fractionation.

Images of gels were captured using a UVP Imagestore 5000 (Ultra-Violet Products, U.K.) and band intensity quantified by Image-J. Results were expressed as the relative change in MMP activity in miR-21 transfected cells compared with miR-SCC scrambled control transfected cells.

2.7.6 Proteomic array procedure

To identify secreted factors differentially expressed by miR-21 and control miR-SCC transfected fibroblasts, PCF or HFFF2 cells were plated in 6 well plates and transfected with MiRIDIAN™ oligonucleotides at a final concentration of 100nM as described in 2.5.2.

The following day, cells were washed once in PBS and immersed in 1ml complete growth medium. After 48 hours CM was harvested and clarified by centrifugation for 5 minutes at 13000 xg. The supernatant was then extracted and frozen immediately at -20^{o}C.

The reagents for the Chemokine Array (RandD Systems Catalogue # ARY017) and Cytokine Array (RandD Systems Catalogue # ARY005) differed slightly, but the protocol was identical and conducted strictly in accordance with manufactures instructions:

500µl CM^{21} and CM^{SCC} from PCF or HFFF2 cells thawed on ice, was diluted in 1ml of the appropriate Array-specific buffer. 15µl of a detection antibody cocktail was added to the diluted CM samples and incubated for 1 hour at room temperature. A further buffer included with the kit was then used to ‘block’ a nitrocellulose membrane, on which selected capture antibodies had been spotted in duplicate.

Subsequently, blocking buffer was aspirated from the nitrocellulose membrane and replaced with the prepared CM/antibody mix. Incubation was then conducted overnight at 4^{o}C on a rocking platform shaker.

The following day, membranes were agitated in wash buffer for 10 minutes on a rocking platform shaker. The wash step was repeated two further times, before membranes were submerged in Streptavadin-HRP antibody diluted 1:2000 in blocking buffer. After 30 minutes incubation at room temperature on a rocking platform shaker, three further washes were conducted. Finally, immunoblots were visualised using the Chemi Reagent Mix supplied with the kit. Membranes were exposed to X-ray film in an autoradiography cassette for between 1 and 10 minutes. Band intensity was subsequently quantified by densitometry using Image-J image processing software.

2.7.7 ENA-78/CXCL5 ELISA assay

Expression of the chemokine ENA-78/CXCL5 was examined at 48 hours in CM from PCF and HFFF2 fibroblasts transiently transfected with either miR-21 or miR-SCC.

The Human ENA-78/CXCl5 Quanitkine® ELISA Assay from RandD systems was used for this purpose in accordance with the manufacturer's instructions. The assay, which employs a highly sensitive and specific sandwich immunoassay technique, was conducted using a 96 well microplate, pre-coated with an anti-ENA-78 capture antibody. 50µl of undiluted CM (CM^{21} or CM^{SCC}) was added to triplicate wells and incubated at room temperature for 2 hours. Wells were then washed 3 times in buffer ensuring complete removal of liquid between each step. 200µl Human ENA-78 enzyme-linked conjugate polyclonal antibody was then added to each well and a further incubation at room temperature was conducted for 2 hours. After 3 further wash/aspiration steps, 200µl substrate solution was added. Incubation was then conducted in darkness and at room temperature to enable colour to develop in proportion with the amount of ENA-78 originally present. After 30 minutes, 50µl of an enzymatic stop solution was added to each well, and the optical density at 450nm measured using a spectrophotometric plate reader (Thermo Scientific, Waltham, MA, USA).

2.7.8 Immunofluorescence

$5x10^4$ PCFs transfected with miR-21 or miR-SCC control plasmid (PCF^{21} vs. PCF^{SCC}) using the protocol described in 2.4.2, were seeded in complete DMEM onto 19mm circular coverslips prepared in 100% ethanol in 12 well plates. After 12 hours

incubation cells were fixed in 4% paraformaldehyde in PBS for 15 minutes, washed 3 times in Tris-Buffered Saline supplemented with 0.1% Triton x-100 (PBS-Triton; Triton x100: Sigma-Aldrich) and incubated for 30 minutes in 3% BSA diluted in TBS-Triton. Coverslips were overturned onto 50µl aliquots of 1:100 polyclonal anti-RECK (Abgent®), or anti-TIMP3 (Abgent®) antibody (Table 2.3) on parafilm and incubated at 4^{o}C overnight in a humidified box. The following day, coverslips were washed 3 times in TBS-Triton, overturned onto 50µl aliquot of 1:500 fluorescent conjugated Alexa Fluor 546 (Invitrogen) secondary antibody and incubated at room temperature for 1 hour. Finally coverslips were mounted onto glass slides using Vectashield Hardset with DAPI (Vector Laboratories; UK) and imaged using an Olympus IX81 fluorescence microscopy platform.

Similar methodology was used to assess α-SMA expression in MRC5^{21} and MRC5^{SCC} fibroblasts: 24 hours after seeding onto 19mm coverslips, MRC5 fibroblasts were treated with complete DMEM or CM extracted from SW480 CRC cells cultured overnight in DMEM, or TGFβ (R&D Systems) in complete DMEM at final concentrations of 2ng/ml or 10ng/ml. 72 hours later, cells were fixed in 4% paraformaldehyde, immunostained using anti-SMA primary antibody (clone 1A4, Sigma) and detected with conjugated Alexa Fluor 546 secondary antibody prior to imaging.

2.8 Immunohistochemistry

All immunohistochemistry was performed commercially by our collaborative colleagues at the Histochemistry research unit, University Hospital Southampton.

Briefly, a monoclonal anti-pan-Cytokeratin antibody (Sigma) and Alexa Fluor 546 conjugate anti-GFP antibody (Invitrogen) was used for immunohistochemistry on FFPE organotypic sections. Sections were mounted on APES coated slides and dried for 24 hours at 37^{o}C then dewaxed and rehydrated. Endogenous peroxidase was blocked for 10 minutes with 0.5% hydrogen peroxidase in methanol. Antigen retrieval was conducted by incubating the sections with protease (Sigma) for 10 min. Primary antibody was applied in TBS (pH 7.6) overnight at 4^{o}C. Swine anti-rabbit IgG biotinylated secondary antibody (Dako) was subsequently applied for 30 min followed by peroxidase-labelled avidin biotin complex (Vectastain Elite ABC Reagent, Vector Laboratories) for 30 min. Slides were washed three times with TBS between applications. Positive staining was visualised using DAB+ (Dako) for 5 min counterstained in Mayer's haematoxylin.

2.9 Tissue Microarray construction and immunostaining

The tissue microarray (TMA) prepared for this study compared patients with stage II CRC, with no significant histological or clinical features of biologically aggressive disease but who subsequently developed metastasis within 5 years with carefully matched patients who remained metastasis free.

Tumour specimens were identified from a database constructed by the author containing comprehensive histological, pathological and patient outcome data (Table 2.5).

TMA construction and immunostaining was performed by University colleagues under the supervision of Professor Gareth Thomas and Mr Alexander Mirnezami.

In brief, haematoxylin and eosin-stained slides from each tissue block were reviewed by a senior consultant pathologist, together with pathological reports to select triplicate areas of representative adenocarcinoma. Microarrays were subsequently assembled using a semiautomatic array machine (ALPHELYS MiniCore 3, Plaisir, France) using a 1-mm core punch, annealed at 40^{o}C.

TMA sections were immunostained using a primary Rabbit polyclonal antibody for RECK and TIMP3 (Table 2.3) pre-optimised by the group for use with paraffin-embedded specimens, using tonsilar tissue as a positive control.

RECK and TIMP3 expression was subsequently scored by an independent investigator blinded to the clinical data. Each core was scored semi-quantitatively on a scale of 1-7 corresponding to absent, low, medium or high intensity staining (0-3) with <25% positivity, 25-50% positivity, 50-75% positivity and >75% positivity (1-4).

2.10 In vivo metastasis studies

Orthotopic tumour models provide enhanced physiological relevance as they feature cancer cells growing in the correct anatomical location.

Orthotopic murine models of CRC require mouse anaesthesia in order to inject a cancer cell suspension into the caecal wall via a laparotomy.[407] However, to our knowledge, data from this study provides the first demonstration of a modified technique, whereby CRC cells are co-implanted orthotopically with stromal elements such as fibroblasts.

All *in vivo* experiments were performed in accordance with the University of Southampton's policy on the humane use of animals in research and the principles of the National Centre for the Replacement, Refinement and Reduction of animals in research.

Animals (SCID/CB17 mice) were housed within the Biological Resources Facility at University Hospital Southampton, and all appropriate licences were in place.

Ethical approval and Trust R and D approval were obtained in advance and the provisions of the Animals (Scientific Procedures) Act of 1986 rigorously observed. Furthermore, welfare standards consistent with all current UK legislation were upheld by the local ethics committee.

The number of mice required for this series of experiments was inferred following a thorough literature review. Two important studies used similar methodology to demonstrate the impact of miR-9 and miR-10b on the metastatic potential of breast cancer cells. One study demonstrated a significant increase in metastatic burden associated with miR-10b expression using 4 miRNA transfected mice and 4 mock transfected mice harvested at 11 weeks ($p=0.015$) but failed to demonstrate a statistically significant difference in a cohort of 6 mice (3 vs. 3) harvested at 9 weeks.[408] Another study described a 50% increase in lung micrometastasis formation associated with miR-9 expression using 8 miRNA transfected mice vs. 8 mice transfected mice at 4 weeks ($p=0.02$).[255]

As no directly equivalent studies have been performed for CRC, it was felt that 6 mice per experiment, harvested at 5 weeks would provide adequate statistical power as well as being financially reasonable and ethically justifiable.

On the day of orthotopic tumour implantation, for each mouse, $1x10^6$ SW620 CRC cells were combined with $5x10^6$ HFFF2 fibroblasts (stably expressing miR-21 or miR-SCC) in a 50µl mix of DMEM and Matrigel™ in equal measure.

Mice were anaesthetised with inhaled isofluorane, and the depth of anaesthesia assessed using toe pinch. The abdomen of the anaesthetised mouse was then shaved and cleaned with betadine before being draped in a sterile fashion. A 2-3 cm abdominal wall incision was made and the caecum exteriorised. A 27G needle was then used to inject the entire 50µl cell suspension into the caecal wall, before the caecum was returned to the abdomen and the abdominal wall closed using interrupted, full thickness sutures.

Once recovered, the animals were weighed regularly and closely observed. After 5 weeks all mice were euthanized and primary tumour, liver, spleen and lungs removed and weighed.

Tissue was subsequently fixed in 10% formalin and embedded in paraffin for further histological analysis.

2.11 In silico analytics

The potential biological relevance of miRNA candidates identified in this study was examined using online software to model potential miRNA/mRNA interactions *in silico*.

Identification of putative miRNA target genes was performed using TargetScan (http://www.targetscan.org/; release 5.1). The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/home.jsp) was used to evaluate enrichment of biological processes and pathways associated with putative miRNA target genes. More specifically, the Gene Ontology Biological Process (FAT), Panther, BioCarta and KEGG annotation categories were assessed against a background of all human genes using the default DAVID parameters.

2.12 Statistical analysis

Analysis and graphical display of miRNA validation data were generated using GraphPad Prism version 4.03 (GraphPad Software Inc., USA). U6B was selected as the endogenous reference gene. Mean triplicate U6B RNA expression was quantified by the ΔΔCt method allowing expression of selected miRNAs to be calculated relative to this reference. Differences between paired tumour and normal tissue samples were expressed as fold-change, using the mean expression value from the normal group as the reference. Differences between recurrence and non-recurrence tumour groups were expressed as fold-change using the mean value from the non-recurrence group as a reference. Statistical analysis was subsequently conducted using paired and un-paired Student's t-test as appropriate.

From the TMA immunohistochemistry experiments (section 2.9), protein expression scores were expressed as mean ± standard error of the mean (s.e.m) from triplicate cores of CRC tissue extracted for each patient represented on the array. Statistical significance was assessed using independent t-tests for comparison between recurrence and non-recurrence tumour groups.

Analysis of clinicopathological patient data was performed using a combination of independent t-tests, Mann–Whitney U, w2- and Fisher's exact tests.

Disease free survival in early stage CRC, was calculated in months from time of surgery to first recurrence of disease. Recurrence was defined by positive radiological imaging (CT, MRI or PET) and/or biopsy information. Deaths from other causes were censored.

Kaplan–Meier plots for analysis of recurrence-free/overall survival levels were generated with IBM SPSS Statistics Version 19 software (SPSS Inc).

Survival analyses were performed using the logrank test and Cox proportional hazards in SPSS. In all cases differences were considered statistically significant at $P < 0.05$.

Whole miRnome microarray data form hybridisation slides, was processed by subtracting background signals from the foreground signals for each feature. Any features which had a non-zero flag or a background subtracted signal <0.5 was set to 'missing.' Expression signals were then log2 transformed and quartile normalised. After

normalisation, any features targeting non-human miRNAs were removed. Data was sorted according to fold change (>1.5) and p-value (<0.05).

Differences between experimental groups in functional assays were expressed as mean +/- s.e.m from multiple experiments.

The threshold level of significance was set to 0.05 for all statistical tests in the study and all t-tests were 2 tailed unless otherwise stated.

3 Results

3.1 Experimental planning, assay development and optimisation

Based on prior profiling and bioinformatic work, epithelial candidate miR-224 was selected for further interrogation in *in vitro* assays of CRC cell phenotype. This was intended to mirror a complementary analysis for miR-153 published by the group.[235]

In addition, miR-21 was selected as it has pleiotropic actions in numerous cancer relevant biological pathways[409] and was amongst the most upregulated stromal miRNAs identified in our QuantimiR™ screen (preliminary data; section 1.7).

In order to effectively interrogate the functional and prognostic significance of these miRNAs, a number of key assays required initiation or optimisation:

3.1.1 Ectopic microRNA expression in colorectal cancer cell lines

To examine the biological impact of epithelial miR-224, ectopic expression was induced in a number of CRC cell lines. DLD1, HCT116 and SW480 cells were selected as they expressed amongst the lowest baseline levels of miR-224 by Taqman® qPCR in a panel of 10 cell lines examined.[410]

Transient transfection of CRC cells with pre-miR-224 constructs from Ambion® was achieved using Lipofectamine 2000™ transfection reagent in a 24-well format (Figure 3.1). Lipofectamine 2000™ and Ambion® oligonucleotides were selected as they had been used previously by the group with good effect to induce ectopic miR-153 expression in SW480 CRC cells.[235] Similarly, transfection with scrambled miR-SCC oligonucleotides from Ambion® was used as a negative control in accordance with group protocols.

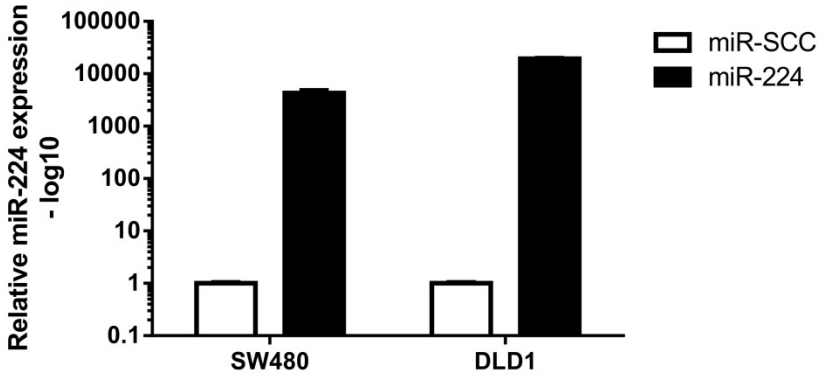


Figure 3.1 Transient induction of precursor miR-224 in CRC cell lines

Relative miR-224 expression by Taqman® qRT-PCR is shown in SW480 and DLD1 CRC cells transfected with pre-miR-224 or control miR-SCC. Cells were harvested at 48 hours post transfection in accordance with Promega® protocols for Lipofectamine 2000™. Results represent mean expression ± s.e.m relative to control miR-SCC transfected cells from triplicate repeat experiments.

As for miR-153, transient miR-224 induction was deemed appropriate for the relatively short duration of *in vitro* CRC cell invasion and proliferation assays. However, the biological impact of neither miR-224 nor miR-153 had previously been examined in the context of more physiologically relevant organotypic co-culture models. Organotypics are 3-dimensional invasion assays, in which cancer epithelial cells and stromal elements such as fibroblasts are co-cultured for 2 weeks in a synthetic gel consisting of type-II collagen and essential ECM components.[404, 411] To achieve ectopic miRNA expression in the requisite number of cells and sustain it over this more protracted time-course, CRC cells were transfected with a miRNA/GFP co-expression plasmid from GeneCopoeia™ (plasmid map; figure 2.1), using the transfection reagent Fugene® HD (Promega).

Fugene® HD was selected because it is specifically formulated for the transfection of plasmid DNA into a wide variety of cell lines, including each of the CRC cell lines used in this study,[412] with high efficiency and low toxicity. GeneCopoeia™'s products were selected because they were the only company identified which supplied prefabricated miRNA expression vectors containing both antibiotic-resistance and GFP-reporter

genes.[413-416] This meant that following transfection, enrichment of miRNA/GFP expressing cells could be conducted under Puromycin selection, monitored visually using fluorescence microscopy.

After transfection with miRNA/GFP co-expression plasmid in a 6 well format, CRC cells were allowed to grow to full confluence. Once confluent, cells were exposed to Puromycin selection. After 48 hours, selection media was removed, the cells washed in PBS and surviving cells expanded as per standard laboratory protocol in complete growth media.

Stably transfected cells were expanded over 14 days until sufficient numbers were available for use in organotypic models. After 14 days $1/10^{th}$ of the cells were harvested and lysed for RNA extraction, to measure candidate miRNA expression by Taqman® qPCR relative to equivalent miR-SCC transfected controls. This was repeated after a further 13 days to demonstrate sustained miRNA over-expression for the entire duration of the organotypic incubation period (Figure 3.2).

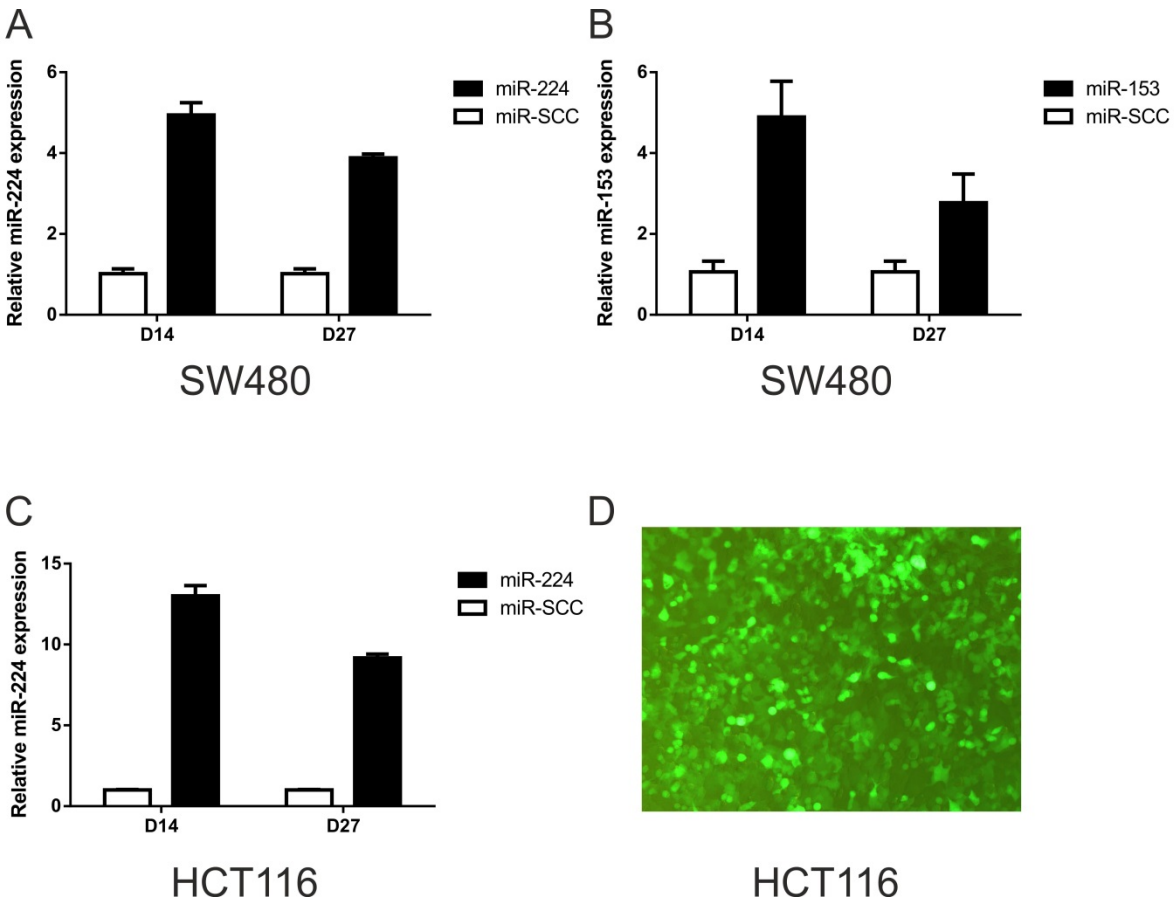


Figure 3.2 Stable ectopic miRNA expression in CRC cell lines quantitated by Taqman®-qRT PCR

Stable (A) miR-224 and (B) miR-153 expression in SW480 cells transfected with a miRNA/GFP co-expression plasmid relative to cells transfected with the equivalent scrambled control plasmid (miR-SCC); (C) Stable miR-224 induction in HCT116 cells relative to control transfected cells; Results represent mean miRNA expression ± s.e.m from triplicate repeat experiments conducted 13 days apart (day 14;D14 and day 27;D27 after Puromycin selection had been removed). (D) HCT116 cells stably expressing miR-224/GFP co-expression plasmid visualised under fluorescence at x10 magnification.

Intriguingly, miRNA over-expression in stably transfected cells was >2 orders of magnitude lower than equivalent transiently transfected cells. However, the purpose of stable induction was to interrogate candidate miRNAs in organotypic models, in highly

physiologically relevant conditions. As we have seen in preliminary data; section 1.7 (figure 1.10), miR-153 and miR-224 expression *in vivo* in LMD CRC epithelium is approximately 5 fold greater than expression in paired normal colonic epithelium. Therefore, for organotypic assays which are conducted over a relatively protracted time course, this low but sustained induction of miRNA expression was considered ideal.

Nevertheless, in order to produce the most flexible system possible, further optimisation of stable candidate miRNA induction was also attempted: For this purpose, CRC cells transfected with a miR-224/GFP or control miR-SCC/GFP co-expression plasmid were subjected to additional selection: 96 hours after Puromycin selection had been removed, a BD FACSARIA II flow cytometer, gated to identify the 5% most highly fluorescent cells was used to isolate a population of 50,000 cells enriched for miR-224 or miR-SCC expression. Once sorted, cells were expanded without further Puromycin selection.

As we see in figure 3.3, in HCT116 CRC cells this produced approximately 35 fold overexpression of miR-224 by Taqman® qPCR, compared with identically treated miR-SCC transfected control cells.

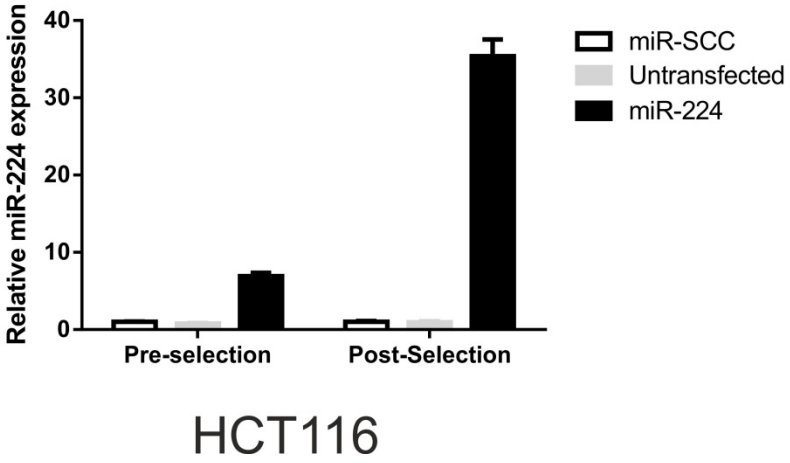


Figure 3.3 Enrichment of miR-224/GFP expressing CRC cells by Fluorescence-activated cell sorting (FACS)

Here, miR-224 expression by Taqman® qRT-PCR is compared between HCT116 CRC cells transfected with miR-224/GFP co-expression plasmid, or miR-SCC/GFP control plasmid pre- and post-sorting by FACS, and un-transfected, un-selected wild-type HCT116 cells.

MiR-224 expression in control miR-SCC transfected cells is used as the reference. Results represent mean expression ± s.e.m from triplicate repeat experiments.

Data presented here illustrates a highly flexible experimental system capable of producing low, moderate or high levels of candidate miRNA induction in CRC cells for use in different assay formats: low or moderate but sustained miRNA induction ideal for organotypic models; and high transient miRNA induction more suited to functional screening assays with the purpose of rapidly excluding miRNA candidates with limited biological significance.

3.1.2 Ectopic microRNA expression in cultured fibroblast cell lines and *ex vivo* fibroblasts from human colonic biopsy specimens

To evaluate the biological significance of stromal miR-21 in *in vitro* functional cell assays and organotypic models, transient miR-21 induction was attempted first in human fibroblast cell lines using pre-miR-21 from Ambion® and the transfection reagent Oligofectamine™ in accordance with a fibroblast transfection protocol optimised by the group.

HFFF2 fibroblasts were selected as they are the cell line of choice for the development of novel organotypics in our laboratory, having been used successfully in the past to model squamous cell carcinoma,[417] basal cell carcinoma[398] and oesophageal adenocarcinoma.[418] MRC5s were selected to provide flexibility in the experimental design, as they are the only other cultured fibroblast cell line identified in the literature to have been used in the context of gastrointestinal tumour modelling.[419]

Unfortunately, results with this initial transfection strategy were unfavourable as miR-21 expression appeared to fall or remain largely unaltered following transfection. Substituting Oligofectamine™ with Lipofectamine 2000™ and using both pre-miR-21 and anti-miR-21 preparations from Ambion® produced similarly mixed and incongruous results.

In an attempt to overcome this critical barrier to my research, oligonucleotides manufactured by Ambion®, were replaced by the equivalent MiRIDIAN™ products by Dharmacon®, including a miR-21 mimic, a miR-21 hairpin inhibitor and a miR-SCC scrambled negative control (Table 2.7).

Reverting to the original group protocol for fibroblast transfection with Oligofectamine™, several hundred fold miR-21 induction and suppression was achieved using MiRIDIAN™ products in both HFFF2 and MRC5 cells, an effect which was observed for several days following transfection (figure 3.4).

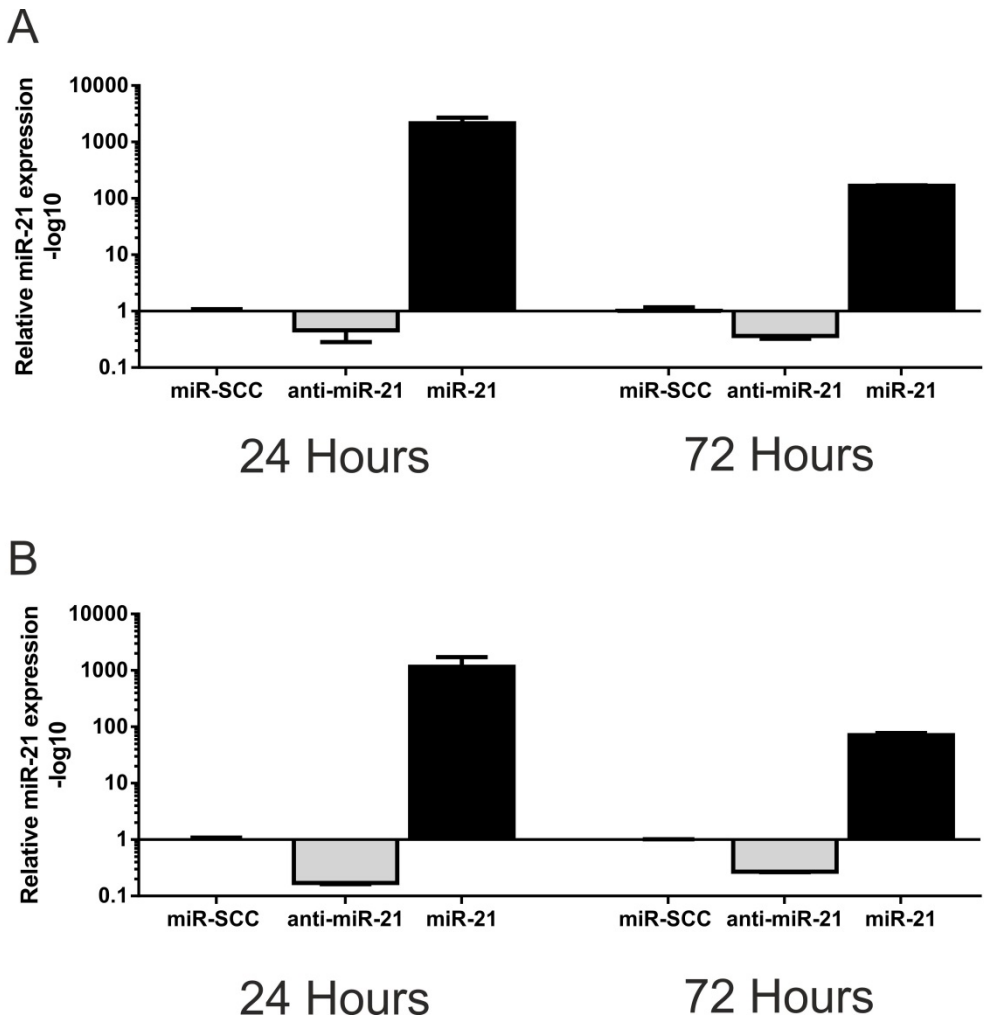


Figure 3.4 miR-21 expression by Taqman® qRT-PCR in transiently transfected fibroblast cell lines

MiR-21 expression in (A) HFFF2 and (B) MRC5 cultured fibroblast cell lines following transfection with pre-miR-21 or anti-miR-21 from Dharmacon®, at 100nM concentration. Results represent mean miR-21 expression ± s.e.m from triplicate repeat experiments. MiR-21 is expressed relative to miR-SCC control transfected cells at 24 hours and 72 hours following transfection with Oligofectamine™.

Having identified a robust method of transiently manipulating miR-21 expression in cultured fibroblast cell lines, attention turned to primary colonic fibroblasts.

A key feature of this research is the use of primary tissue wherever possible, to better reflect *in vivo* conditions in the experimental design. HFFF2 fibroblasts are derived

from human foetal foreskin tissue and MRC5 fibroblasts from human foetal lung tissue. Their ubiquitous use in tissue culture reflects their versatility and predictability under experimental conditions. It should not however be assumed that they behave in identical fashion to anatomically accurate primary cells.

It was therefore deemed essential to also induce ectopic miR-21 expression in *ex vivo* human colonic fibroblasts. For this purpose, a panel of non-cancer associated primary colon fibroblasts (PCFs) were extracted from the stroma of unaffected sections of fresh human CRC resection specimens. During this process, which is summarised in figure 3.6 but discussed in detail in the methods section and in Bullock et al., 2014,[420] fibroblasts spontaneously exit from colonic biopsy specimens after 5-7 days incubation in fibroblast specific growth media. In the absence of specific inhibitors, fibroblasts tend to outgrow other cell types and are easily recognised under light-microscopy by their flat, elongated morphology, and lamellipodia protrusions which give them a spindle-like appearance (figure 3.5).[421]

PCFs were assigned a number from 1-16, to denote the patient from which they had been isolated.

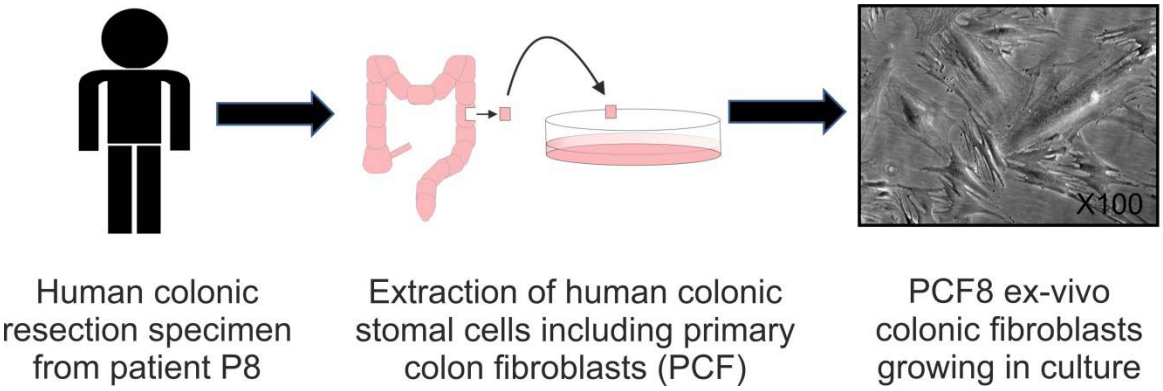


Figure 3.5 Primary Colon Fibroblast (PCF) extraction and culture

To maximise the physiological relevance of this research, anatomically accurate ex vivo colonic fibroblasts were used in various experiments. Fibroblasts within the colonic resection specimens of several patients were extracted from unaffected tissue >5cm from the tumour.

To select primary fibroblasts for use in further studies, it was first necessary to assess whether significant differences in biology and function existed between cells recruited from different patients. To achieve this, the capacity of PCFs to induce CRC invasion in transwell assays was assessed. Crucially however, only PCF7, PCF8, PCF9, PCF15 and PCF16 grew sufficiently robustly under culture conditions and were present in sufficient numbers to be included in this analysis:

$1x10^5$ PCFs were plated in 6 well plates in complete DMEM. After 24 hours, culture medium was removed and following 3 washes in PBS, replaced with serum free DMEM supplemented with 1% BSA. After a further 24 hours, this conditioned medium (CM) was clarified by centrifugation and transferred in aliquots to triplicate wells of a 24 well plate. Subsequently, Matrigel™ coated transwell invasion chambers, into which SW480 CRC cells had been seeded, were inserted into each well containing CM. CRC cells which had successfully invaded through the synthetic Matrigel™ basement membrane, were counted after 72 hours (figure 3.6A).

Interestingly, PCF15 and PCF16 cells induced significantly greater SW480 CRC invasion than non-cancer associated primary cells from all other patients. This implies

that although the use of physiologically relevant primary tissue is desirable, significant intra-patient variability may also be expected.

Next, endogenous miR-21 expression was quantitated in PCF7, PCF8, PCF9, PCF15 and PCF16 cells by Taqman® qPCR relative to wild-type HFFF2 cells. This revealed only relatively small (<1.5-fold) variations in miR-21 expression between the various PCF cell lines (figure 3.7).

Subsequent to this analysis, PCF8 and PCF9 cells were selected for use in further studies because they express relatively low baseline levels of miR-21 and induce low to medium CRC invasion, making them ideal to assess the biological impact of ectopic stromal miR-21 upregulation.

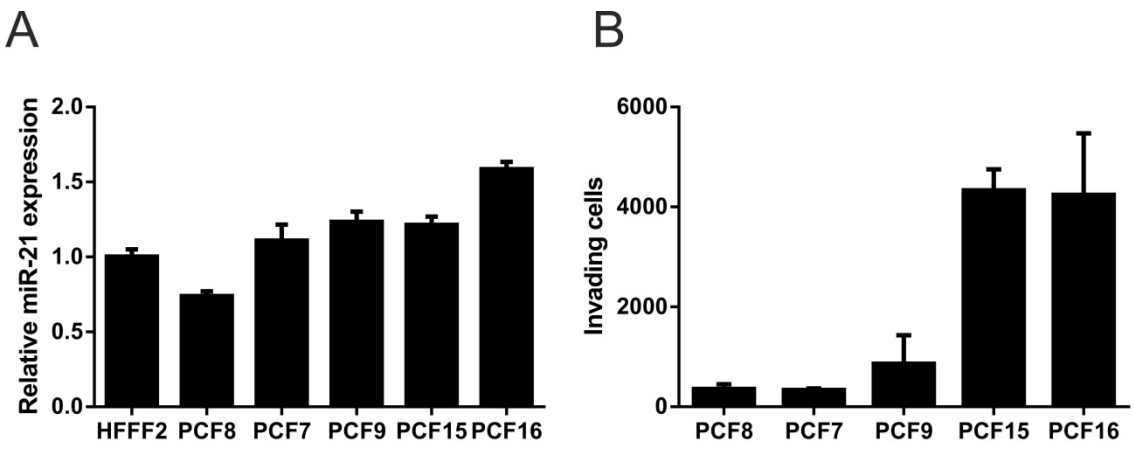


Figure 3.6 Selection of primary colon fibroblasts for experimental use

(A) Endogenous miR-21 expression by Taqman®-qPCR in a panel of PCFs relative to HFFF2, a cultured human fibroblasts cell line. (B) SW480 CRC cell invasion assay conducted over 72 hours using culture media supernatant extracted from various PCFs as chemo-attractant. Results represent mean miR-21 expression (A) or mean number of invading cells (B) from triplicate repeat experiments ± s.e.m.

The next step was to induce transient ectopic miR-21 overexpression and suppression in selected PCF cell lines. As with MRC5 and HFFF2 fibroblasts, the use of Ambion® oligonucleotides produced incongruous results both with Oligofectamine™ and Lipofectamine 2000™ transfection reagents.

However, MiRIDIAN™ products from Dharmacon®, produced several hundred fold miR-21 induction and suppression in PCF cells for at least 72 hours following transfection with Oligofectamine™ (figure 3.7).

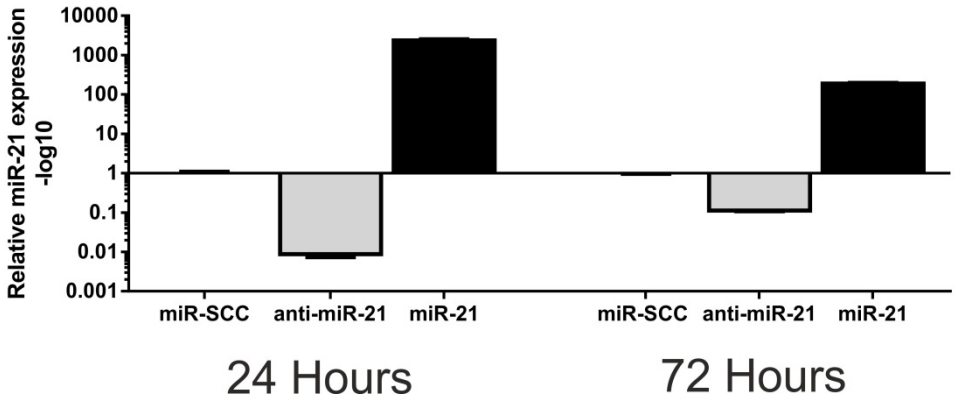


Figure 3.7 Taqman® qRT-PCR quantitation of miR-21 expression in transiently transfected PCF9 fibroblasts

MiR-21 expression by Taqman® qRT-PCR in PCFs following transfection with pre-miR-21 or anti-miR-21 from Dharmacon® at 100nM concentration. Results represent mean miR-21 expression ± s.e.m from triplicate repeat experiments. MiR-21 expression is relative to miR-SCC control transfected cells 24 hours and 72 hours following transfection with Oligofectamine™.

To achieve sustained miR-21 upregulation over time, fibroblasts were transfected with a miRNA/GFP co-expression plasmid from GeneCopoeia™ containing a Puromycin resistance gene. On the advice of colleagues, Xfect™, a transfection reagent from Clontech™ formulated to deliver DNA into difficult to transfect mammalian cells, was used in the first instance and in accordance with the manufacturer's instructions.

The inclusion of an antibiotic resistance gene meant that cells capable of transmitting the plasmid in a stable fashion could subsequently be enriched by Puromycin selection. The minimum cytotoxic dose for HFFF2 and MRC5 fibroblasts was calculated in advance using Puromycin dose response curves (figure 3.8).

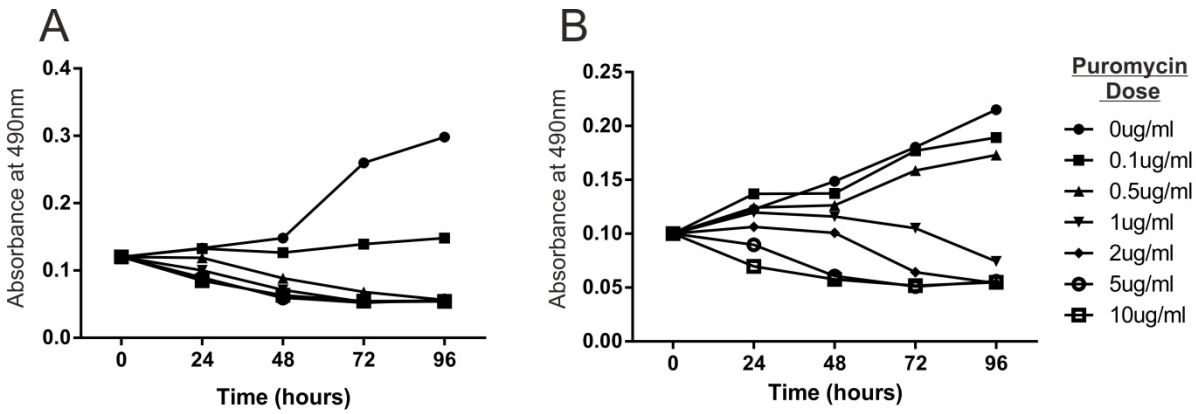


Figure 3.8 Puromycin dose response curves for the selection of stably transfected fibroblast cell lines

Puromycin dose response curve for (A) HFFF2 and (B) MRC5 cultured fibroblast cell lines. Using the colorimetric MTS assay, absorbance of light at 490nm provides a proxy measure of viable cell numbers over time following exposure to Puromycin in increasing doses.

For MRC5 fibroblasts this transfection and selection strategy was immediately successful. Transfection with 8µg of plasmid DNA using Xfect™ in a 6-well format achieved approximately 60% transfection efficiency. This was assessed visually at 48 hours post-transfection by calculating the ratio of GFP expressing to non-expressing cells. Cells were subsequently enriched for expression of miR-21/GFP or miR-SCC/GFP by exposure for 2 weeks to Puromycin at a concentration of 2µg/ml in complete growth medium (figure 3.9).

However, gene expression analysis on the day Puromycin selection was removed, revealed an apparent paradox, as stable expression of the miR-21/GFP plasmid seemingly produced down-regulation of miR-21 compared with miR-SCC transfected cells (figure 3.9). Concern that this represented activation of an intracellular autoregulatory mechanism meant that quantification of miR-21 by Taqman®qRT-PCR was considered an insufficiently precise measure of transfection efficiency in stably transfected fibroblasts.

An alternative strategy was to measure expression of known miR-21 mRNA targets. Assangani and colleagues used mutated/unmutated 3'UTR luciferase reporter assays to

demonstrate in epithelial CRC cell lines, that translation of PDCD4 mRNA is supressed directly by miR-21.[230] Similarly, Gabriely et al., demonstrated that RECK is a direct target and TIMP3 a possible indirect target of miR-21 in Glioma models.[284]

In the current study MRC5 fibroblasts stably expressing the miR-21/GFP co-expression plasmid ($MRC5^{21}$ cells) also expressed downregulated PDCD4, RECK and TIMP3 mRNA compared with control miR-SCC/GFP expressing cells ($MRC5^{SCC}$). As a consequence, expression of direct miR-21 targets (PDCD4 or RECK) became the preferred method for measuring the efficiency of stable miR-21 induction in fibroblasts.

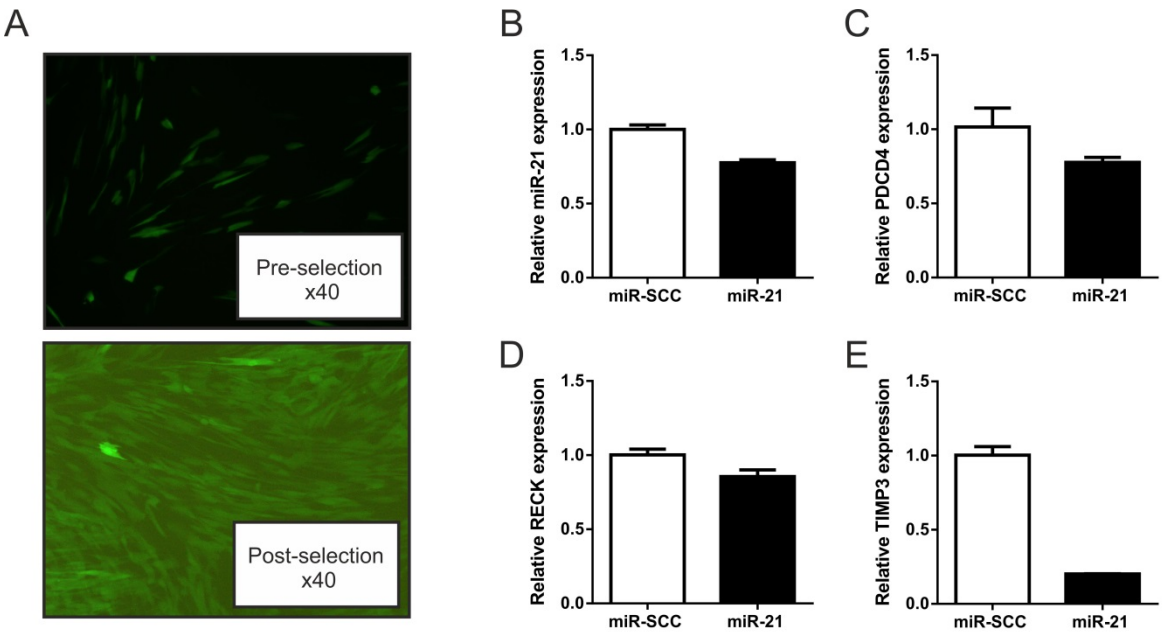


Figure 3.9 Selection of stable miR-21/GFP and miR/SCC expressing MRC5 fibroblasts

(A) MRC5 fibroblasts in live culture following transfection with miR/GFP co-expression plasmid. Note enrichment of GFP +ve cells following selection with Puromycin for 2 weeks.

Expression of (B) miR-21, (C) PDCD4, (D) RECK and (E) TIMP3 in MRC5 cells stably expressing the miR-21/GFP co-expression plasmid, compared with cells expressing control miR-SCC. Results represent mean expression from triplicate repeat experiments ± s.e.m.

In contrast to MRC5 fibroblasts, the initial transfection of HFFF2 fibroblasts with plasmid DNA using Xfect™ was not effective. The proportion of transfected cells assessed visually under fluorescence was approximately 20% (figure 3.10). Furthermore, the rate of cell proliferation was slow and transmission of the GFP-tagged plasmid inefficient, particularly under Puromycin selection. This made the subsequent expansion of antibiotic resistant clones particularly problematic.

Identifying this as an opportunity to develop a new skill by capitalising on expertise already present within our collaborative group,[398] a lentiviral delivery system (Lenti-Pac™ from GeneCopoeia™) was used to infect HFFF2 cells with miR-21/GFP or miR-SCC/GFP co-expression plasmid, rather than optimising the transfection protocol

further. This proved an effective solution, as 48 hours following infection with 30µl viral supernatant, the ratio of GFP expressing to non-expressing HFFF2 cells was approximately 5:1 for both miR-21 and control miR-SCC plasmids (figure 3.10).

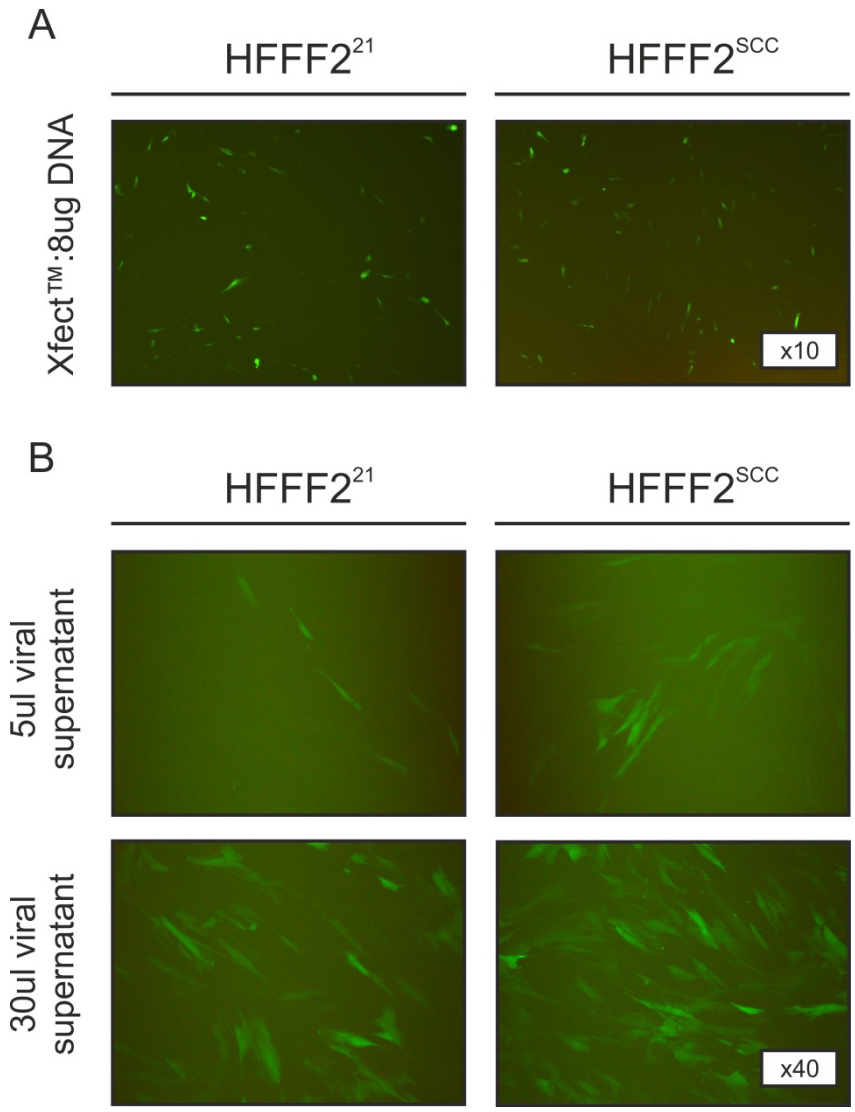


Figure 3.10 Optimisation of miRNA induction in HFFF2 fibroblasts with miRNA/GFP co-expression plasmid

(A) HFFF2 cells transfected with the highest attempted dose of plasmid DNA (8µg) using Xfect™ transfection reagent: 48 hours post transfection with miR-21/GFP or miR-SCC/GFP co-expression plasmid, only approximately 20% of cells were fluorescent. (B) At low doses (5µl per well) lentiviral supernatant (containing Lenti-Pac™ particles from GeneCopoeia™) produced similar results to transfection (in A), but at higher doses (30µl per well) excellent transduction efficiency was achieved with approximately 80% of cells expressing green fluorescence at 48 hours. Note that the image display HFFF2 cells at approximately 80% confluence.

Exposure to Puromycin at a concentration of 0.5µg/ml for 2 weeks further enriched both populations of GFP+ve cells and on the day Puromycin selection was removed, expression of the miR-21 targets PDCD4 and RECK were heavily supressed in cells containing the miR-21/GFP co-expression plasmid ($HFFF2^{21}$ cells) compared with miR-SCC/GFP expressing control cells ($HFFF2^{SCC}$) (figure 3.11).

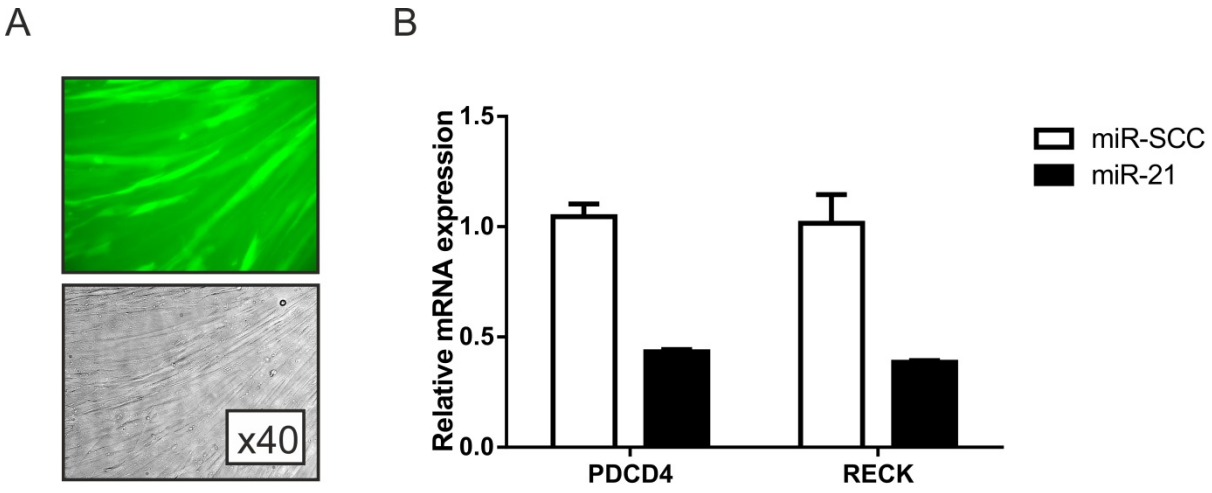


Figure 3.11 PDCD4 and RECK expression in stable miR-21 transfected HFFF2 fibroblasts

(A) HFFF2 cells enriched for expression of miR-21/GFP co-expression plasmid imaged under fluorescence and normal light microscopy show approximately 100% of cells contain the plasmid miRNA expression construct. (B) Relative PDCD4 and RECK mRNA expression in HFFF2 cells stably expressing miR-21/GFP plasmid compared with stable miR-SCC/GFP expressing controls. Results represent mean expression from triplicate repeat experiments ± s.e.m.

As with epithelial CRC cell lines, the primary purpose of inducing stable ectopic miRNA expression in fibroblasts was to examine the impact of miRNA deregulation in physiologically relevant models of CRC progression.

To ensure that ectopic miR-21 expression in HFFF2 fibroblasts was sustained, stably transfected cells were expanded for 5 days after Puromycin selection had been removed, before $1/10^{th}$ of the cells were harvested and lysed for RNA extraction. PDCD4

suppression, as a proxy measure of miR-21 upregulation, was subsequently quantitated by Taqman® qPCR in HFFF2[21] cells relative to equivalent miR-SCC transfected controls. This was repeated after 5 and 13 days to demonstrate sustained ectopic miRNA expression for at least the entire duration of an organotypic incubation period (Figure 3.12).

An equivalent experiment was conducted for stably transfected MRC5 fibroblasts, however the miR-21 transfected cohort was lost to infection at an early stage and data from all time points is not available (Figure 3.12).

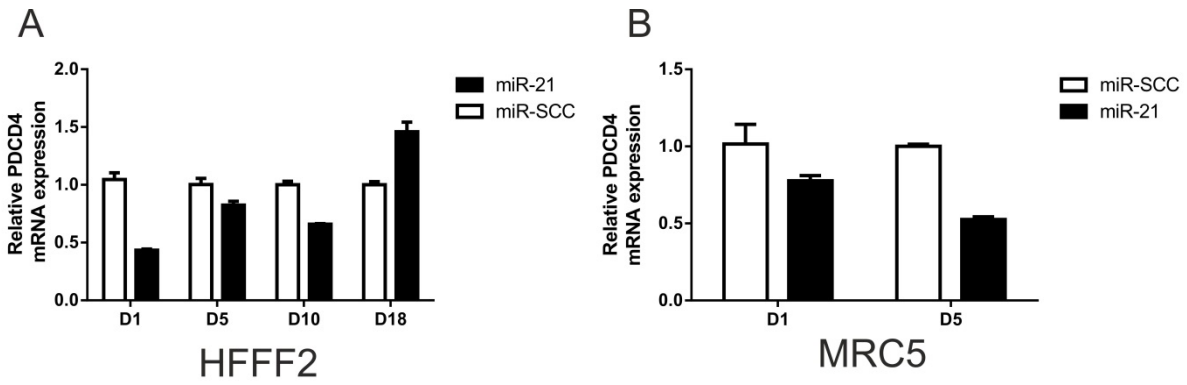


Figure 3.12 PDCD4 expression in stable miR-21 transfected fibroblasts over time

PDCD4 mRNA as a proxy measure of ectopic miR-21 expression in (A) HFFF2 and (B) MRC5 fibroblasts quantitated by Taqman®-qRT PCR. PDCD4 mRNA in fibroblasts transfected with a miR-21/GFP co-expression plasmid is expressed relative to control cells transfected with miR-SCC/GFP plasmid. Results represent mean miRNA expression ± s.e.m from triplicate repeat experiments. Analysis for HFFF2 cells was conducted at day 1 (D1); 5 (D5); 10 (D10) and 18 (D18) after Puromycin selection had been removed. For MRC5 fibroblasts data is available for D1 and D5.

Surprisingly we see in figure 3.12, that PDCD4 mRNA suppression is lost in HFFF2[21] fibroblasts after a relatively short interval, and in fact PDCD4 expression appears upregulated in HFFF2[21] cells compared with HFFF2[SCC] cells 18 days after Puromycin selection has been removed. The significance of this is not clear however, it is

reassuring that the within the plasmid vector used for ectopic miR-21 and miR-SCC expression, the miRNA gene is located next to the GFP reporter gene, and both are driven from the same CMV promoter sequence (figure 2.1). Thus it may be reasonable to assume that the miRNA of interest continues to be expressed in cells which are positive for GFP. Furthermore, PDCD4 is a key regulator of biological activity within the cell and is involved particularly in apoptosis and cell turnover pathways.[422] It may therefore be essential for cell homeostasis for compensatory mechanisms to counter the loss of PDCD4 activity in the context of heavy miR-21 overexpression.

In summary, this section describes the optimisation of various methodologies designed to produce a highly flexible platform for ongoing research. Crucially, transient and stable induction of miR-21 in a variety of fibroblast cell lines will permit the impact of stromal miR-21 deregulation to be examined both *in vitro* and *in vivo*, and for these findings to be rapidly validated across multiple experimental formats.

3.1.3 Developing a 3-Dimensional organotypic model of colorectal cancer.

Organotypics are tissue-engineered models in which stromal constituents such as fibroblasts are 3-dimensionally co-cultured with cancer epithelial cells in a collagen gel containing essential extracellular matrix components. They are highly manipulatable experimental tools which enable invasion and cancer-stroma interactions to be studied in near-physiological conditions.[420]

As CRC invasion is a complex biological process, it was crucial to examine the impact of stromal and epithelial miRNA manipulation in conditions which resemble the situation *in vivo.* With this in mind, one of the early priorities of this project was to develop for the first time, a working organotypic CRC model.

Initially, various epithelial cancer cells lines were tested in 3-D co-culture with *ex vivo* PCF8 fibroblasts, to identify combinations which effectively reconstitute the conditions for CRC invasion. A primary fibroblast cell line was selected in the first instance to maximise the physiological relevance of the model (figure 3.13).

HCT116 CRC epithelial cells did not appear to thrive in organotypic culture. Although unicellular invasion pockets were occasionally noted, frequently the epithelial layer would detach entirely from stroma during fixation and processing, compromising the quality of the final result (figure 3.13A).

In contrast, DLD1 (figure 3.13B) and HT29 (figure 3.13C) CRC cells did produce a healthy, well stratified epithelial layer however; invasion in the presence of PCF8 fibroblasts was almost entirely absent. This could perhaps have been anticipated as DLD1 cells display a strongly epithelial morphology and HT29 cells originate from a low grade colonic tumour specimen.

Under identical conditions, AAC1/82 CRC cells also did not invade, but instead organised into an acinar pattern characteristic of well-differentiated adenocarcinoma. Intriguingly, the conservation of this glandular colonic morphology may represent an opportunity in future to study tumours during the earliest phases of development and not just CRC invasion and progression (figure 3.13D).

Although small pockets of invading cells were frequently identified in organotypics containing SW620 CRC cells (figure 3.13E), the only truly robust model of CRC invasion was achieved using a combination of PCF8 fibroblasts and SW480 cells. Crucially, this cellular juxtaposition produced very well defined invasive tumour islands consisting of numerous multicellular assemblies extending deep into stroma (figure 3.13F). This is consistent with the origins of SW480 cells which were recruited from a high grade (III/IV) human colonic adenocarcinoma specimen.

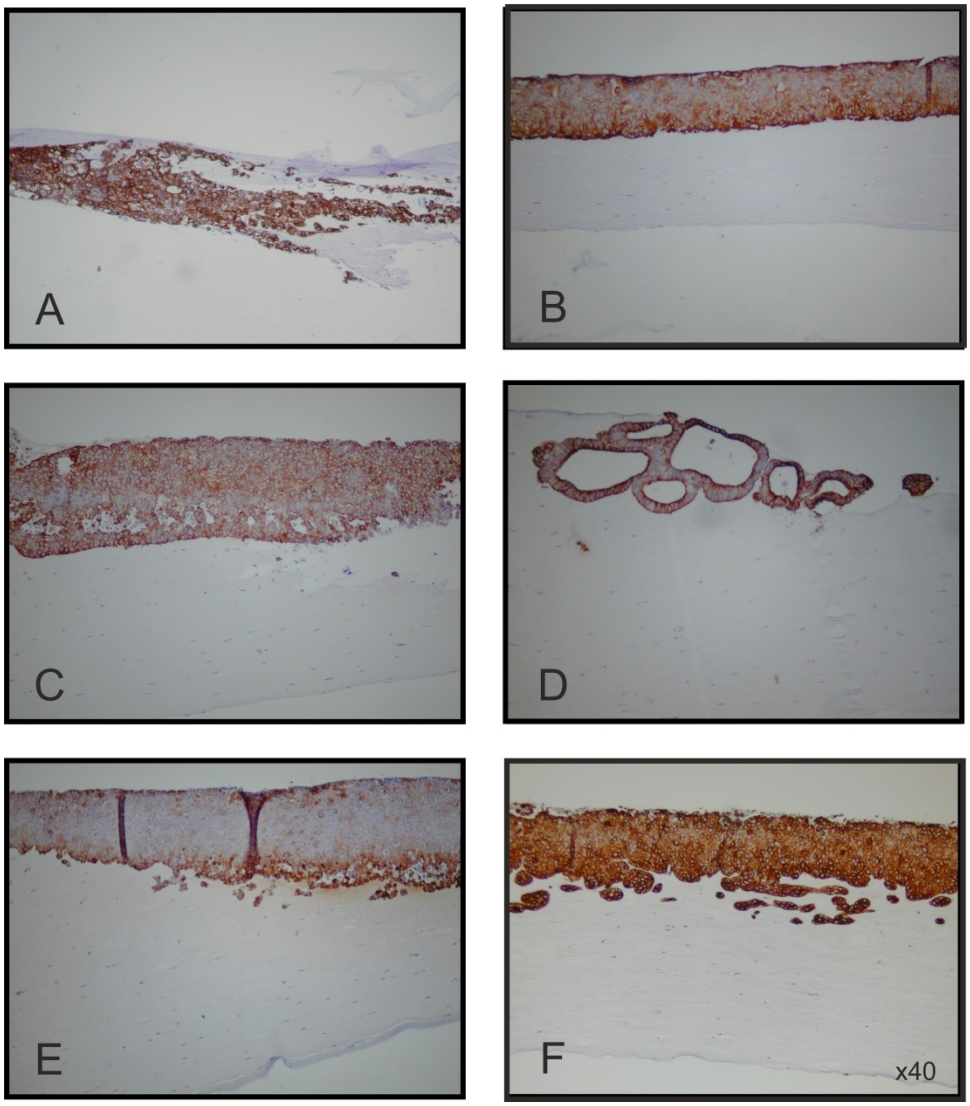


Figure 3.13 CRC Organotypic development (I)

Representative cross-sections of Organotypic co-cultures in which PCF8 human ex vivo colonic fibroblasts in the stroma are juxtaposed with an epithelial cell layer consisting of various CRC cell lines including (A) HCT116s; (B) DLD1s; (C) HT29s; (D) AAC1/82s; (E) SW620s; and (F) SW480s. Models were produced in triplicate and cultured for 14 days before being bisected, fixed in formalin, sectioned and stained. Immuno-staining with a pan-cytokeratin antibody produces a brown chromogenic reaction which highlights epithelial cancer cells.

Prompted by this success, several other primary and cultured fibroblast cell lines were also tested in combination with SW480 CRC cells (figure 3.14).

HFFF2 fibroblasts induced similarly strong invasion to PCF8 fibroblasts, in contrast to other fibroblasts including *ex vivo* fibroblasts, which induced only moderate (MRC5s) or weak (PCF6; PCF9) invasion (figure 3.14). Relative invasion across various organotypics was measured using a semi-quantitative score known as the 'invasion index'.[404]

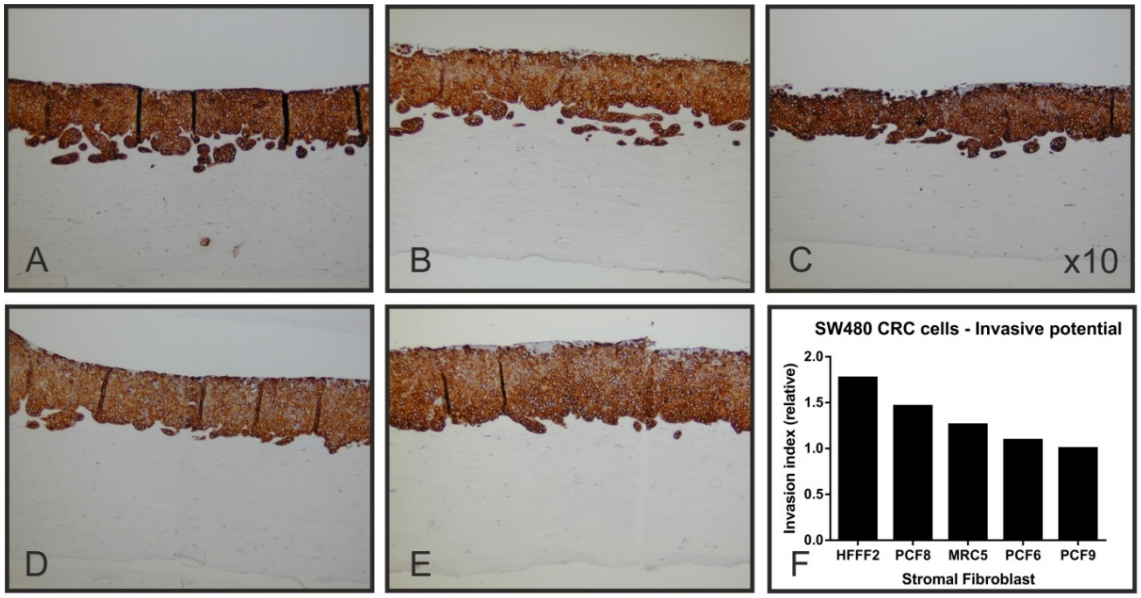


Figure 3.14 CRC Organotypic development (II)

Representative cross-sections of Organotypic co-cultures in which (A) HFFF2; (B) PCF8; (C) MRC5; (D) PCF6 and (E) PCF9 fibroblasts in the stroma are juxtaposed with an epithelial cell layer consisting of SW480 CRC cells. Models were produced in triplicate and cultured for 14 days before being bisected, fixed in formalin, sectioned and stained. Immuno-staining with a pan-cytokeratin antibody produces a brown chromogenic reaction which highlights epithelial cancer cells. (F) For the purpose of comparison, the extent of invasion achieved by SW480 CRC cells in the presence of various stromal fibroblasts is measured using a semi-quantitative score known as the invasion index. Here the mean invasion index is presented from triplicate repeat experiments and expressed relative to the organotypic demonstrating the least invasion.

In summary, data presented in this section suggests that CRC and specifically CRC invasion can be effectively replicated in synthetic tumour models, by juxtaposing cultured CRC cells and stromal fibroblasts in 3-dimensions.

3.1.4 Laser microdissection

One of the key objectives of the current study is to establish for the first time, whether separately profiling miRNA expression in tumour epithelium and stroma, has prognostic utility in cancer.

In order to characterise miRNA expression in different tumour compartments, it was first necessary to isolate CRC epithelium from stroma. This was achieved using Laser Microdissection (LMD), a technique used extensively within the host group.

To ensure LMD was efficient in my own hands, a FFPE CRC specimen, mounted on a membrane coated slide and stained with Cresyl Violet, was laser dissected using the AS LMD platform from Leica. Stromal and epithelial tumour compartments were collected separately and total RNA was extracted. Subsequently, qPCR assays were conducted to quantify expression of epithelial and mesenchymal specific markers in the microdissected tissue.

Compared with the corresponding epithelial tissue, microdissected stroma expressed approximately 4-fold higher levels of mesenchymal cell-specific vimentin (VIM) mRNA and fibroblast-specific protein 1 (FSP1; S100 calcium-binding protein A4, S100A4) and approximately 2-fold lower levels of epithelium specific E-Cadherin (CDH1) (figure 3.15).

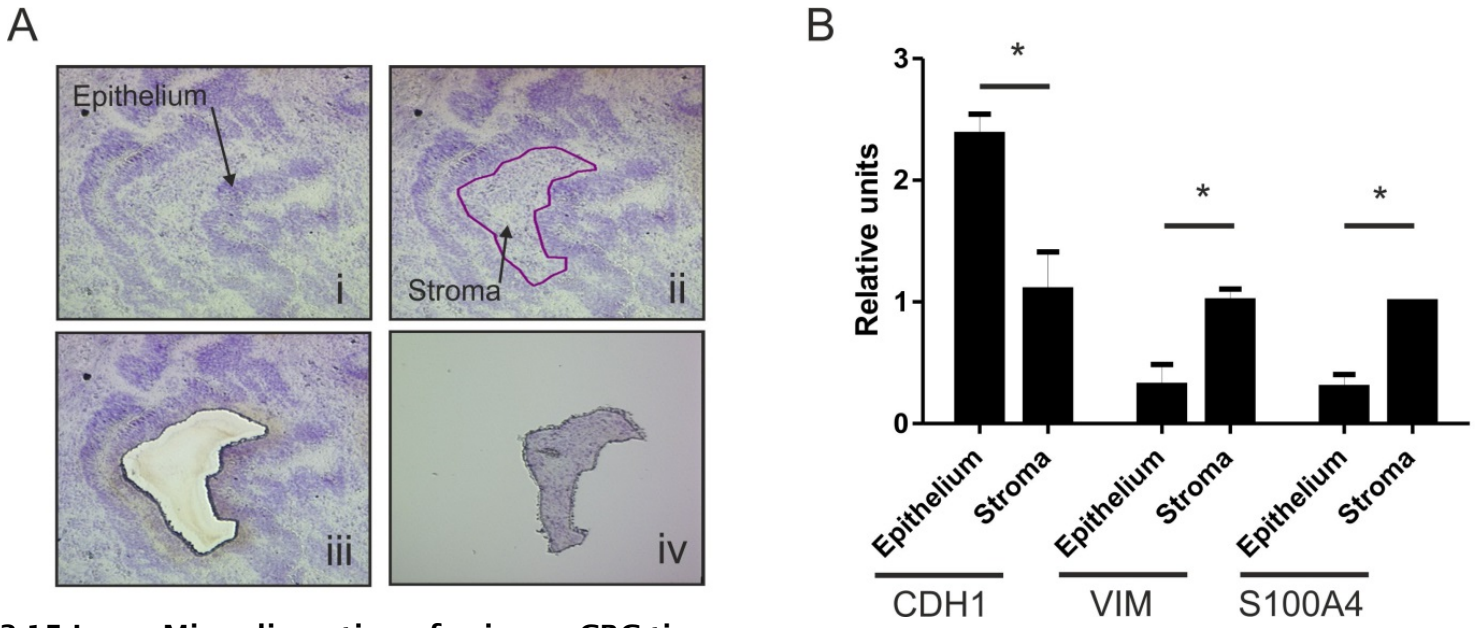


Figure 3.15 Laser Microdissection of primary CRC tissue

*(A) LMD was performed on FFPE CRC specimens to isolate stroma from epithelial tissue. Cresyl violet staining was used to highlight epithelium (i); the area of interest was then defined (ii) before laser dissection occurred (iii) and the cut piece was transported to a collection device (iv). (B) To prove the efficiency of LMD, expression of mesenchymal cell-specific mRNA vimentin (VIM), FSP1 mRNA (S100A4), and epithelium specific mRNA E-Cadherin (CDH1) were quantified in LMD epithelium by Taqman qPCR and expressed relative to the corresponding LMD stromal tissue (*P<0.05)*

3.1.5 Results discussion

In the current study, the first and fourth objectives are to identify and validate candidate miRNAs deregulated in CRC, and to establish their prognostic utility.

Previous studies have characterised miRNA expression in whole tumour sections, lymph nodes, metastases, serum and even faeces of patients with CRC.[183, 311] As discussed in section 1.3.5.2, working with each of these tissues presents different challenges, and it is noteworthy, that despite early promise, no study has yet produced a robust prognostication tool capable of translation to the clinical setting, based on miRNA expression in CRC.

Notwithstanding, one advantage of focusing on corrupt miRNAs in primary tumour tissue, rather than serum, faeces or metastases, is that miRNA candidates identified in this context, may not only hold prognostic significance, but are also most likely to relevant to underlying tumour biology.

At the last count at least 28 studies had characterised differential miRNA expression between CRC tissue and normal colonic tissue (Introduction; Table 1.1). Crucially however, all but 2 of these studies neglected potentially important biological differences between cancer cells and their supportive tumour microenvironment by profiling bulk tumour specimens, rather than epithelium and stroma separately.

The remaining studies used laser microdissection (LMD) to isolate stroma from epithelial tissue prior to profiling however, in both cases the authors failed to subsequently examine the prognostic significance of the deregulated miRNAs which they had identified.[296, 348]

It would therefore, be highly novel and potentially clinically advantageous to correlate expression of deregulated miRNAs in CRC stroma and epithelium with patient outcomes independently of one another. As LMD is an essential component of this, it was important to prove in advance that it could be performed with sufficient precision, by demonstrating appropriate expression of epithelium and stroma-specific markers in microdissected tissue (figure 3.15).

The second objective of this study is to determine the biological consequences of deregulated miRNAs, both in CRC epithelium and stroma. In order to achieve this, it was necessary to induce ectopic expression and suppression of candidate miRNAs in CRC cells and fibroblast cell lines, and *ex vivo* human colon fibroblasts.

Although challenging, the optimisation of both stable and transient transfection techniques to induce and suppress miRNA expression in a range of CRC cancer cells and fibroblasts, afforded me an excellent and very flexible platform on which to conduct this research.

A further innovation to facilitate study of the biological consequences of deregulated miRNAs, and any cross-talk between tumour compartments, was the development of CRC organotypic models.

Organotypic co-cultures are 3-dimensional reconstructions of the *in vivo* tumour microenvironment.[404] Principally conceived as a method of measuring tumour invasion, organotypics reduce reliance on animal models, and avoid the shortcomings of other *in vitro* techniques such as transwell assays, in which invading cells are forced artificially into a mono-dispersed state.[411]

The inclusion of stromal cells, such as fibroblasts, reflects the essential role of the tumour microenvironment in regulating malignant invasion and metastasis. Crucially, anatomically accurate *ex vivo* fibroblasts were used here, as well as cultured fibroblast cell lines, in order to maximise the physiological relevance of the model.

Intriguingly, organotypic models constructed, incubated and processed in identical fashion induced invasion from SW480 CRC cells to a very different extent, depending on the fibroblast content of the stroma. This provided a striking visual demonstration of the importance of the stroma during CRC invasion. However, perhaps more importantly, these organotypic models also allowed us to ‘freeze-frame’ a key moment in the metastatic cascade, when a subset of malignant epithelial cells which had acquired the capacity to invade, first became discernable against a background of otherwise well-stratified non invading malignant epithelial cells.

In contrast to SW480 cells which were highly invasive, AAC1/82 CRC cells organised into a well differentiated acinar pattern, and DLD1 and HT29 cells organised into well

stratified layers with very little invasion evident. This further illustrates the flexibility of an experimental system which may be adapted to model tumourogenesis or metastatic progression; or high or low grade disease, simply by modifying the CRC cell line included in the epithelial layer.

Having engineered these CRC models, they have subsequently been used in a variety of novel ways within the University of Southampton Cancer Sciences Unit, to examine the impact on tumour progression of chemical inhibitors and targeted gene alterations.[235, 420, 423]

In the next phase of development, the aim will be to extract both stromal fibroblasts and malignant epithelial cells from colonic resection specimens, to produce personalised tumour facsimiles for individual patients.

3.2 The role of epithelial microRNA candidates during colorectal cancer progression

MiR-153 was amongst the most highly upregulated miRNA candidates in a comparison of metastatic CRC (SW620) cells compared with paired non-metastatic (SW480) cells; and miR-224 was highly unregulated in LMD CRC epithelium compared with paired normal colonic epithelium (preliminary data; section 1.7).

In the following sections, the functional impact of deregulated miR-153 and miR-224 during CRC progression will be examined *in vitro*.

3.2.1 The functional impact of ectopic miR-153 expression in vitro

MiR-153 has been the subject of intense interrogation by the host group. In brief, miR-153 appears to promote CRC progression *in vitro*, by enhancing cellular invasiveness through an indirect MMP9 induction pathway.[235]

Prompted by this initial assessment and in order to more closely recreate and model *in vivo* circumstances, the effect of stably transfected GFP-tagged miR-153 in SW480 cells was examined using a 3-dimensional organotypic co-culture with HFFF2 fibroblasts (figure 3.16). To our knowledge, this represents the first published use of organotypics to assess the impact of miRNA manipulation in cancer of any kind, and the first experimental use of a synthetic CRC tissue model in our institution.[235] This entirely novel approach powerfully demonstrated that ectopic expression of miR-153 but not control miR-SCC expression resulted in increased invasion of SW480 cells into the underlying stroma.

Although stable overexpression was achieved in only 75% of cells, this had beneficial consequences, as immunostaining against GFP illustrated enrichment of miR-153 expressing cells at the invasive front, supporting the notion that miR-153 may be a key regulator of the invasive phenotype in CRC.

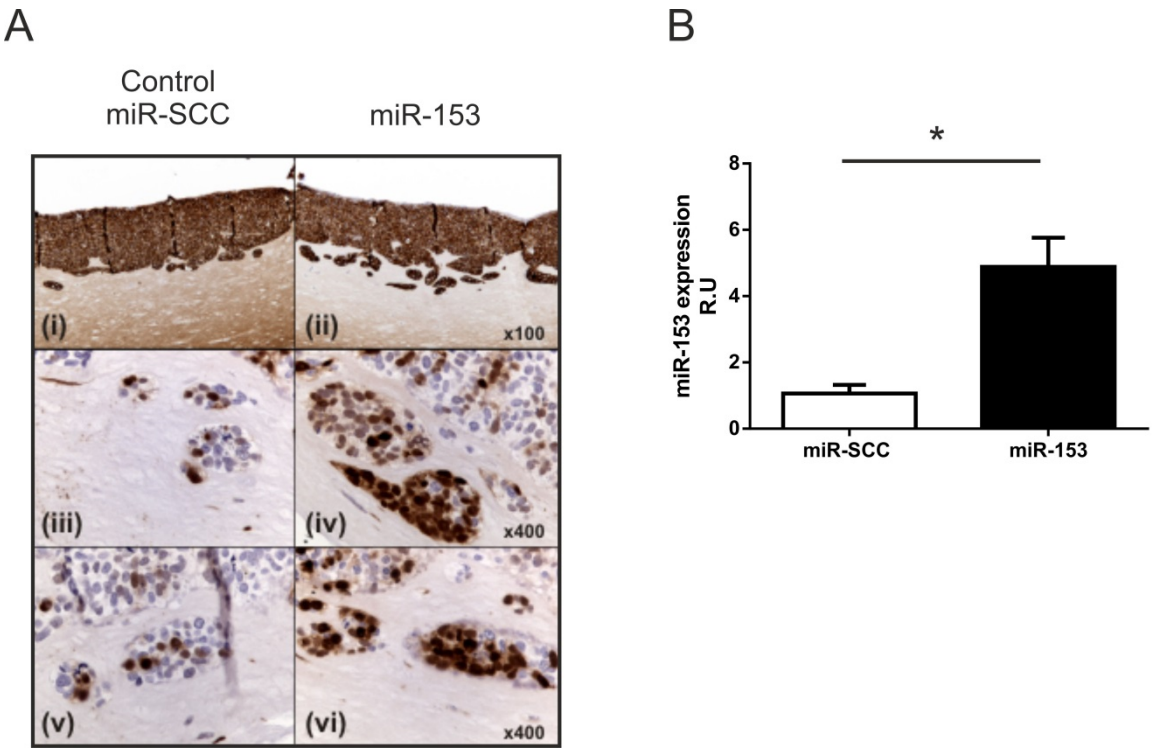


Figure 3.16 3-dimensional organotypic co-cultures of miR-153 transfected SW480 cells and human foetal fibroblast cells

*(A) SW480 cells were stably transfected with miR-153/GFP co-expression plasmids. After 14 days sections of organotypic gels were immunostained with (i, ii) anti-cytokeratin antibody or (iii-vi) anti-GFP antibody. Results show increased invasiveness of miR-153-overexpressing SW480 cells and enrichment of transfected (GFP+) cells at the invasive front compared with control miRNA. (B) Relative miR-153 expression by Taqman® qPCR in SW480 CRC cells stably expressing miR-153 compared with miR-SCC control transfected cells. Results represent the ratio of means ± s.e.m from triplicate repeat experiments. *P<0.05.*

Furthermore, these results emphasise the value of organotypics in miRNA research. As 3-D co-culture models are more physiologically relevant than other invasion assays, they provide a rapid platform to screen miRNA candidates for biological function, bridging the gap between *in vitro* and *in vivo* methodologies.

3.2.2 The functional impact of ectopic miR-224 expression in vitro

The phenotype of CRC cell lines transfected with miR-224 was characterised using a panel of *in vitro* assays designed to screen miRNAs identified through profiling, and rapidly exclude non-pathologically relevant candidates. The assays selected for this purpose were quick, reproducible and extensively used within the host group.

MiR-224 overexpression in HCT116 cells did not enhance cellular proliferation compared with controls in an MTS cell viability assay conducted at multiple time points (figure 3.17).

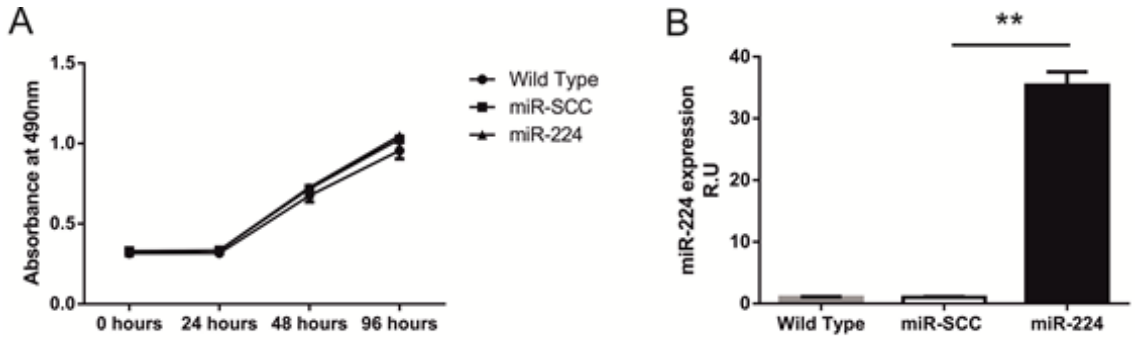


Figure 3.17 Cellular proliferation in miR-224 transfected CRC cells

*(A) MTS cell viability assay comparing HCT116 CRC cells in which miR-224 expression has been ectopically induced, with control transfected cells and non-transfected wild-type cells. Results represent mean ± s.e.m from triplicate repeat experiments. (B) Relative miR-224 expression by Taqman® qPCR in HCT116 CRC cells used in this assay. Results represent the ratio of means ± s.e.m from triplicate repeat experiments. **P<0.005.*

Furthermore, compared with control transfected cells, no significant differences were identified in transwell invasion assays, in a panel of CRC cells in which miR-224 expression had been ectopically induced (figure 3.18).

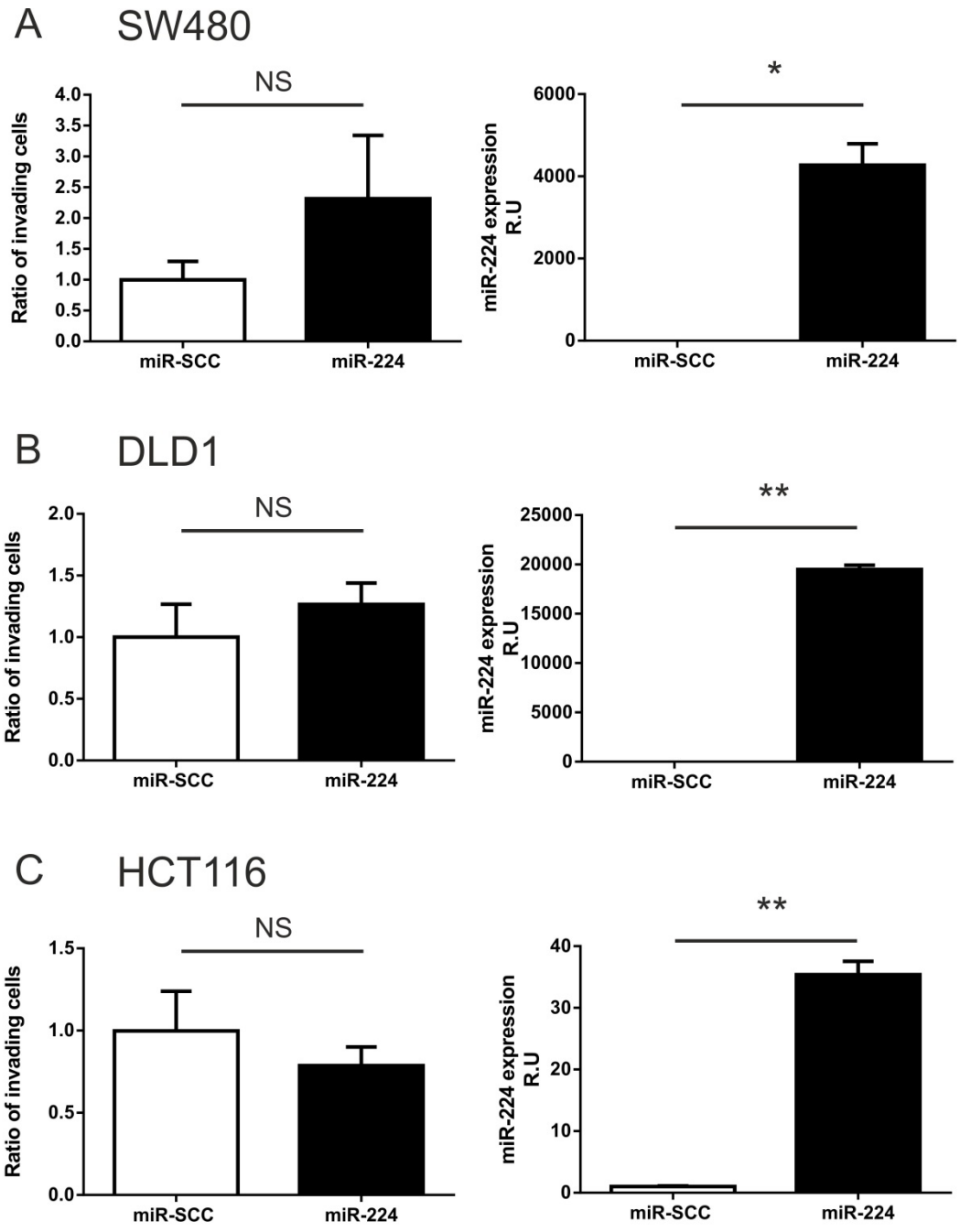


Figure 3.18 Invasion in miR-224 transfected CRC cells: Transwell invasion assay

*Up-regulation of miRNA-224 in (A) SW480; (B) DLD1 and (C) HCT116 cells does not significantly enhance CRC invasion in vitro. To account for differences in cell numbers between experiments, data has been normalised as a percentage of control transfected cells. Results represent mean +/- s.e.m from triplicate repeat experiments. NS = Not Significant; *P<0.05; **P<0.005.*

To corroborate these findings in a more physiologically relevant setting, organotypic models were constructed using SW480 and HCT116 CRC cells stably transfected with miR-224, or control miR-SCC. Once more, the use of HCT116 CRC cells in organotypic culture was a technical failure, as the epithelial layer detached completely from stroma during fixation and processing (data not shown).

By contrast, SW480 cells stably expressing ectopic miR-224 or control miR-SCC invaded well in organotypic culture, but no significant differences were identified between the 2 groups (figure 3.19).

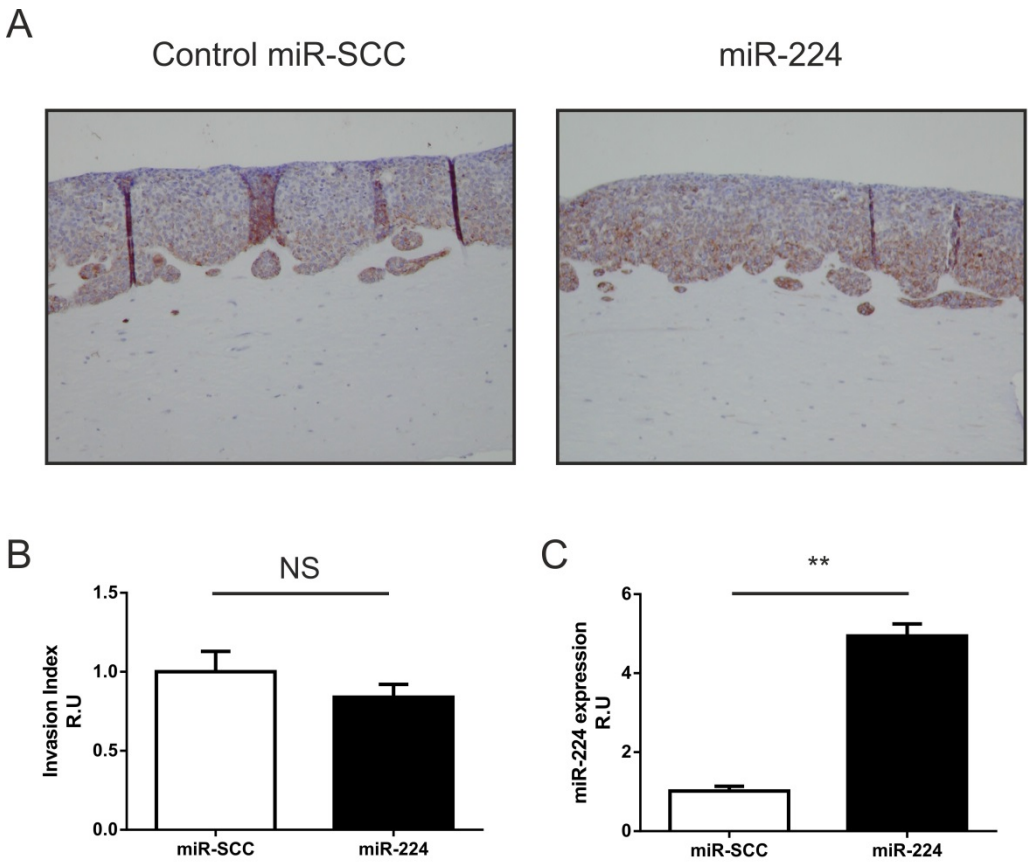


Figure 3.19 miR-224 dependent CRC cell invasion in organotypic models

*(A) Representative sections from organotypic models constructed with HFFF2 fibroblasts in the stromal layer. The malignant epithelium consists of SW480 CRC cells stably expressing either ectopic miR-224 or control miR-SCC. After 14 days incubation organotypics were fixed in formalin, sectioned and immunostained with a pan-cytokeratin antibody to highlight malignant epithelial cells. (B) The mean invasion index ± s.e.m from three independent experiments has been normalised with mean invasion from the control group used as reference (C) In this study miR-224 expression by Taqman® qPCR was approximately 4.9-fold overexpressed in miR-224 transfected SW480 CRC cells compared with controls. NS=Not significant; **P<0.005.*

Given these consistently negative *in vitro* findings, further interrogation of the potential impact of epithelial miR-224 on CRC progression was temporarily suspended.

3.2.3 Results discussion

The impact of over-expressed miR-153 and miR-224 on CRC proliferation and invasion was assessed by transfecting CRC cell lines with miR-specific/GFP co-expression plasmids and/or pre-miR oligonucleotides. Screening studies subsequently enabled rapid interrogation of candidate miRNA function.

MiR-153 is a poorly understood miRNA, first detected in brain tissue where it is implicated in neurodegenerative disorders.[424, 425] Although it has also been implicated in the pathogenesis of endometrial adenocarcinoma[426] and prostate cancer[427] it has not previously been identified in CRC.

Here, upregulated miR-153 expression promoted a more invasive phenotype in organotypic CRC models. Furthermore, organotypic sections immunostained with an anti-GFP antibody demonstrated enrichment of miR-153 expressing cells at the tumour-stroma interface; evidence that individually transfected miR-153-positive CRC cells have enhanced invasive capacities. This data corroborated, and was published alongside a detailed mechanistic analysis exploring the role of miR-153 in CRC progression. In the study, co-authored by members of the host group, miR-153 enhanced CRC invasiveness by indirectly activating MMP9, but not by enhancing *MMP9* mRNA transcriptional levels.[235] MMP9 is a collagenase involved in ECM turnover, and upregulated expression has previously been identified as an independent predictor of overall, cancer specific and disease-free survival in CRC.[428, 429]

MiR-153 may also promote CRC progression by activating chemoresistance pathways mediated through its target, the transcription factor FOXO3.[235] FOXO3 is an important tumour suppressor gene which is downregulated in advanced stage CRC compared with early stage disease and which correlates inversely with miR-153 expression *in vivo*.[231, 235]

Paradoxically, other data suggests that miR-153 expression may also be closely associated with an epithelial cell phenotype. MiR-153 is supressed in oral Squamous Cell Carcinoma (SCC) cells undergoing EMT in response to TGFβ treatment, and in the presence of ectopic miR-153 expression, this transition is abrogated. In fact, miR-153 directly targets 2 key regulators of the EMT-MET axis, SNAIL and ZEB2, which would

suggest that in SCC at least, miR-153 may exert a protective, anti-metastatic influence.[430]

The current literature regarding miR-224 is equally contradictory. Recently, miR-224 has been identified as a potential regulator of chemoresistance pathways in human lung adenocarcinoma cells, and a multifaceted oncogene in hepatocellular carcinoma.[431-433]

In CRC specifically, Zhang and colleagues,[239] corroborated our finding that miR-224 is upregulated in CRC compared with paired normal tissue, but also suggested that ectopic miR-224 activity in SW480 CRC promotes both CRC invasion and proliferation by targeting SMAD4, a key mediator of TGFβ signalling and a putative tumour suppressor in CRC pathways.[434, 435] However, contrary analysis published in *Gastroenterology* in 2013, suggested that miR-224 inhibition and not induction, was associated with enhanced SW480 CRC cell proliferation *in vitro* and increased metastasis *in vivo* in xenograft mouse models of CRC.[436]

In the current study, ectopic miR-224 expression in two from three CRC cell lines did appear to promote invasion in transwell assays to a minor degree, but these analyses did not reach statistical significance. Notably, ectopic miR-224 expression did not promote invasion in organotypic models, or drive CRC cell proliferation *in vitro*. It is essential therefore to conclude, that data presented here supports neither of the two conflicting hypotheses identified in the literature regarding the biological impact of deregulated miR-224 expression during CRC progression.

In summary, the findings of the current study suggest that over-expression of miR-153, may have a role in promoting disease progression in CRC through enhanced cellular invasion. This adds to the weight of evidence implicating miRNAs as pathogenic actors during cancer progression and metastasis. However, contrasting data implying that miR-153 is active in a highly-conserved molecular cascade which may in-fact limit metastatic potential, suggests that further mechanistic study is required to bring the true relevance of miR-153 into sharp relief. Equally, the role of miR-224 remains unclear and further analysis of biological function both *in vitro* and *in vivo* remains a high priority.

3.3 The role of stromal microRNA candidates during colorectal cancer progression

Next, attention turned to miR-21 which was amongst the most upregulated miRNAs in CRC stroma in the QuantimiR™ comparison with paired normal colonic stroma (preliminary data; section 1.7).

3.3.1 miR-21 expression localises to fibroblast-like cells in the CRC stroma

Mir-21 has been shown to be upregulated in various cancers including CRC.[437-441] As most of these studies utilised pieces of tumour tissue containing tumour and non-tumour cells for the detection of miR-21 expression, the relative contribution of stromal/cancer cells to the observed miR-21 upregulation was not clearly established. To identify the source of overexpressed miR-21 in CRC, *in situ* hybridization (ISH) was used. Highly sensitive locked-nucleic acid (LNA) probes showed specific and predominant staining in fibroblast-like cells in the stroma of CRC specimens examined. Crucially, tumour epithelium and benign tissue expressed miR-21 only sporadically in weakly staining mononuclear cells (figure 3.20). This assay was conducted in the commercial sector on our behalf, by Bioneer (Horsholm; Denmark), and we are currently developing capacity to conduct the work 'in-house'.

To quantify overexpression of miR-21, qPCR was performed using Taqman® probes. Total RNA was extracted from the 10 paired CRC and normal colonic tissue specimens used by the group in preliminary QuantimiR™ profiling studies (preliminary data; section1.7). Mean miR-21 expression was 4.0-fold higher in the LMD stroma of CRC specimens compared with paired normal tissue ($P<0.05$; figure 3.20).

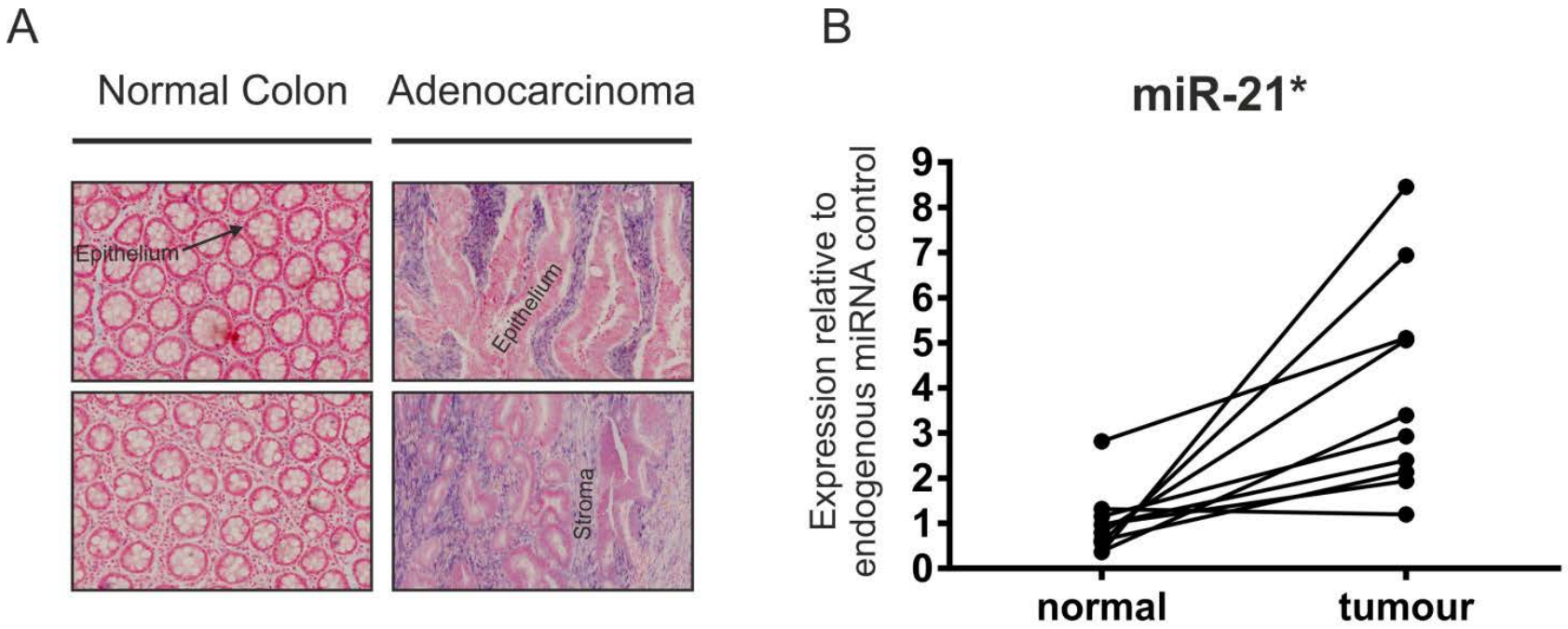


Figure 3.20 miR-21 expression in CRC stroma

*(A) ISH of representative CRC and normal colonic tissue sections using LNA probes against miR-21. MiR-21 positivity is characterised by an intense blue chromogenic reaction.(B) High sensitivity Taqman® qPCR using total RNA extracted from LMD tissue of patients with CRC (n=10) reveals that stromal miR-21 expression successfully separates tumour from paired normal tissue.*P<0.05.*

3.3.2 The impact of stable ectopic miR-21 overexpression in immortalised stromal fibroblasts

To assess the biological significance of upregulated stromal miR-21, its ectopic expression was stably induced in an established human fibroblast cell line (MRC5). MiR-21 overexpressing MRC5 cells ($MRC5^{21}$) underwent striking morphological change, adopting enlarged and elongated forms, which was accompanied by minor increases in α-SMA expression compared to identical passage scrambled control transfected ($MRC5^{SCC}$) cells (figure 3.21). Fibroblasts showing similar morphological features are considered as myofibroblasts and characterised by increased α-SMA containing intracellular filaments. Myofibroblasts are often observed in the tumour microenvironment and their presence has been proposed as an independent marker of poor prognosis.[442]

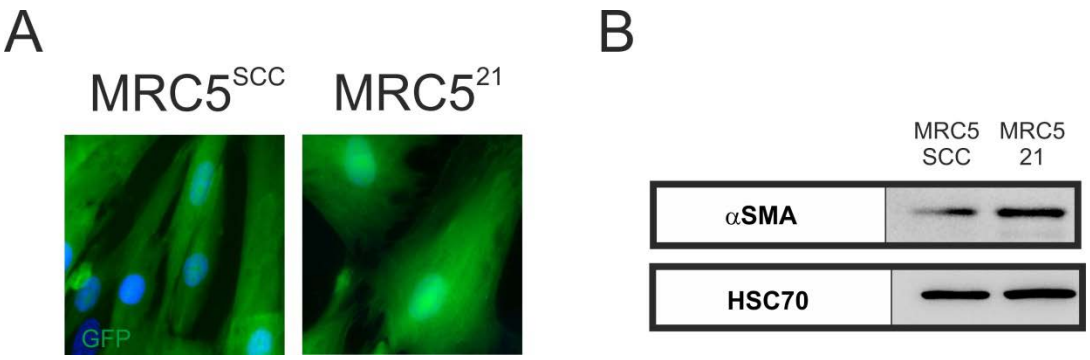


Figure 3.21 Ectopic miR-21 expression in fibroblasts

(A) Fluorescence from MRC5 fibroblasts stably transfected with miR-21/GFP or control miR-SCC/GFP co-expression plasmids highlight the morphological changes associated with ectopic miR-21 expression in fibroblasts (x40 magnification). (B) Western blot analysis show minor increases in αSMA expression in MRC5 fibroblasts stably transfected with miR-21 compared with controls.

Secreted molecules from cancer cells such as TGFβ are responsible for myofibroblast differentiation.[98] Published data also suggests that miR-21 inhibition using specific

antisense oligonucleotides prevents MRC5 fibroblasts from undergoing transdifferentiation in response to TGFβ.[299]

To assess whether miR-21 overexpression primes myofibroblast transdifferentiation or provokes an intermediate differentiation state, $MRC5^{21}$ and $MRC5^{SCC}$ cells were treated with conditioned media (CM) extracted from SW480 CRC cells (CM^{SW480}) or TGFβ at 2ng/ml or 10ng/ml final concentration. After 3 days, α-SMA expression was assessed by immunofluorescence, revealing that stress fibre formation had occurred in a significantly greater proportion of $MRC5^{21}$ cells than $MRC5^{SCC}$ cells in response to CM^{SW480} and low dose TGFβ. As anticipated, treatment with higher TGFβ doses induced large numbers of both $MRC5^{SCC}$ and $MRC5^{21}$ to undergo myofibroblast transdifferentiation. Stress fibre deposition was not found in significant numbers of $MRC5^{21}$ or $MRC5^{SCC}$ cells in the untreated group (figure 3.22).

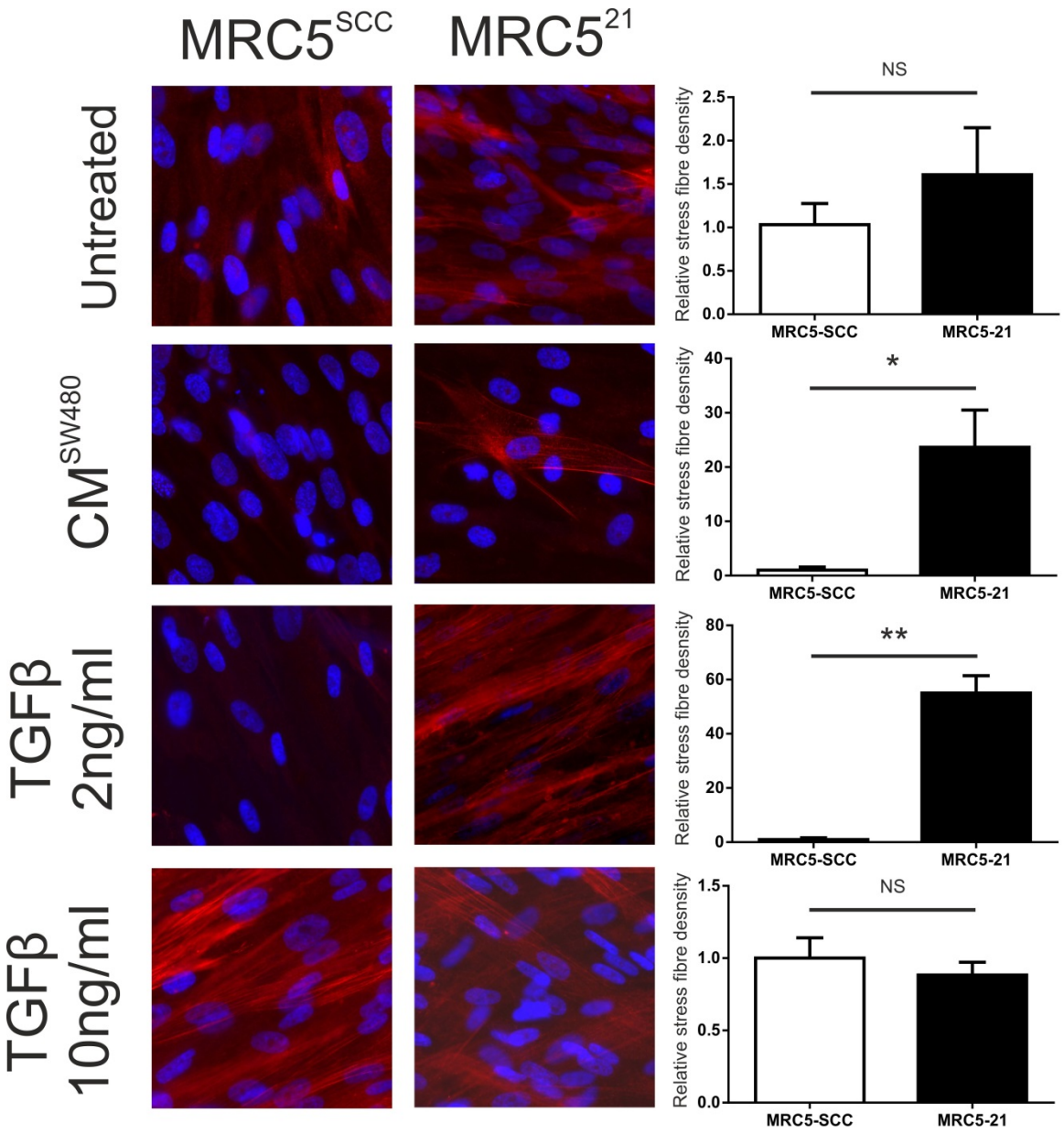


Figure 3.22 Immunofluorescence staining for αSMA in MRC5 fibroblasts stably expressing either miR-21 or miR-SCC

*MiR-21 but not miR-SCC is associated with α-SMA stress fibre formation following treatment with low doses of TGFβ (2ng/ml) or culture medium extracted from SW480 CRC cells (CM^{SW480}). High doses of TGBβ (10ng/ml) also induce stress fibre formation deposition in miR-SCC transfected cells, which serves as a positive control. NS = Not Significant; *P<0.05; **P<0.005. (x40 magnification).*

These results suggest that although miR-21 expression alone is insufficient to drive myofibroblast transdifferentiation, it may condition/facilitate fibroblasts to acquire the myofibroblast state.

3.3.3 The functional impact on CRC cells of ectopic miR-21 expression in fibroblasts

The reciprocal interaction between cancer cells and tumour stroma is a contributing factor in carcinoma progression. In the following sections, the effect of stromal miR-21 manipulation on CRC cell fate is examined.

3.3.3.1 Medium supernatant from miR-21 over-expressing fibroblasts increases the proliferative capacity of CRC cells

After a 24 or 48 hour incubation with conditioned medium from $MRC5^{21}$ cells (CM^{21}), the mean cell density of SW480 cells increased 40% (*P=0.03*) and 93% (*P=0.01*) compared to controls (CM from $MRC5^{SCC}$; CM^{SCC}) respectively. Under the same conditions, another CRC cell line (DLD1), showed an 18% (t=24 hours; not significant) and 66% (t=48 hours; *P=0.01*) mean cell density increase compared to controls (figure 3.23).

3.3.3.2 Medium supernatant from miR-21 over-expressing cells protects CRC cells from Oxaliplatin induced apoptosis

MiRNAs have important regulatory roles in target pathways associated with chemoresistance.[443, 444] Emerging data also suggests that stromal fibroblasts can influence chemosensitivity of tumour cells indirectly by modulating the tumour micro-environment, mitigating the impact of cytotoxic stress.[445, 446]

Thus, the comparative effect of CM^{21} or CM^{SCC} treatment on Oxaliplatin mediated apoptosis was evaluated in SW480 CRC cells using the Annexin V/Propidium iodide (AnnV/PI) double staining method: Crucially, Oxaliplatin, a first-line cytotoxic agent for the treatment of patients with stage III and IV CRC, was less effective at inducing apoptosis and cell death in CM^{21} treated CRC cells compared with CM^{SCC} treated controls (figure 3.23).

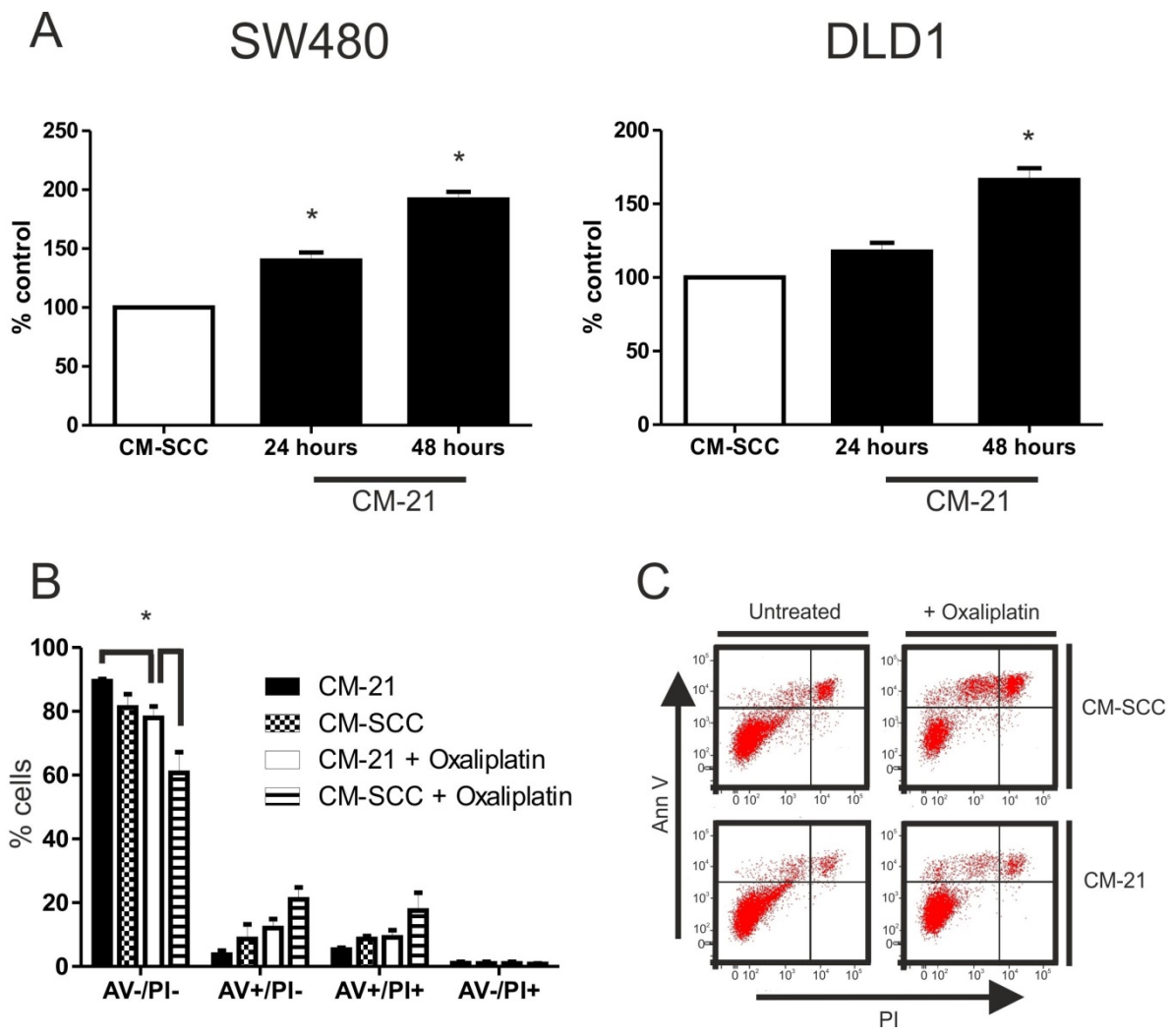


Figure 3.23 The functional impact of ectopic stromal miR-21 expression

*(A) MTS cell proliferation and viability assay of SW480 and DLD1 CRC cells following treatment with CM^{21} or CM^{SCC}. Results represent mean cell density ± s.e.m after 24 or 48 hours incubation, from experiments repeated in triplicate. (B) Analysis of apoptosis in SW480 CRC cells pre-treated with CM^{21} or CM^{SCC} in the presence or absence of oxaliplatin at 30μg/ml. Columns represent percentage of live (AV-/PI-), early apoptotic (AV+/PI-) and late apoptotic (AV+/PI+) cells in each treatment group. Data presented represents results from three independent experiments expressed as means ± s.e.m. (C) Representative FACS plot indicating the quadrants used to calculate the proportion of live and apoptotic cells in B. *P≤0.05.*

3.3.3.3 Medium supernatant from miR-21 over-expressing fibroblasts enhances CRC cell invasion

Increased motility and invasion are critical physiological processes in the course of metastasis. To assess if stromal miR-21 alters invasive properties of CRC cells, CM from miR-21 and miR-SCC stably transfected MRC5 cells (CM^{21}/CM^{SCC}) was used as the chemo-attractant in transwell invasion assays. CM^{21} was associated with 1.7 (*P<0.02*) and 1.8 fold (*P=0.03*) increases in invasiveness of SW480 and DLD1 CRC epithelial cells respectively compared with CM^{SCC} controls (figure 3.24). In order to examine invasiveness in more physiological relevant conditions, organotypics were created by embedding $MRC5^{21}$ or $MRC5^{SCC}$ cells into gels containing extracellular matrix components (Matrigel™ and collagen), on to which CRC cells were seeded. MRC5 fibroblasts were chosen initially for this purpose, because they induce only moderate invasion from SW480 CRC cells in co-culture under wild-type conditions (figure 3.14).

Results showed a 2.5 fold (*p<0.005*) increase in the invasion of SW480 cells into the stroma containing $MRC5^{21}$ cells compared with controls. GFP positive cells within the stroma confirm the presence of fibroblasts stably transfected with CMV driven miRNA/GFP co-expression plasmid (figure 3.24).

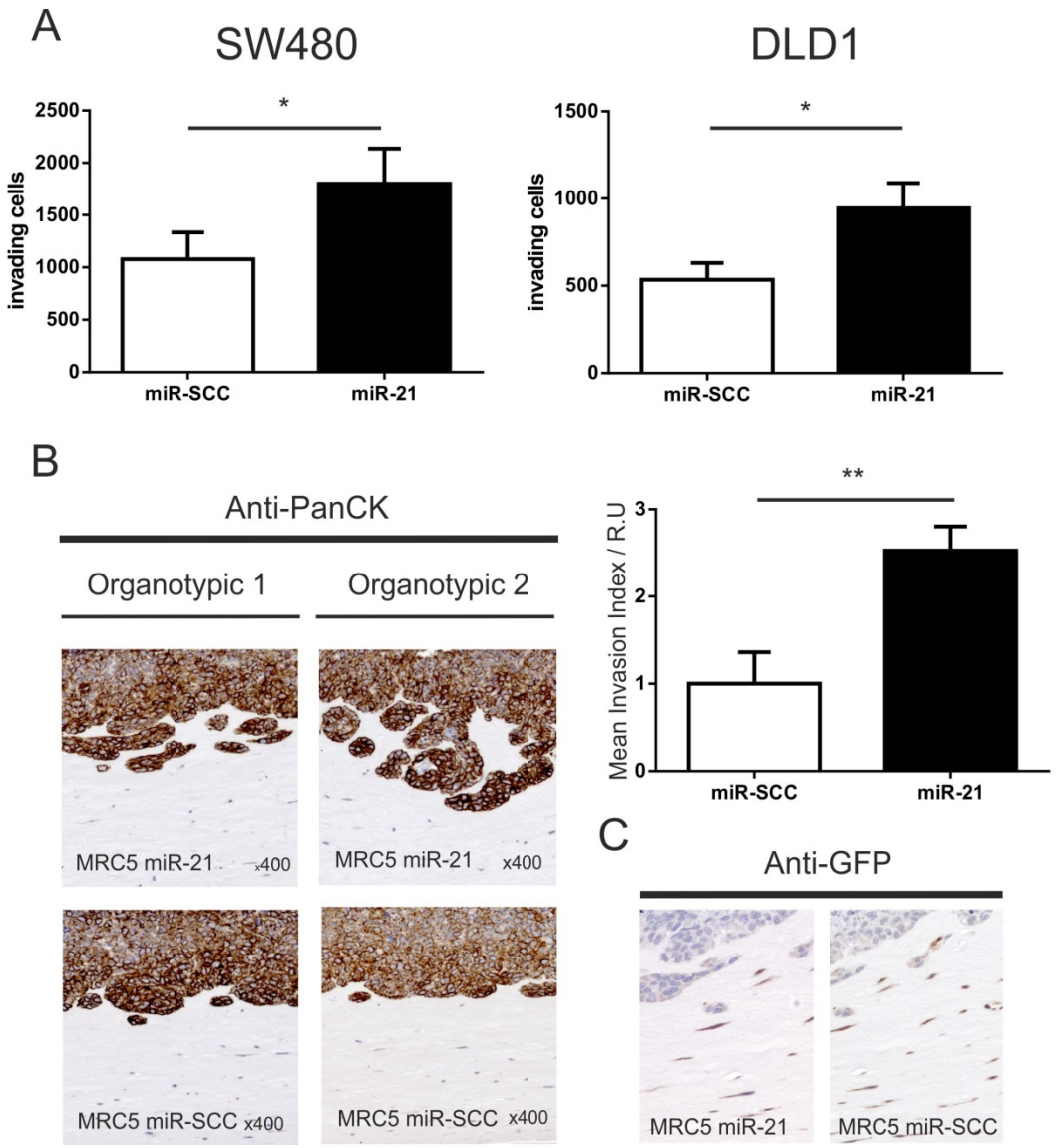

A
SW480
DLD1
invading cells
2500
2000
1500
1000
500
0
1500
1000
500
0
*
*
miR-SCC
miR-21
miR-SCC
miR-21
B
Anti-PanCK
Organotypic 1
Organotypic 2
MRC5 miR-21 x400
MRC5 miR-21 x400
MRC5 miR-SCC x400
MRC5 miR-SCC x400
**
Mean Invasion Index / R.U
3
2
1
0
miR-SCC
miR-21
C
Anti-GFP
MRC5 miR-21
MRC5 miR-SCC

Figure 3.24 The impact of ectopic stromal miR-21 expression on CRC cell invasion (I)

*(A) CM^{21} from stably transfected MRC5 fibroblasts, when used as chemoattractant in in vitro transwell invasion assays is associated with increased invasiveness in CRC cell lines (SW480 and DLD1) compared with controls (CM^{SCC}). Data presented represents results from three independent experiments (mean ± s.e.m; *P<0.05). (B) Representative sections of organotypic co-culture models containing $MRC5^{21}$ and $MRC5^{SCC}$ cells within the stroma. Immunohistochemical staining with anti-pan-Cytokeratin antibody highlights malignant epithelial SW480 CRC cells. The invasive index is increased 2.5-fold in the presence of $MRC5^{21}$ cells compared with control transfected $MRC5^{SCC}$ cells (ratio of means ± s.e.m from triplicate repeats; **P<0.005). (C) Staining for GFP, which is co-expressed by miR-21 and miR-SCC plasmids, confirms the presence of stably transfected fibroblasts within the stroma. GFP positivity is characterised by a brown chromogenic reaction.*

These results suggest that stromal expression of miR-21 may induce enhanced motility and invasion of CRC cells. To further interrogate this hypothesis organotypic models were constructed using a second miR-21 transfected fibroblast cell line.

As we see in figure 3.25 strong SW480 invasion is induced by HFFF2 fibroblasts regardless of miR-21 expression status. Across 3 repeat experiments, invasion was slightly increased (1.2-fold; p=0.06) in organotypics containing miR-21 transfected HFFF2s compared with miR-SCC control transfected cells but this result was not statistically significant. This may mean that miR-21 overexpression in HFFF2 fibroblasts does not substantially impact on the invasive capacity of SW480 CRC cells. Alternatively, in the context of organotypic models, the effect of relatively small increases in miR-21 expression in HFFF2 cells may be masked against a background of strong baseline SW480 cell invasion.

It is also interesting to note that miR-21 overexpression in both MRC5 fibroblasts and HFFF2 fibroblast is not sufficient to induce invasion from DLD1 CRC cells in organotypic culture (figure 3.25).

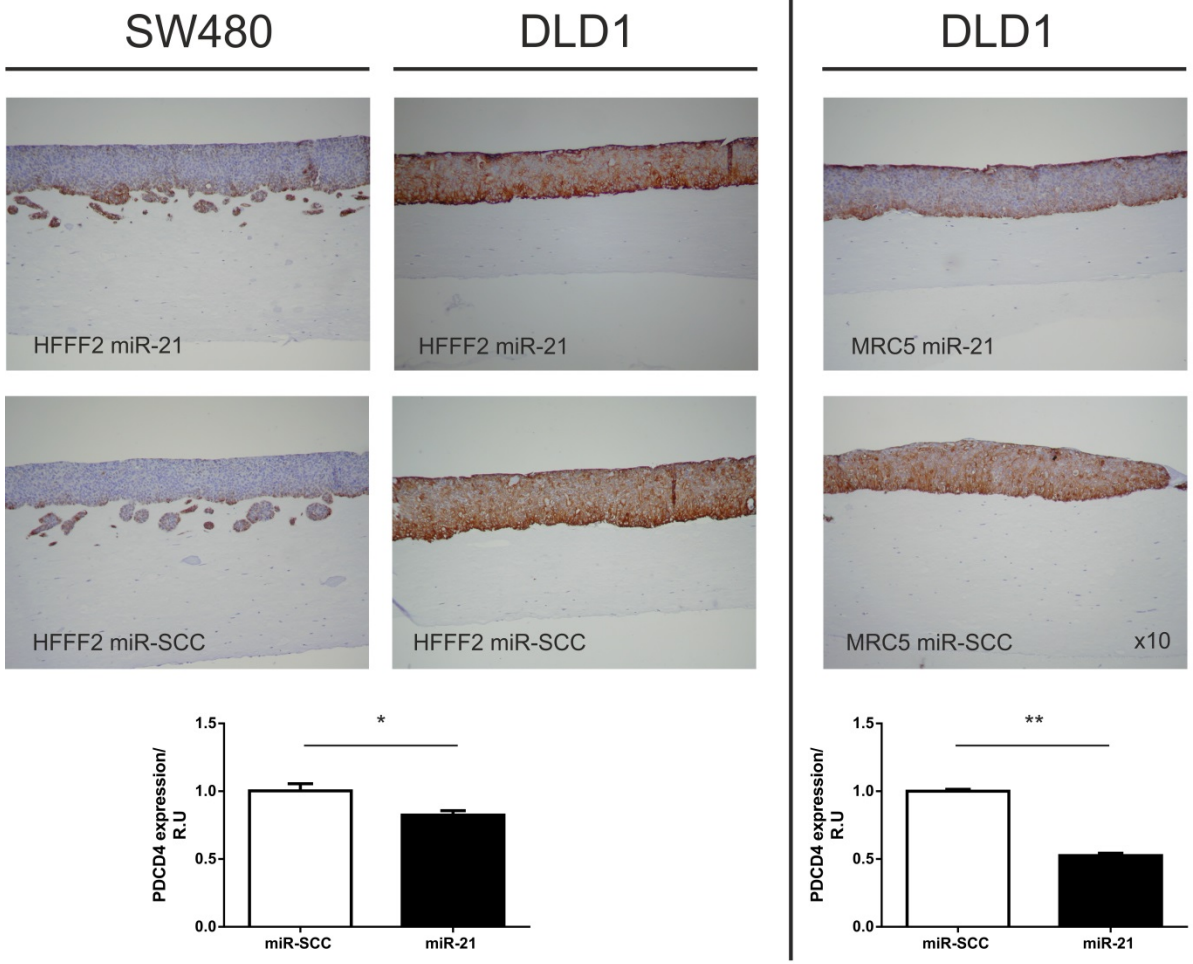


Figure 3.25 The impact of ectopic stromal miR-21 expression on CRC cell invasion (II)

*Representative sections of organotypic co-culture models repeated in triplicate and containing fibroblasts expressing ectopic miR-21 or control miR-SCC in the stroma. PDCD4 mRNA by Taqman® qPCR is used as a proxy measure of stromal miR-21 expression relative to control transfected stroma on the first day of co-culture incubation with SW480 or DLD1 CRC cells *p<0.05; **p<0.005. Immunohistochemical staining with anti-pan-Cytokeratin antibody highlights malignant epithelial cells.*

Crucially however, these results do not tally with *in vitro* data from transwell assays. In figure 3.26 we see that CM^{21} from transiently transfected HFFF2 fibroblasts and PCF9 *ex vivo* fibroblasts is associated with 1.7-fold and 1.9-fold increases in invasiveness of SW480 CRC respectively compared with CM^{SCC} controls. Equally, in figure 3.26, CM^{21} from MRC5 fibroblasts boosts DLD1 invasion 1.8-fold. These inconsistencies may reflect the different time-course over which these two invasion assay formats are conducted, or indeed the fact that transient fibroblast transfection for transwell assays affects far greater miR-21 induction than stable transfection with plasmid DNA (Figure 3.4 and 3.7).

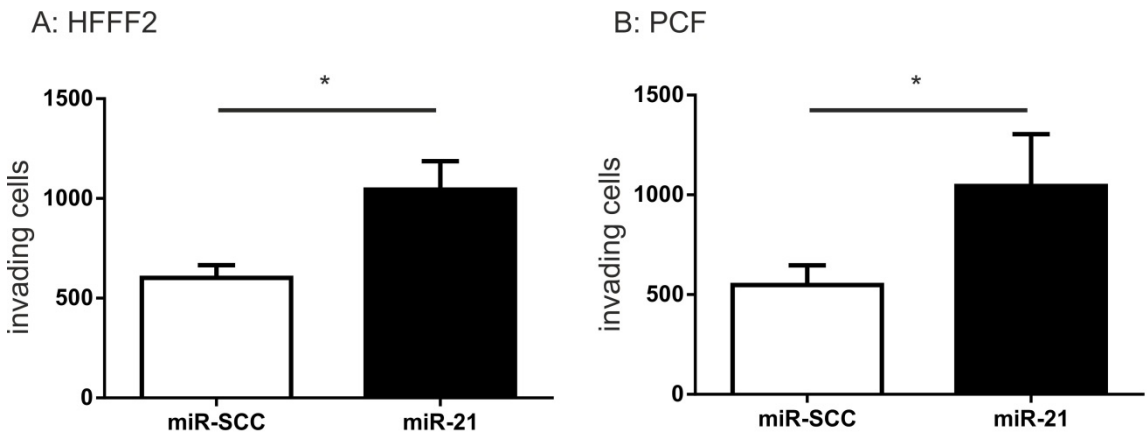


Figure 3.26 The impact of ectopic stromal miR-21 expression on CRC cell invasion (III)

*CM from transient miR-21 transfected (A) HFFF2 and (B) PCF9 fibroblasts is associated with increased SW480 CRC cell invasion in transwell assays compared with CM from miR-SCC transfected controls. Results represent mean ± s.e.m from a single experiment (A) and three independent experiments (B). *P<0.05.*

Importantly, these data validate to a large extent the decision to use MRC5 fibroblasts in the first instance to assess the biological impact of deregulated stromal miR-21 expression. The very fact that wild-type MRC5 fibroblasts provoke little spontaneous invasion from SW480 CRC cells, makes them ideally suited for use in organotypic assays in which ectopic stromal miR-21 expression is induced. Conversely, HFFF2 cells which induce strong invasion from SW480 cells in 3-Dimensional co-culture may in future be better suited to assessing the impact of miR-21 suppression. However, at the

time this study was conducted, anti-miR-21 expression plasmids were not commercially available and stable miR-21 suppression in fibroblasts has not yet been attempted.

3.3.4 MiR-21 targets Matrix Metalloproteinase inhibitors (MMPi) in the CRC stroma

To streamline the search for mechanistic explanations for the novel observation that miR-21 overexpression in fibroblasts enhances CRC invasiveness, focus turned to regulators of ECM remodelling known to be targeted by miR-21 in other contexts. RECK and TIMP3 are both negative regulators of MMP activity. RECK is downregulated in the majority of CRC specimens compared with normal tissue and low expression is associated with poor outcome;[447] it is a membrane anchored glycoprotein and its main function is to inhibit MMP2, MMP9, and MT1-MMP.[448] TIMP3 is a secreted protein and binds elements of the ECM and has been shown to be a potent inhibitor of invasion.[449] MiR-21 has been shown to target RECK mRNA directly in mutated/unmutated 3'UTR luciferase reporter assays using several cell lines, and in numerous tumour contexts, [284, 450, 451] however, regulation of TIMP3 by miR-21 is not thought to be direct despite exhibiting 2 conserved 3'UTR binding sites for miR-21 (figure 3.27).[284]

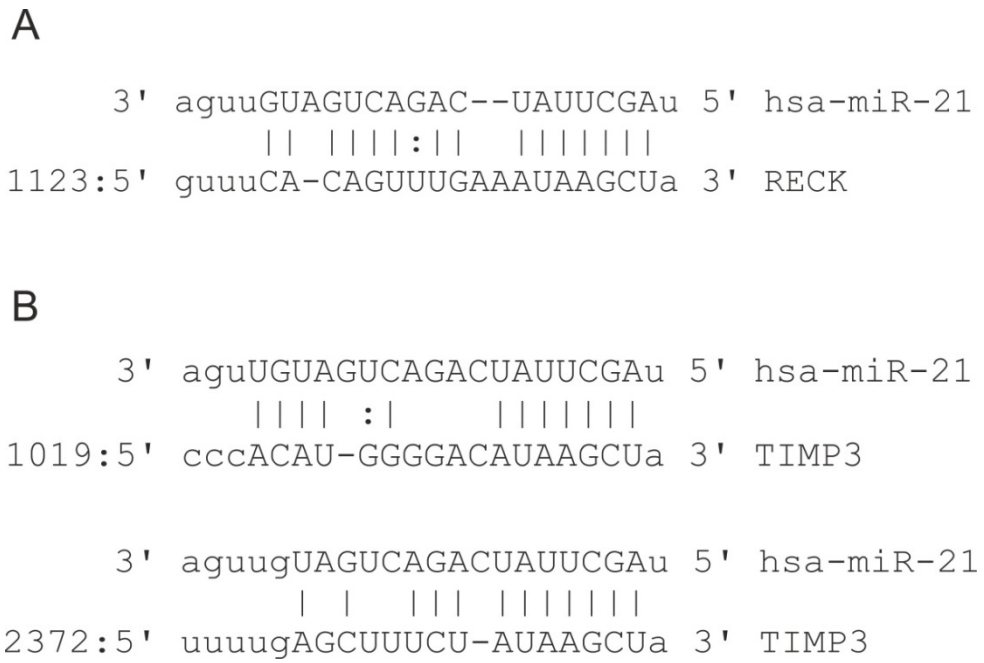


Figure 3. 27 RECK and TIMP3 are putative miR-21 targets in the stroma

Highly conserved putative miR-21 binding sites within (A) RECK and (B) TIMP3 mRNA 3'UTR sequences identified by in silico analysis with miRNA target prediction software from www.microrna.org (august 2010 release).[452, 453]

3.3.4.1 MiR-21 targets RECK in the CRC stroma

To assess whether miR-21 expressing fibroblasts express reduced RECK, mRNA was quantified by Taqman® qPCR and protein by Western blot analysis in stably transfected MRC5 fibroblasts and control transfected cells. These results provide mechanistic explanation why stromal expression of miR-21 is a contributing factor to invasiveness of CRC tumours (figure 3.28). In order to validate the functional consequences of miR-21 expression in relation to MMP activation, sections of organotypic cultures were stained for RECK. This revealed fibroblast specific expression of this protein in $MRC5^{SCC}$ but not $MRC5^{21}$ specimens (figure 3.28) consistent with the previous findings that $MRC5^{21}$ containing stroma significantly enhanced invasion of SW480 CRC cells (figure 3.24).

To observe the relationship between miR-21 and its target RECK in a more physiological setting, primary colon fibroblasts were transiently transfected with miR-21/GFP or miR-SCC/GFP co-expression plasmid using the transfection reagent Xfect™ from Clontech.

As for HFFF2 cells (figure 3.10), transfecting PCF9 fibroblasts with plasmid DNA was inefficient, resulting in <10% of cells displaying positive fluorescence at 48 hours. Although it would have been difficult to select and expand miR-21/GFP or miR-SCC/GFP positive PCF9 clones in this situation, low transfection efficiency did present an opportunity to visually compare RECK expression in cells expressing miR-21 with adjacent non-transfected cells. Thus, forty eight hours after transfection with miR-21/GFP or miR-SCC co-expression plasmid, PCF cells were fixed for the purpose of immunofluorescence labelling.

As we see in figure 3.28 non-transfected cells and cells transfected with the miR-SCC scrambled control plasmid demonstrate baseline levels of RECK expression. In contrast, miR-21 transfected cells display decreased RECK expression in proportion with transfection efficiency as cells exhibiting the most intense GFP signal are also associated with the most diminished RECK fluorescence signal.

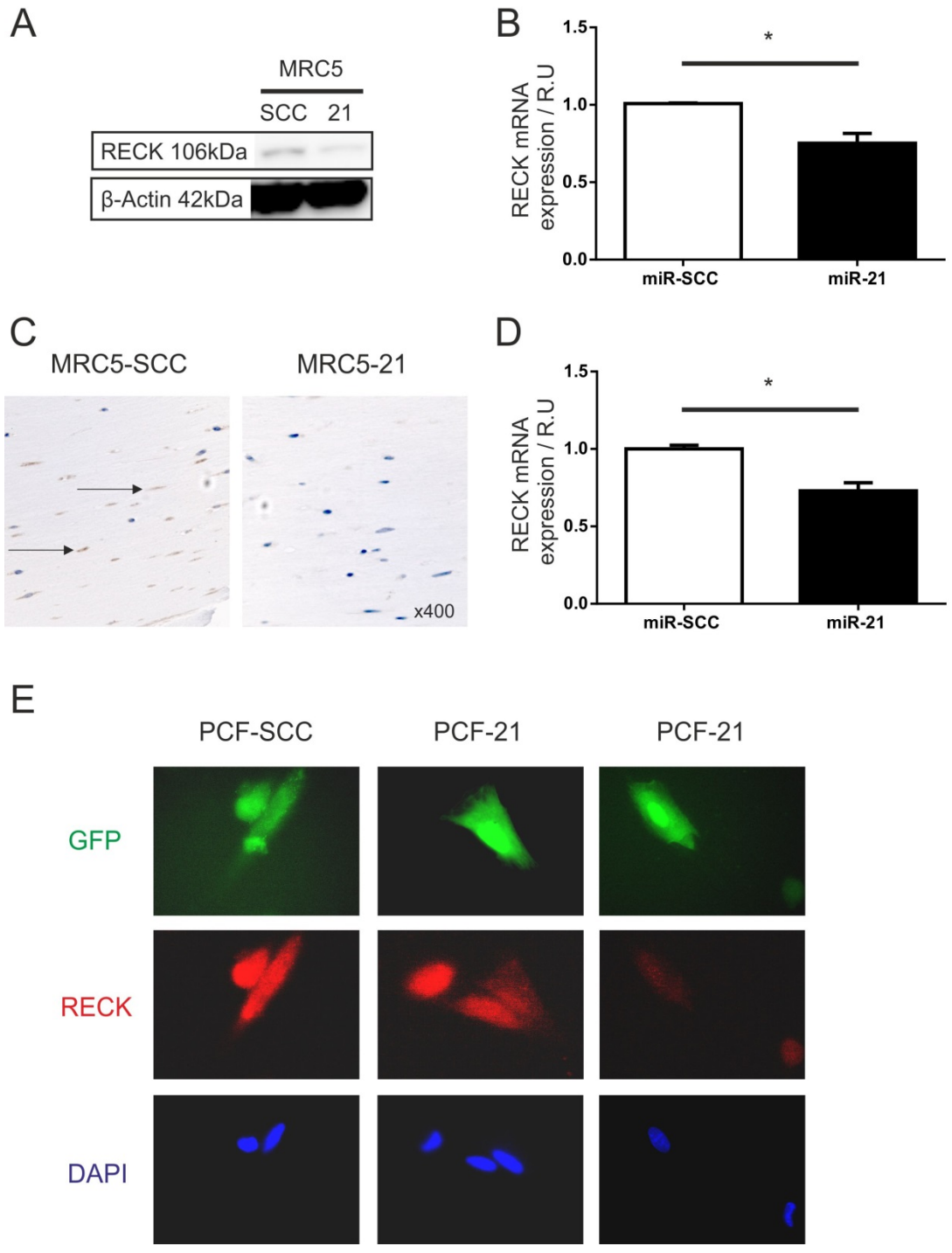

A
MRC5
SCC 21
RECK 106kDa
β-Actin 42kDa
B
RECK mRNA expression / R.U
1.5
1.0
0.5
0.0
*
miR-SCC
miR-21
C
MRC5-SCC
MRC5-21
x400
D
RECK mRNA expression / R.U
1.5
1.0
0.5
0.0
*
miR-SCC
miR-21
E
PCF-SCC
PCF-21
PCF-21
GFP
RECK
DAPI

Figure 3.28 miR-21 upregulation in fibroblasts leads to downregulated expression of MMP inhibitor RECK

*(A) Western blot and (B) Taqman® qPCR analysis for RECK protein and mRNA expression in $MRC5^{21}$ or $MRC5^{SCC}$ cells; * represents mean ± s.e.m of four independent repeat experiments $p<0.05$. (C) Representative sections of organotypic stroma containing $MRC5^{21}$ or $MRC5^{SCC}$ cells immunostained with anti-RECK antibody. Positive brown cytoplasmic staining in stroma (indicated with arrows) containing $MRC5^{SCC}$ fibroblasts contrasts with scant RECK expression in stroma containing $MRC5^{21}$ cells. (D) Taqman® qPCR analysis for RECK mRNA expression in miR-21 transfected PCF9 cells expressed as mean ± s.e.m from an experiment repeated in triplicate *$P<0.05$ (E) Immunofluorescence for RECK protein expression in ex vivo PCF9 fibroblasts transfected with either a miR-21/GFP or miR-SCC/GFP co-expression plasmid at x40 magnification.*

3.3.4.2 MiR-21 dependent TIMP3 downregulation is not consistently observed in stromal fibroblasts

TIMP3 is likely to be a downstream effector of miR-21 signalling, as TIMP3 mRNA is responsive to miR-21 manipulation in glioma[284] and renal cell carcinoma[454] models. However, this interaction may not be direct, as despite exhibiting 2 conserved putative 3'UTR binding sites, TIMP3 mRNA expression is not rescued by site specific mutation in the presence of miR-21.[284]

In the current study, TIMP3 protein and mRNA expression were downregulated by ectopic miR-21 expression in MRC5 fibroblasts by western blotting and Taqman® qPCR respectively (figure 3.29). Similarly, qPCR demonstrated downregulated TIMP3 mRNA expression in HFFF2 fibroblasts transiently transfected with miR-21. Furthermore, in organotypic models in which stable miR-21 overexpression had been induced in MRC5 fibroblasts, TIMP3 expression was notably diminished compared with control transfected cells (figure 3.29).

In contrast, transient transfection of PCFs with miR-21 did not significantly alter expression of TIMP3 mRNA, nor was TIMP3 protein reduced in miR-21 expressing cells compared with controls in immunofluorescence assays.

This conflicting data reflects ongoing uncertainty in the literature regarding the nature of miR-21 and TIMP3 interaction *in vivo*.

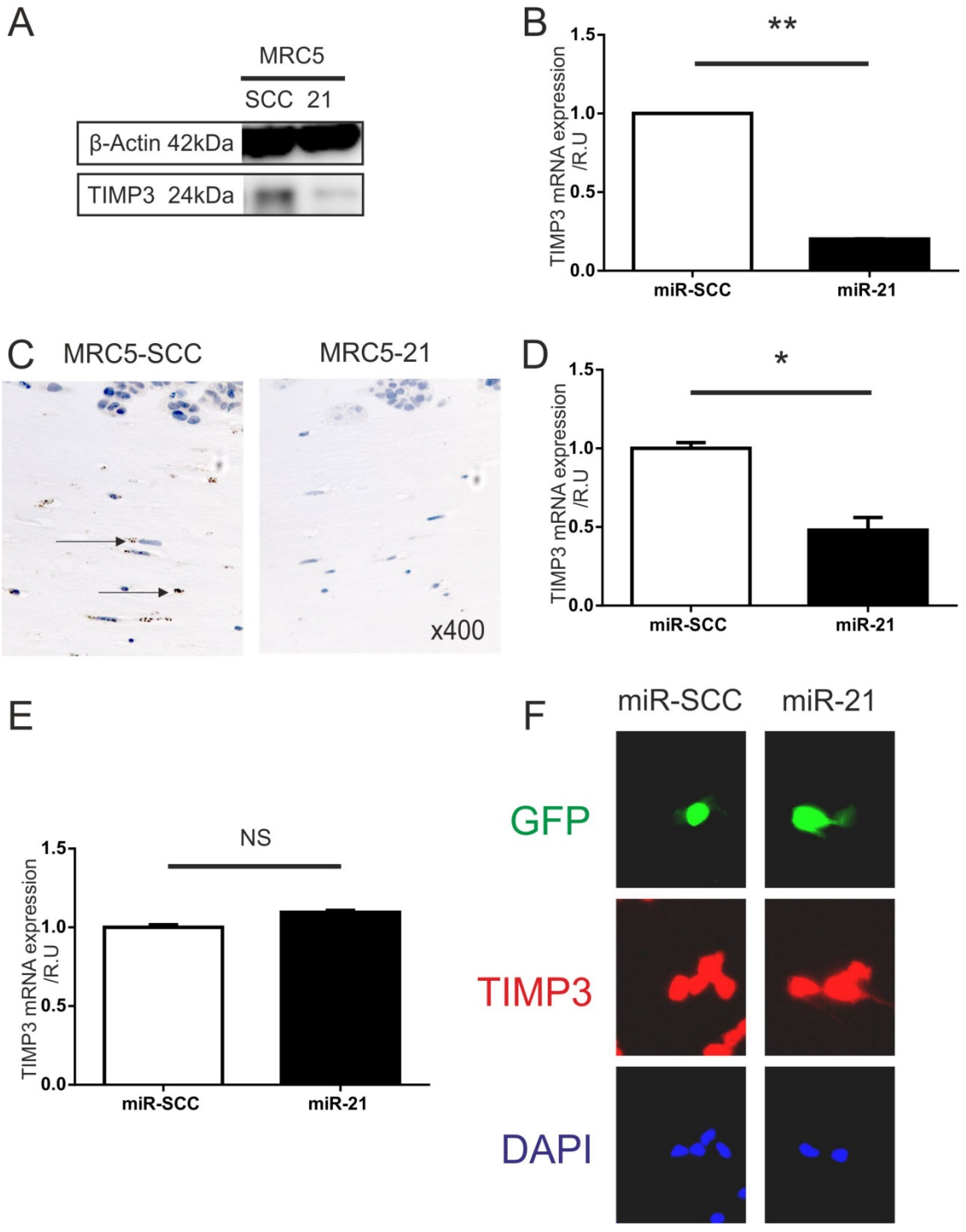


Figure 3.29 miR-21 upregulation in MRC5 fibroblasts, but not PCF9 fibroblasts, leads to downregulated expression of TIMP3

(A) Western blot and (B) Taqman® qPCR analysis for TIMP3 protein and mRNA expression in MRC5^{21} and MRC5^{SCC} cells. (C) Representative sections of organotypic stroma containing MRC5^{21} or MRC5^{SCC} cells immunostained with anti-TIMP3 antibody. Positive brown cytoplasmic staining in stroma (indicated with arrows) containing MRC5^{SCC} fibroblasts contrasts with scant TIMP3 expression in stroma containing MRC5^{21}cells.

*Taqman® qPCR analysis for TIMP3 mRNA in (D) HFFF2 and; (E) PCF9 fibroblasts transiently transfected with miR-21. (F) Immunofluorescence staining for TIMP3 in ex vivo colonic fibroblasts transfected with either a miR-21/GFP or miR-SCC/GFP co-expression plasmid at x40 magnification. MiR-21 +ve cells, as well as control transfected and non-transfected cells, express TIMP3 in similar abundance. NS=Not Significant; *P<0.05; **P<0.005.*

3.3.4.3 MiR-21 dependent MMPi downregulation is associated with a reciprocal rise in MMP2 activity

The key action of RECK and TIMP3 is to inhibit the otherwise unchecked activity of MMPs involved in the breakdown of ECM. To assess whether RECK and TIMP3 downregulation in response to ectopic miR-21 expression in MRC5 fibroblasts leads to increased MMP activity, CM^{21} and CM^{SCC} were analysed by gelatin zymography. In this assay, a dominant band corresponding to MMP2 was consistently detected and miR-21 upregulation led to significantly increased gelatinase activity compared with controls (figure 3.30).

Interestingly MMP9 activity was absent in CM associated with MRC5 fibroblasts in both miR-21 transfected and miR-SCC transfected groups, although this is consistent with previous reports suggesting stromal MMP9 expression is restricted to neutrophils and macrophages in CRC.[455] (figure 3.30)

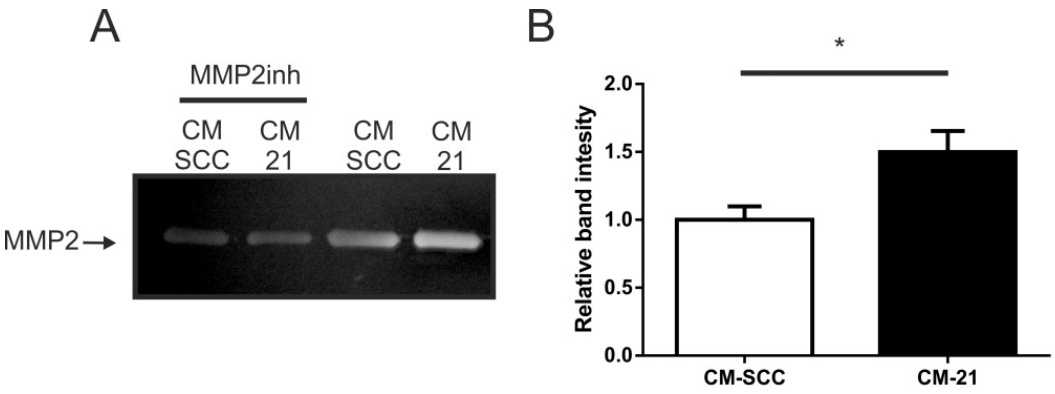


Figure 3.30 Ectopic miR-21 expression in MRC5 fibroblasts leads to upregulated MMP2 activity

*(A) Representative experiment showing zymographic activity in CM^{21} or CM^{SCC}, with and without MMP2 inhibitor (MMP2inh). (B) Assessment of MMP2 activity by determination of zymographic band intensity. Results are expressed as a ratio of MMP2 activity in CM^{21} compared with CM^{SCC} in three independent experiments. *P<0.05.*

3.3.4.4 SW480 CRC cell invasion is MMP2 dependent

To address whether up-regulated MMP2 activity in response to ectopic miR-21 expression in fibroblasts is relevant during CRC invasion, a highly specific soluble MMP2 inhibitor (Merck)[456-458] was used in transwell assays.

To determine the ideal working concentration of MMP2 inhibitor, 10mM stock dissolved in DMSO and diluted in complete DMEM to between 0µM and 100µM final concentration was used as chemoattractant. Results show that as little as 2.5µM concentrations of MMP2 inhibitor produces effective inhibition of SW480 CRC cell invasion in transwells (figure 3.31), and below 50µM, treatment of SW480 cells with MMP2 inhibitor in DMSO has very little cytotoxic impact (figure 3.32).

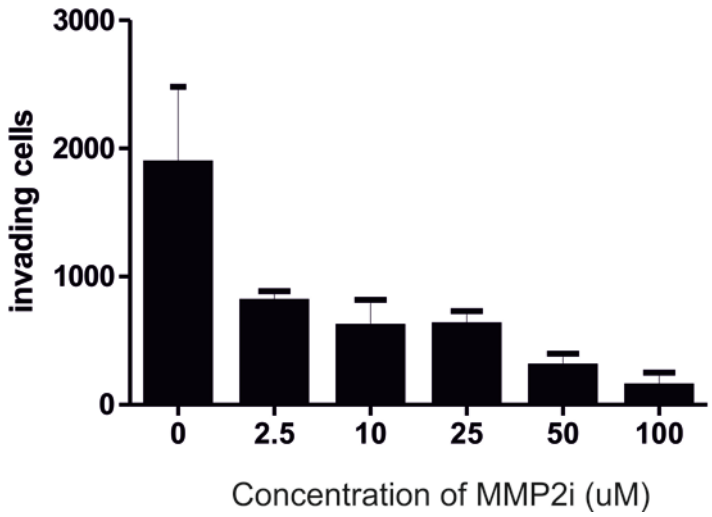


Figure 3.31 Optimisation of MMP2 inhibitor (MMP2i) working concentration – impact on cellular invasion

The impact of increasing concentrations of MMP2 inhibitor (MMP2i) on SW480 CRC cell invasion in vitro in Transwell assays.

In parallel experiments, SW480 CRC plated at identical densities to those seeded in transwell invasion assays ($5x10^4$ in 200µl in 96 well plates), were treated for 24 hours with complete DMEM to which stock MMP2 inhibitor solution had been added at final concentrations of 25µM , 50µM and 200µM. The proportion of live and apoptotic cells were then quantified by FACS sorting using the Annexin V/PI double staining method (figure 3.32).

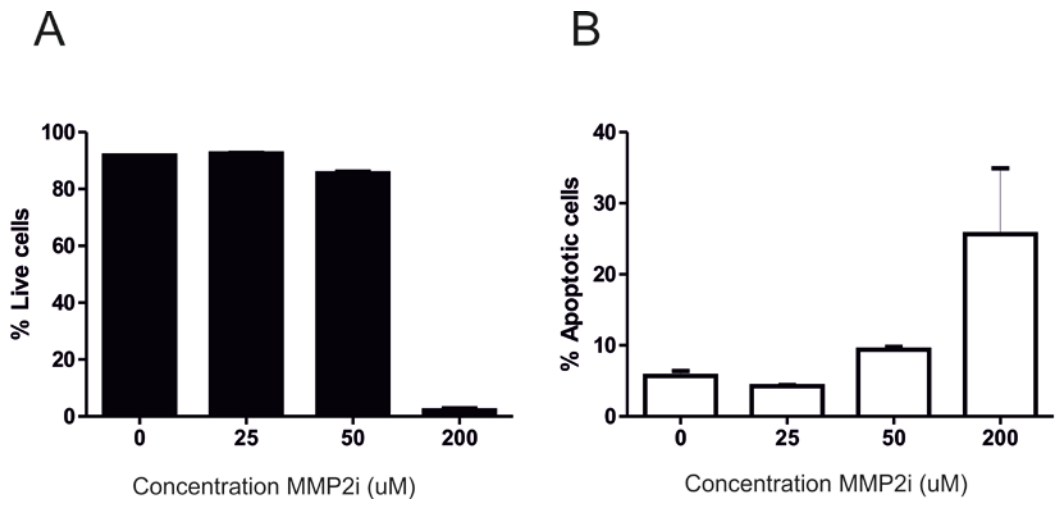


Figure 3.32 Optimisation of MMP2 inhibitor (MMP2i) working concentration – impact on cellular apoptosis

SW480 CRC cells were treated with MMP2i in DMSO diluted from 10mM stock using complete DMEM. After 24 hours cells were analysed by FACS to quantify the proportion of (A) live cells and (B) apoptotic cells. Results represent mean +/- s.e.m of triplicate repeat experiments.

This preliminary work was essential given the cytotoxic effect of DMSO on CRC cell lines.[459] Having conducted these experiments, we could be confident that any apparent impact of low dose MMP2 inhibitor on CRC invasion was not the result of DMSO induced apoptosis.

3.3.4.5 MiR-21 dependent CRC cell invasion is MMP2 dependent

Importantly, the increased invasive effect associated with MRC5-CM[21] compared with CM[SCC] was partially abrogated in the presence of 25 μM MMP2 inhibitor suggesting that stromal miR-21 dependent SW480 CRC cell invasion is mediated in-part by increased MMP2 activity (figure 3.33).

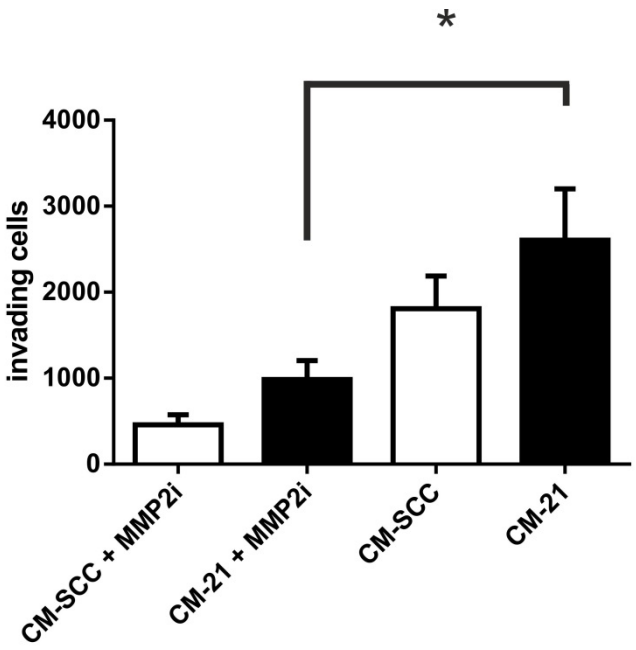


Figure 3.33 miR-21 dependent CRC cell invasion is MMP2 dependent

*Transwell invasion assay using CM^{21} and CM^{SCC} as chemoattractant, with and without MMP2 inhibitor (MMP2i) at a final concentration of 25μM. Results represent the mean number +/- s.e.m of invading SW480 cells; *p<0.05.*

3.3.5 Upregulated stromal miR-21 expression does not induce Epithelial to Mesenchymal transition in CRC epithelial cells

EMT is a key prometastatic event as transformed epithelial cells adopt mesenchymal characteristics and acquire the capacity to break free from their sister cells and invade the ECM. EMT is regulated by various intracellular signalling pathways including TGFβ which triggers internalisation of epithelial markers such as E-Cadherin and upregulated expression of mesenchymal markers including Vimentin and N-Cadherin.[253] TGFβ is also an important trigger of fibroblast-to-myofibroblast transdifferentiation[98] and furthermore miR-21 is strongly upregulated in MRC5 fibroblasts in response to TGFβ treatment.[299] To assess whether miR-21 pathobiology links EMT in transformed epithelial CRC cells and myofibroblast transdifferentiation in cancer-associated stroma, $1.5x10^5$ DLD1 CRC cells (which express a strongly epithelial phenotype), plated in 6 well plates were treated for 72 hours with MRC5 associated

CM^{21} or CM^{SCC}. Cells were then lysed and their proteins fractionated in reducing conditions and examined by western blot for expression of the epithelial and mesenchymal markers.

In this study, DLD1 cells continued to express high levels of the epithelial marker E-Cadherin regardless of whether they had been treated with CM^{21} or CM^{SCC}(figure 3.34). In addition, N-Cadherin expression was absent in both treatment groups (result not shown). This suggests that EMT activation is not a dominant mechanism by which stromal miR-21 overexpression triggers enhanced epithelial invasiveness.

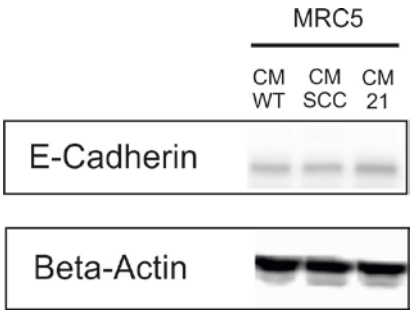


Figure 3.34 Stromal miR-21 deregulation does not provoke EMT in CRC epithelial cells

Representative Western blot of DLD1 colorectal cancer cells treated with MRC5-CM^{21}, CM^{SCC} or CM from non-transfected wild-type control cells (CM^{WT}). Results demonstrate cellular expression of the epithelial marker E-Cadherin. The experiment was repeated twice.

3.3.6 Chemokine and cytokine profiles in the tumour microenvironment are reprogrammed by ectopic miR-21 expression in stromal fibroblasts

One of the most interesting observations so far in this research is how different stromal fibroblast cell lines and even primary fibroblasts from different patients, appear to support CRC cell invasion to a highly variable degree. In figure 3.14 for example, SW480 CRC are barely invasive in 3-D co-culture with PCF6 and PCF9 cells, however in the presence of MRC5 cells they invade well, and invade better still in the presence of HFFF2 and PCF8 fibroblasts. As fibroblasts and CRC epithelial cells are not physically connected in organotypic co-culture, this suggests secreted factors must play an important role.

Interestingly, a recent proteomic analysis identified a pro-oncogenic secretome comprised of MMPs, chemokines and cytokines, produced by cancer associated mammary fibroblasts and regulated by a stromal miRNA (miR-320).[300] In a complementary gene-array study, chemokines were the most highly deregulated genes in fibroblasts transfected with anti-miR-214, a miRNA identified as being significantly downregulated in ovarian tumour stroma compared with normal ovarian stroma.[302]

This novel but expanding research theme resonates strongly with the objectives of the current study. Hence, we used a highly accessible bench-top antibody-based protein array to characterise expression of a panel of cancer relevant chemokines and cytokines in CM from *ex vivo* colonic PCF8 cells transfected with miR-21 or control miR-SCC (figure 3.35).

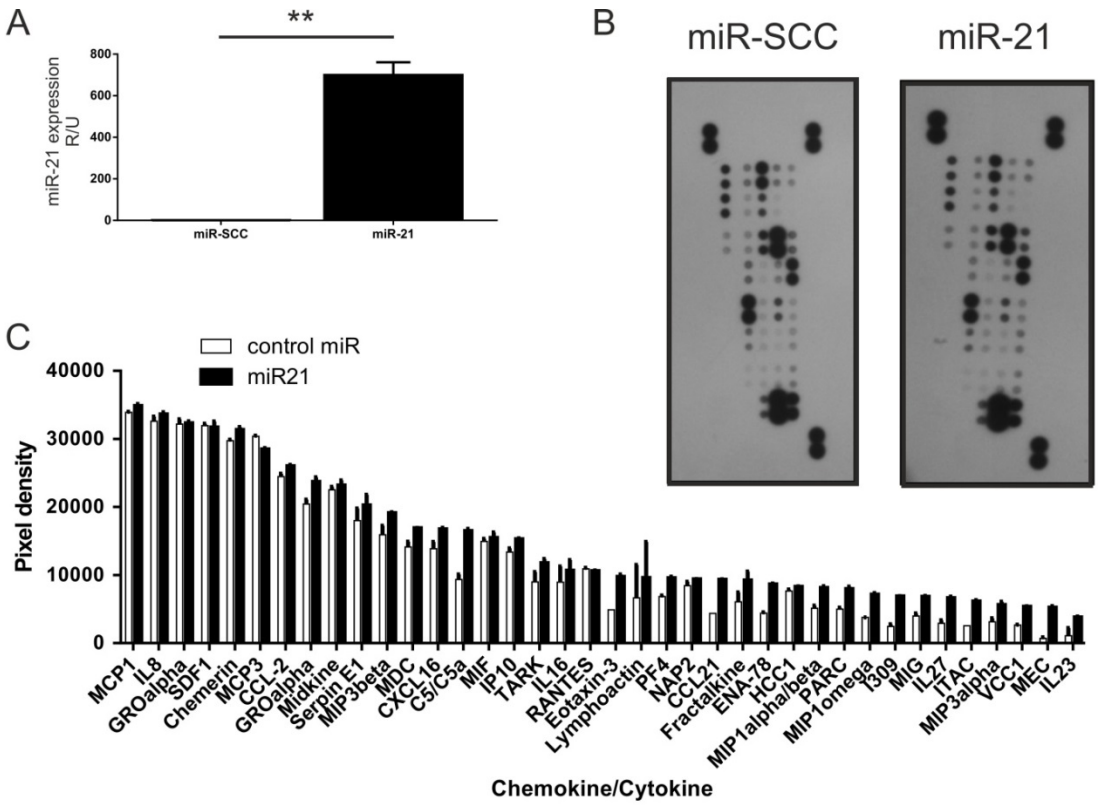


Figure 3.35 Expression of a panel of secreted cancer relevant chemokines and cytokines expressed in conditioned media from miR-21 transfected PCFs compared with miR-SCC control transfected cells

*(A) Transfection of cells with precursor miR-21 using the transfection reagent Oligofectamine™ resulted in approximately 700-fold increase in miR-21 expression. Results represent mean ± s.e.m miR-21 expression by qPCR from a single experiment repeated in triplicate, relative to expression in control miR-SCC transfected cells. **P<0.005*

(B) Expression arrays for chemokines and cytokines in CM from miR-21 transfected cells and control cells. (C) Tabulated results represent mean expression, calculated using an objective visual 'pixel density' score, from an experiment consisting of duplicate technical repeats.

Nine secreted factors were deregulated by a factor >2 in CM from miR-21 transfected fibroblasts compared with control transfected cells (Table 3.1). Among the most

deregulated were CCL28, IL23 and CCL1, all of which are implicated in regulatory T-cell mediated tumour tolerance and angiogenesis pathways.[460-462]

Rank	Secreted factor	Fold change
1	MEC/CCL28	7.4
2	IL23	3.5
3	I-309/CCL1	2.8
4	I-TAC/CXCL11	2.4
5	IL27	2.3
6	6Ckine/CCL21	2.1
7	VCC-1/CXCL17	2.0
8	Eotaxin-3/CCL26	2.0
9	ENA-78/CXCL5	2.0

Table 3.1 Chemokines and Cytokines upregulated by a factor >2 in conditioned media from miR-21 transfected PCF fibroblasts compared with miR-SCC control transfected cells

In order to prioritise the most biologically relevant events, a second proteomic screen was conducted, this time using HFFF2 cells to identify secreted factors under putative miR-21 control in multiple anatomical contexts (figure 3.36).

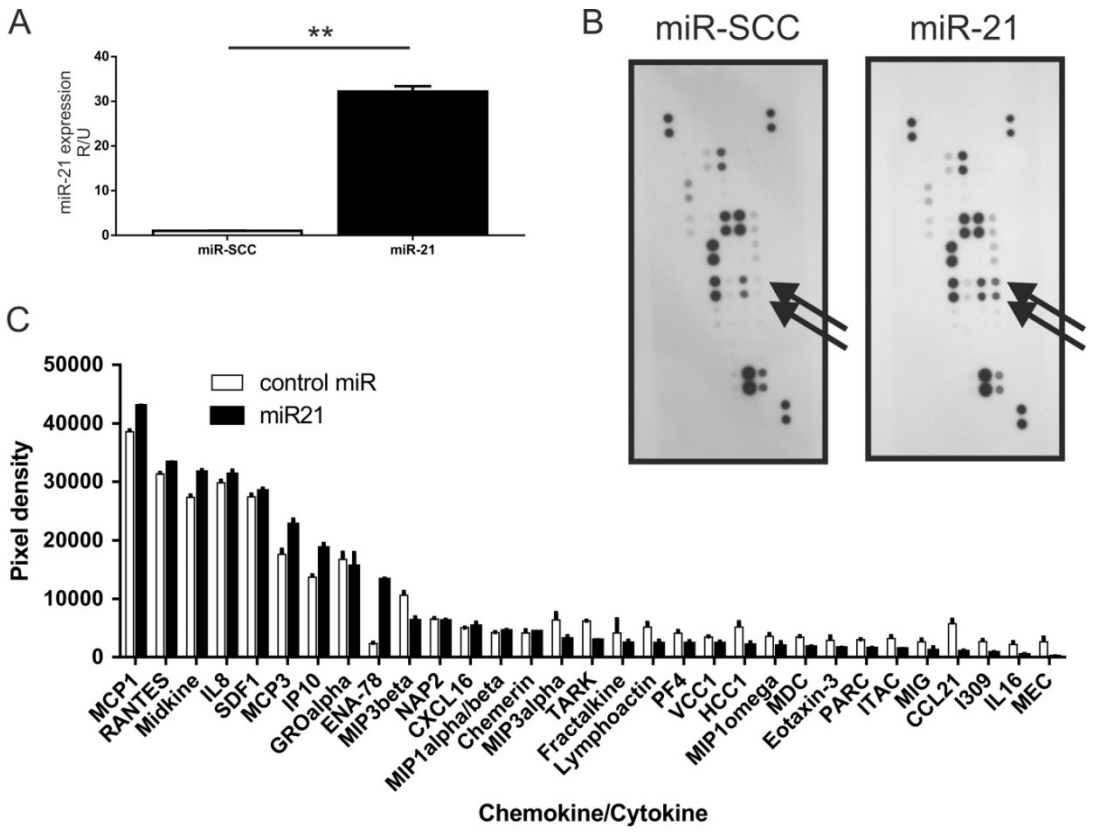


Figure 3.36 Expression of a panel of secreted cancer relevant chemokines and cytokines expressed in CM from miR-21 transfected HFFF2 fibroblasts compared with miR-SCC control transfected cells

*(A) Transfection of cells with precursor miR-21 using the transfection reagent Oligofectamine™ resulted in an approximately 30-fold increase in miR-21 expression. Results represent mean ± s.e.m miR-21 expression by qPCR from a single experiment repeated in triplicate, relative to expression in control transfected miR-SCC cells **P<0.005. (B) Expression of chemokines and cytokines in CM from miR-21 transfected cells and control cells. The grid coordinates corresponding to ENA-78/CXCL5 are indicated by arrows. (C) Tabulated results represent mean expression, calculated using an objective visual 'pixel density' score, from an experiment consisting of duplicate technical repeats.*

Upregulated miR-21 did not appear to impact on the expression of secreted factors in CM from PCF and HFFF2 fibroblasts with a high degree of consistency. This heterogeneous response chimes somewhat with the highly variable impact on CRC cell invasion of different stromal fibroblasts (figure 3.14), and of ectopic miR-21 expression in various fibroblasts in co-culture (figure 3.24 vs. figure 3.25).

However, one chemokine was upregulated more than 2-fold in both miR-21 transfected PCFs and HFFF2 fibroblasts: ENA-78 (epithelial neutrophil activating peptide-78; CXCL5), is a potent chemoattractant and activator of neutrophil function, which acts with considerable homology with IL-8, through the IL-8 receptor system.[463]

Validation by ELISA, using CM from miR-21 transfected fibroblasts confirmed significant upregulation ($P<0.05$) of ENA-78/CXCL5 by a factor of x4.6 and x2.7 in PCF and HFFF2 cells respectively, relative to controls (figure 3.37).

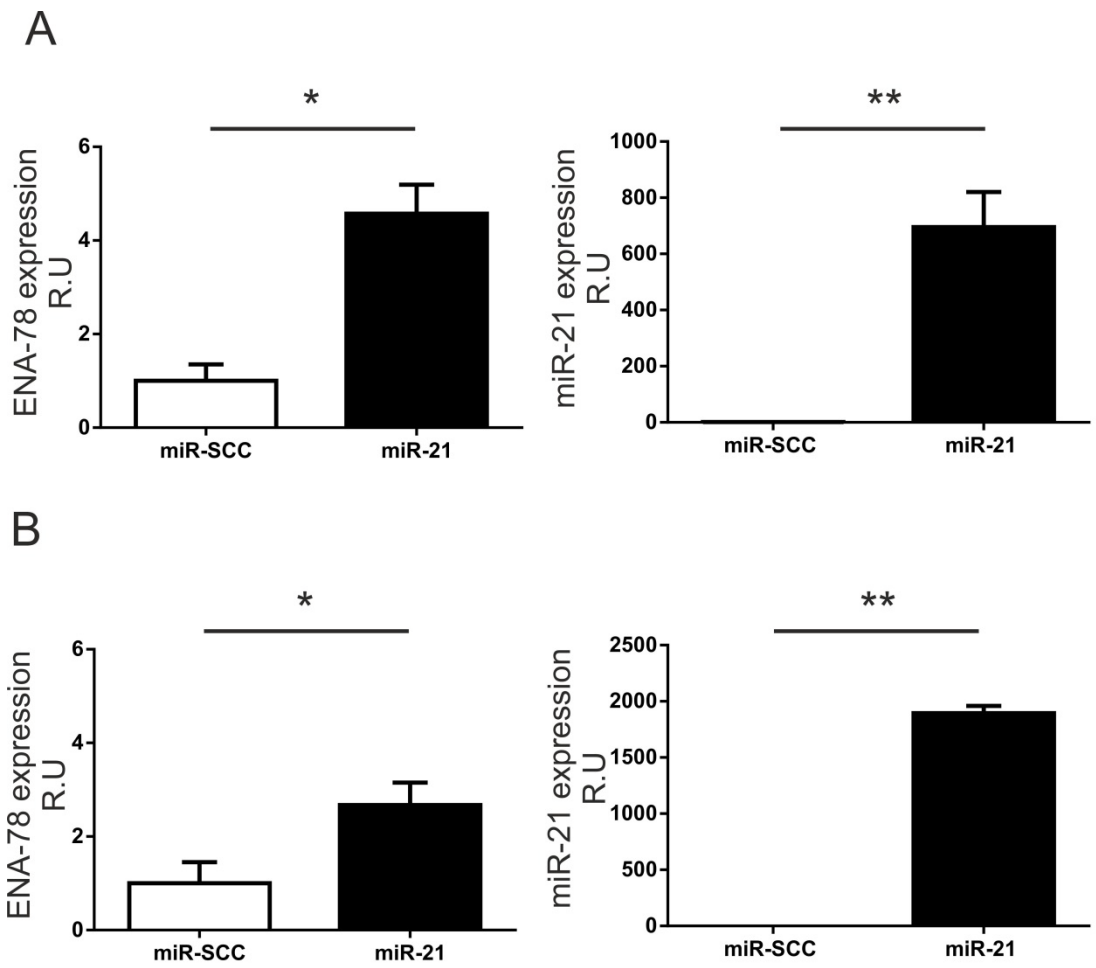


Figure 3.37 ELISA assay for ENA-78/CXCL5 expression in miR-21 transfected fibroblasts

*ENA-78/CXCL5 ELISA assays confirm that ENA-78/CXCL5 protein is overexpressed in CM from miR-21 transfected (A) PCF and (B) HFFF2 fibroblasts compared with control miR-SCC transfected cells. Result represents mean expression from at least 3 repeat experiments. ENA-78/CXCL5 expression is presented as a proportion of expression in the control group. Relative miR-21 expression by Taqman®-qPCR from a representative experimental repeat is also shown *P<0.05; **P≤0.005.*

Together these results suggest that miR-21 upregulation in CRC stroma may reprogram the tumour microenvironment by affecting significant corruption of cancer-associated fibroblast secretory functions. Furthermore, these initial observations provide insight into the biology of permissive stromal-cell to cancer-cell signalling, which may facilitate malignant invasion and metastatic progression *in vivo*.

3.3.7 Results discussion

Recent data from Bronisz and colleagues powerfully demonstrate that downregulated miR-320 expression in mammary stromal fibroblasts activates a pro-oncogenic secretome capable of reprograming the tumour micro-environment to support tumour invasion and angiogenesis.[300] Their analysis of regulatory mechanisms involved in fibroblast differentiation and phenotype, points to a metastasis promoting role for miRNAs in cancer-associated stroma.

To identify stromal miRNAs with putative roles in CRC tumourogenesis and metastasis, our group profiled miRNA expression in LMD CRC specimens using qRT-PCR miRNA micro-arrays. This work broadly corroborated data already published by Nishida *et al*.,[296] which compared miRNA expression in non-case matched tumour and normal stromal specimens and which will be discussed in detail in a later section.

In the current study one of the highest scoring stromal miRNA candidates was miR-21 associated with a 4.0 mean fold increase in the stroma of CRC specimens compared with normal tissue.

Studies of various solid organ tumours have linked elevated miR-21 expression to advancing tumour stage,[311, 337, 346] venous invasion,[346] lymph node and distant metastases,[337, 346] poor disease free survival (DFS)[346, 350] and poor overall survival (OS).[311, 346] In epithelial CRC cell lines, miR-21 correlated inversely with target protein PDCD4 expression and anti-miR-21 reduced invasiveness and capacity to form metastases in a chicken-embryo-metastasis assay.[230]

In order to identify specific miR-21 over-expressing cell types within CRC stroma, ISH was used with highly specific LNA probes. This confirmed that miR-21 overexpression arises in fibroblast-like cells and that crucially, normal tissue and tumour epithelium did not appear to express miR-21 except in sporadically occurring, weakly staining mononuclear cells.

To assess the biological relevance of deregulated miR-21 expression in stromal fibroblasts, over-expression was induced in cultured fibroblast cell lines and primary human fibroblasts. CM extracted from transfected fibroblasts was then used in assays of tumour-cell function:

Results validated across various CRC cell lines demonstrated that treatment with CM from miR-21 transfected fibroblasts is associated with increased proliferation and reduced Oxaliplatin induced apoptosis compared with treatment with CM from scrambled control transfected cells.

This is perhaps unsurprising as miR-21 is a known anti-apoptotic factor; anti-sense inhibition of miR-21 induces apoptosis in cooperation with EGFR-tyrosine kinase inhibitors[440] and enhances sensitivity to radiotherapy and chemotherapy in several tumour contexts.[441, 464, 465] MiR-21 upregulation also enhances proliferation in pancreatic, glioblastoma and colorectal cancer cell lines. [437-439] Crucially however, none of the chemo-resistance or pro-proliferation mechanisms previously described are stroma dependent, and as observations from this study reveal that miR-21 up-regulation occurs exclusively in stromal fibroblasts and not CRC epithelial cells, functional data presented here becomes increasingly relevant.

Yao and colleagues demonstrated that miR-21 is upregulated during TGFβ dependent fibroblast-to-myofibroblast trans-differentiation and that downregulation of miR-21 with specific anti-sense inhibitors prevents trans-differentiation of MRC5 fibroblasts in response to TGFβ.[299]

Myofibroblasts are recognised to be important stromal actors, promoting tumour invasion, proliferation and resistance to chemotherapy, which raised the possibility that the observed functional impact of miR-21 on hallmark processes of tumour progression may be linked to myofibroblast trans-differentiation in the stroma: In this study, changes in cell morphology and increased α-SMA expression, both features of myofibroblast differentiation status, were observed in immortalised MRC5 fibroblasts transfected with miR-21 but not control transfected cells. Although suggestive of myofibroblast differentiation status, in the absence of significant stress fibre deposition, it was essential to conclude that miR-21 overexpression alone is insufficient to drive fibroblast-to-myofibroblast transdifferentiation.

Nevertheless, fibroblasts in which miR-21 had been ectopically induced did share a number of tumour-promoting characteristics with myofibroblasts. Treatment with CM^{21} was associated with increased CRC cell invasiveness in *in vitro* transwell assays and organotypic models. To characterise the mechanism underlying this novel pro-invasive

effect, attention turned to regulators of ECM remodelling known to be targeted by miR-21.

RECK and TIMP3 have been shown to be targeted by miR-21 in glioma models, and as negative regulators of MMP activity, both inhibit tumour invasion.[284] RECK is a membrane bound and TIMP3 an ECM bound regulator of several MMPs including MMP2, MMP9 and MT1-MMP.[448 282, 283]

In this study, miR-21 negatively regulated RECK and TIMP3 in cultured fibroblasts and RECK alone in PCFs.

Both RECK and TIMP3 are known to regulate MMP2 via a number of pre and post transcriptional mechanisms and are involved in other cancer relevant biological pathways (reviewed by Clark JCM *et al.*,[283] and Cruz-Monoz W *et al.*,[282]). In the current study, ectopic miR-21 expression in MRC5 fibroblasts increased MMP2 activity and by using specific inhibitors to show invasion by SW480 CRC cells was MMP2 dependent, a possible mechanism through which upregulated stromal miR-21 expression increases invasion *in vitro* was identified.

In cardiac fibroblasts miR-21 induced MMP2 expression is associated with a PTEN dependent mechanism,[466] and although PTEN is also a miR-21 target, focus remained on this alternative mechanism because *in vivo* MMP2 and RECK expression are inversely correlated and have prognostic relevance in CRC.[467]

It is also of significance, that TIMP3 and RECK do not only impact upon MMP activity, but also play various roles in other cancer relevant biological pathways: TIMP3 overexpression in CRC cells results in a serum-dependent decrease in proliferation, *in vivo* loss of tumourogenicity,[468] and increased apoptosis,[469] and both RECK and TIMP3 inhibit angiogenesis and tumour growth in other malignancies.[283, 470]

As RECK is downregulated in the majority of CRC specimens compared with normal tissue and low expression is associated with poor outcome,[447] it may be of further prognostic value, to study the balance of miR-21 and RECK expression human CRC specimens. Equally, stromal TIMP3 expression is an independent predictor of outcome in CRC,[471] and as such, examining the correlation with miR-21 expression in CRC

tissue may identify a clinical benefit as well as providing insight into the true nature of their interaction *in vivo*.

To identify further mechanisms through which deregulated stromal miR-21 expression may impact on CRC cell function, the protein content of CM from miR-21 transfected fibroblasts was examined using an antibody based profiling array. Significant differences were observed between miR-21 transfected and miR-SCC control transfected cells, including upregulated expression of ENA-78/CXCL5.

ENA-78/CXCL5 is a pro-inflammatory cytokine, which stimulates angiogenesis, malignant invasion, cancer cell proliferation and metastatic progression in various tumour contexts.[472-476] In CRC, high serum ENA-78/CXCL5 is also associated with liver metastasis and poor overall survival.[476]

Here, ENA-78/CXCL5 was significantly upregulated by miR-21 both in PCF and HFFF2 fibroblasts and as such, may provide a link between miRNAs, the stromal inflammatory response and CRC progression.

To identify the relevance of these findings in the clinical context requires further mechanistic interrogation and corroborative study however; two prior studies have examined this theme in some depth. Mitra and colleagues identified deregulated miRNAs in stromal fibroblasts which impacted the protein composition of the tumour microenvironment in ovarian cancer. They demonstrated that miR-214 was significantly downregulated in tumour associated fibroblasts compared with paired normal fibroblasts, and that miR-214 exerted direct transcriptional control over expression of the chemokine RANTES/CCL5. They further demonstrated that RANTES/CCL5 contributed to tumour-cell recruitment and growth in nude-mouse models.[302]

Furthermore, in a seminal paper, Calon and colleagues demonstrated that IL-11 produced by TGFβ treated CAFs promoted metastatic progression in colon cancer. Although not necessarily miRNA dependent, the mechanism described potentially resolves the paradox by which TGFβ-driven tumour progression may occur despite mutational inactivation of TGFβ signalling in colon cancer cells.[134]

In the current study however, IL-11 was not present on the antibody based protein array utilised, and although represented, RANTES/CCL5 expression did not respond to miR-21 induction in either PCF or HFFF2 fibroblast lines.

Nevertheless, pending successful validation, several other interesting candidate chemokine/cytokines under putative miR-21 control were identified and will be the focus of future interrogation. In the first instance, MEC/CCL28 expression will be validated using ELISA methodology in a panel of fibroblasts in which ectopic miR-21 has been induced.

MEC/CCL28 is interesting not only because it is the chemokine most upregulated by ectopic miR-21 expression in primary fibroblasts, but also because it is a recognised pro-angiogenic factor known to corrupt the protective immune response to cancer: In ovarian cancer cells for example, MEC/CCL28 release is triggered by hypoxia and leads in-turn to greater recruitment of tumour-protective regulatory T-cells.[460] However in CRC, immunohistochemistry studies have identified that MEC/CCL28 expression in colonic epithelium is diminished during the transition to adenocarcinoma.[477] Focusing of the stromal contribution of MEC/CCL28 expression in CRC may therefore provide insight to help resolve this apparent discrepancy.

For ENA-78/CXCL5 a series of further experiments are also planned, to study the relationship with miR-21 expression in greater detail. However, further proteomic screening studies will be conducted ahead of this, using CM from fibroblasts transfected with antimiR-21 rather than pre-miR-21. This approach combined with *in silico* analysis has the advantage of potentially identifying direct miR-21 chemokine/cytokine targets which may impact on the capacity for CRC cells to invade and progress through the metastatic cascade. From this amalgamated data-set it will be possible to better prioritise the order in which chemokine/cytokine targets are studied.

In summary, these data demonstrate that deregulation of the key oncomiR miR-21 is a stromal phenomenon in CRC. Ectopic expression of miR-21 in fibroblasts modulates the cytotoxic impact of Oxaliplatin, promotes CRC proliferation and enhances malignant invasion by downregulating RECK and TIMP3 expression and increasing MMP2 activity. This novel, fibroblast mediated mechanism may be linked to the profound phenotypic alterations, and changed secretory functions potentiated by miR-21

overexpression and associated with myofibroblast transdifferentiation. This highlights the importance of the stroma in CRC progression and presents an interesting perspective for the development of new, stroma-targeted drugs.

A description of the major findings presented in this chapter is provided in figure 3.38.

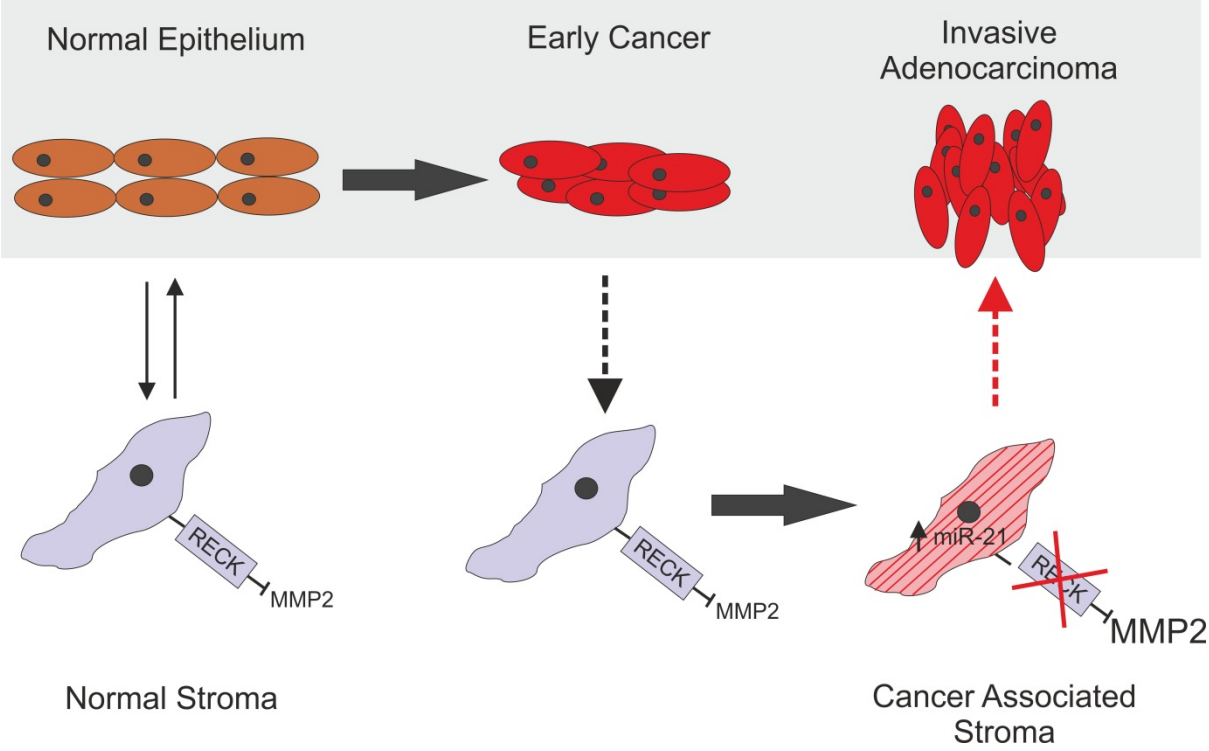


Figure 3.38 Proposed impact of deregulated miR-21 expression in CRC-associated stroma

Reciprocal signalling between stromal and epithelial cells (thin black arrows; left panel) is essential for normal tissue homeostasis. Early in cancer, this process is corrupted and secreted factors from transformed epithelial cells (dotted black line; central panel) initiate profound changes in adjacent stromal cells. Upregulated miR-21 expression in stromal fibroblasts supports TGFβ dependent fibroblast-to-myofibroblast transdifferentiation (thick black arrow) and causes downregulated expression of RECK and possibly TIMP3. CAF-derived factors are important mediators of tumour progression (dotted red arrow; right panel), and although in the absence of TGFβ, miR-21 is not sufficient to initiate myofibroblast transdifferentiation, upregulated miR-21 does promote tumour proliferation and chemoresitance; enhancing tumour invasion though upregulated MMP2 activity but not through EMT trigger pathways.

3.4 The impact of deregulated stromal miR-21 expression *in vivo*

In previous chapters, the functional and mechanistic impact of deregulated miRNAs during CRC progression was examined. However, a key question remains; whether candidate miRNAs promote metastasis *in vivo.*

Numerous *in vivo* tumour models have previously been described, including carcinogen induced and transgenic varieties however, for the study of CRC progression the tumour implantation model is generally preferred.[478] Usually, this involves implanting CRC cell lines, or *ex vivo* colonic tissue xenografts subcutaneously into recipient mice. To prevent xenograft rejection immunocompromised mice are used either in the form of nude mice, which are athymic *Foxn1*^{nu} (winged-helix/forkhead transcription factor) homozygous gene knockouts incapable of generating T-lymphocytes[479]; or SCID mice which carry a *Prkdc* (protein kinse, DNA activated catalytic polypeptide) mutation which leads to disrupted T and B cell maturation processes.[480]

Subcutaneous xenograft tumour implantation provides an inexpensive, flexible, and highly predictable experimental system.[478] However, a major disadvantage to this approach is that the subcutaneous microenvironment differs significantly from that of the colon and as a consequence, spontaneous CRC metastases rarely arise.

In order therefore to recreate physiologically meaningful interaction between CRC cells and surrounding tissues, and more predictably induce metastases, the development of orthotopic models was essential.[481-483] In recent times, the capacity to generate orthotopic mouse CRC tumour models has been achieved within the Biomedical Research Facility at the University of Southampton. It was therefore possible to investigate whether stromal miR-21 promotes metastasis *in vivo,* by co-implanting SW620 CRC cells and HFFF2 fibroblasts (stably transfected with a miR-21 or scrambled control vector) directly into the caecum of SCID mice at laparotomy.

As we see in figure 3.39, significantly greater primary colonic and metastatic liver tumours were observed compared with controls, with the majority of liver tissue replaced by metastatic deposits in the miR-21 transfected group. These data further supports a pro-metastatic function of miR-21 in CRC stroma.

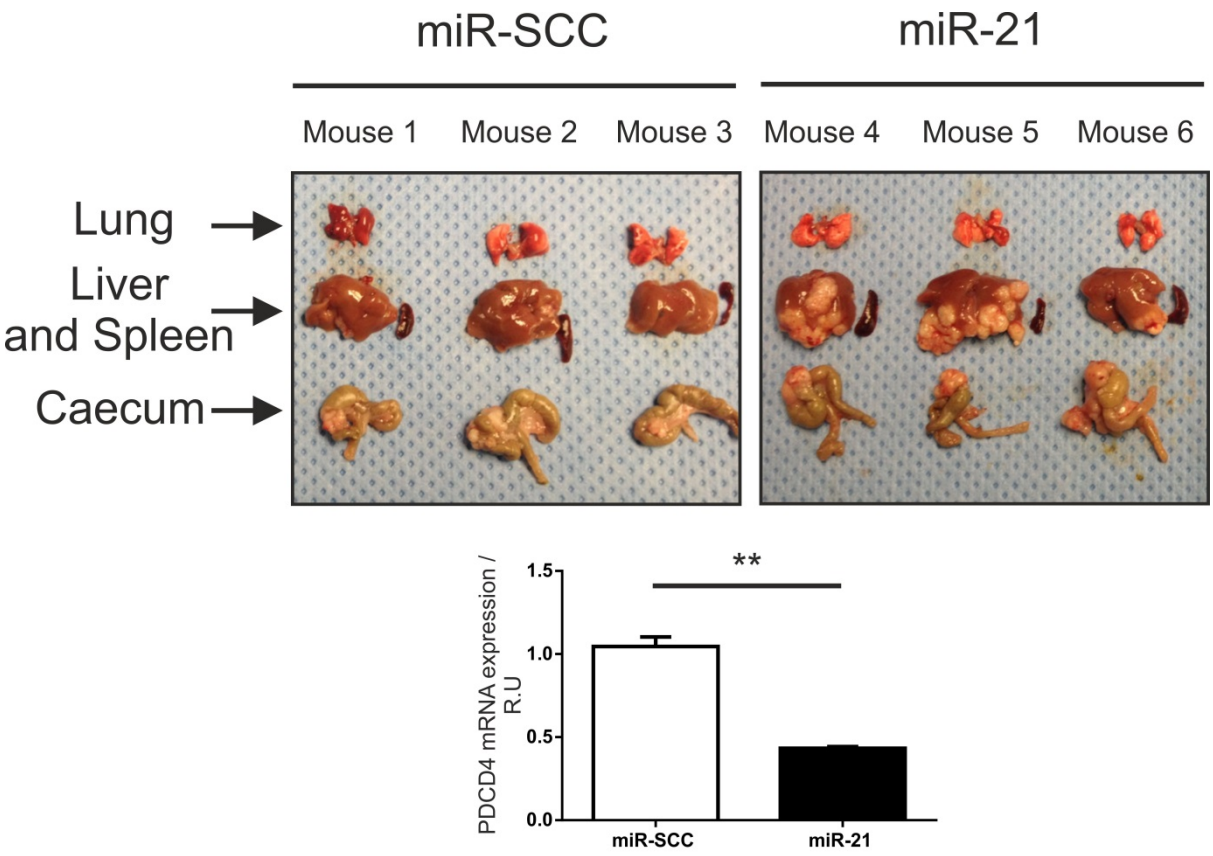


Figure 3.39 Stromal miR-21 promotes CRC growth and metastasis in vivo

*Orthotopic co-injections of SW620 CRC cells with HFFF2 fibroblasts stably transfected with miR-21 or control miR-SCC ($1x10^6$ SW620 vs. $5x10^6$ HFFF2). Visual assessment clearly shows greater caecal tumour formation and metastatic replacement to liver relative to controls. PDCD4 suppression shown by Taqman® qPCR at the time of tumour implantation serves as a proxy measure of relative miR-21expression between miR-21 and miR-SCC control transfected HFFF2 cells **P<0.005.*

As no prior research was identified in the literature to provide methodological guidance, HFFF2 fibroblasts were selected empirically for this study as they best supported invasion in organotypic CRC models. SW480 cells were the most invasive CRC cell line in organotypic culture and they have been used in murine xenograft models in the past[484] however; SW620 CRC cells were preferred, because their use had already been optimised by the group in the context of our novel orthotopic system.

Here, *in vivo* experiments were repeated twice. Tumours are currently being assessed for size, stromal response, and stromal, muscular and vascular invasion. Liver and lungs will be dissected and subjected to histological evaluation to determine the number of pulmonary deposits and the degree of liver replacement.

Although this work is ongoing, it holds sufficient promise to merit examination of the impact of stromal miR-21 antagonism on the ability of CRC cells to metastasise *in vivo*.

In preparation, the impact on SW620 CRC cell invasion of miR-21 downregulation in HFFF2 fibroblasts was examined *in vitro* using an anti-sense miR-21 inhibitor. Crucially, CM from antimiR-21 transfected fibroblasts induced significantly less invasion in transwell assays than CM from miR-SCC transfected controls (figure 3.40).

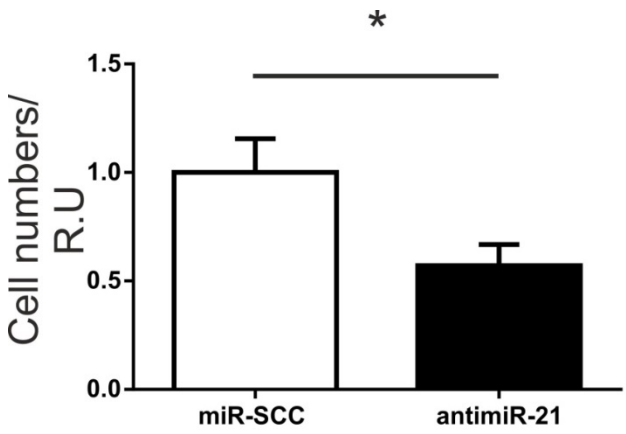


Figure 3.40 Anti-miR-21 expression in stromal fibroblasts inhibits SW620 CRC cell invasion in vitro

*Transwell invasion assay shows mean ± s.e.m numbers of invading SW620 CRC cells in CM from miR-SCC control and miR-21 transfected fibroblasts expressed as a ratio. Results represent a single triplicate repeat experiment *P<0.05. HFFF2 cells were transiently transfected with antimiR-21 and control miR-SCC from Dharmacon® using the transfection reagent Oligofectamine™.*

3.4.1 Results discussion

Murine models of cancer progression are an increasingly important tool in miRNA research. In breast cancer, orthotopic tumour implantation involves subcutaneous injection of cancer cells into the anterior chest wall fat-pads of mice. Early studies demonstrated significantly increased metastatic potential of breast cancer cells transfected with miR-9 and miR-10b,[255, 408] and more recently miR-30a and miR-19a-3p have been shown to inhibit breast cancer growth and metastasis using this methodology.[485, 486]

In CRC, subcutaneous implantation of tumour xenografts, along with inducible and transgenic CRC models are experimentally useful however; heterotopic tumour growth and lack of spontaneous metastasis compromises their physiological relevance.[214, 436, 487, 488]

The biological integrity of murine CRC xenograft models may be improved by co-implanting cancer cells with stromal elements such as fibroblasts to better mimic the *in vivo* tumour microenvironment.[300, 489-491] Furthermore, malignant cells may be injected orthotopically, directly into the caecum of mice; a technique which is being used with increasing frequency.[492-495] However, to our knowledge, data presented here is the first example of cancer cell and fibroblast co-implantation in orthotopic murine models of CRC, and the first documented use *in vivo* of miRNA manipulation in the context of CRC stroma.

In addition, a powerful visual demonstration of the impact of deregulated stromal miR-21 is provided, as a dramatic increase in metastatic tumour burden is seen with orthotopically implanted SW620 CRC cells in the presence of miR-21 transfected fibroblasts compared with controls.

A further recent development in miRNA research is the use of orthotopic nude-mouse tumour models to evaluate the therapeutic potential of inhibitory miRNA antagomiRs.[375, 496]

In the current study, the functional impact on CRC cells of miR-21 inhibition in fibroblasts was examined in transwell assays, and notably the invasive capacity of SW620 cells was significantly impaired relative to controls. Unfortunately, the

unmodified commercial antagomiRs used in this experiment degrade very quickly after systemic administration, and may not be appropriate for use *in vivo* in murine metastasis models.[497]

An alternative is to use 'locked nucleic acid' (LNA) miRNA analogues with a phosphorothiolate backbone in which the ribose ring is 'locked' by a methylene bridge. This makes subsequent Watson-Crick base-paring with complementary oligonucleotides highly stable and resistant to degradation.[498]

For this purpose, I am collaborating with Dr Jon Watts and Dr Ali Tavassoli at the Department of Chemistry at the University of Southampton to develop antimiR-21 and miR-SCC scrambled control LNA oligonucleotides.

This will enable future studies designed to examine the functional impact of miR-21 antagonism in stromal fibroblasts on CRC metastasis *in vitro* and *in vivo*, forming the basis of a nascent research theme to evaluate the potential therapeutic utility of antimiR-21 LNA oligonucleotides which target the CRC stroma.

3.5 Identification and validation of novel stromal and epithelial microRNA candidates relevant during CRC progression

Epithelial miR-153 and miR-224 and stromal miR-21 were identified in a comparison of LMD CRC tissue and paired normal colonic tissue using a QuantimiR™-qPCR profiling array. Crucially, this approach was designed to uncover with high precision miRNAs involved in tumourogenesis, rather than to offer specific insight into the biology of CRC progression. Furthermore, the QuantimiR™ platform, although highly sensitive and specific, is comprised of only 95 cancer-relevant miRNA primer-probes.

To address this issue, and gain a deeper understanding of miRNA mediated effects in CRC progression, miRNA profiling was broadened to encompass miRnome-wide gene expression alterations using the 7th generation miRCURY™ LNA high-throughput microarray (figure 3.41). Secondly, a further examination of deregulated miRNA expression was conducted at various clinically relevant stages of CRC progression.

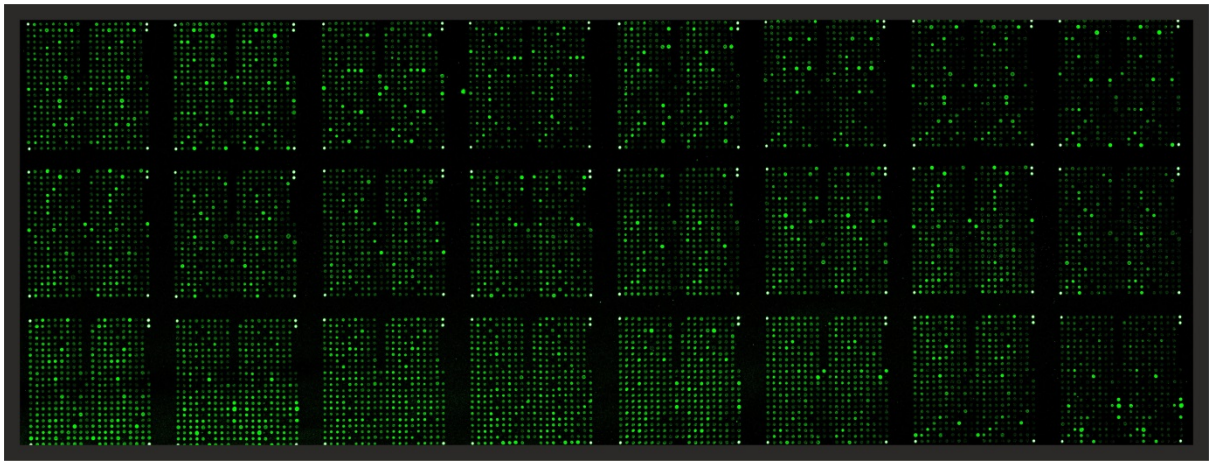


Figure 3.41 Representative image of the miRCURY™ LNA miRNA screening array containing >1500 human miRNAs capture probes

3.5.1 The relevance of stromal and epithelial microRNAs differentially expressed in stage-II CRC with and without metastasis at 5 years

To identify stromal and epithelial miRNAs with specific relevance during the metastatic cascade, 50 consecutive and formalin fixed paraffin embedded (FFPE) CRC specimens were obtained from tissue archives at University Hospital Southampton. This cohort comprised 25 patients with stage II (nodal/visceral metastasis negative) disease who subsequently developed metastasis within 5 years and 25 patients with stage II disease who remained metastasis free in that time (Demographic data in Material and Methods; Table 2.3). Pathological verification of diagnosis and staging was in accordance with the Association of Coloproctology of Great Britain and Ireland guidelines on the management of CRC.[401]

3.5.1.1 MiRnome-wide gene expression analysis in LMD epithelial and stromal tissue from stage II CRC specimens with and without metastasis at 5 years

From this cohort of 50 patients with stage II disease, 20 carefully matched patients with (stage II-R) and without recurrence (stage II-NR), were selected for miRNA array based gene expression analysis (Demographic data in Material and Methods; Table 2.4). Each FFPE CRC specimen was subjected to LMD and total-RNA was extracted separately from epithelial and stromal tumour strata. Global epithelial and stromal miRNA expression profiling was conducted using the miRCURY™ LNA screening platform. Data was sorted by fold change and p values >0.05 were excluded from further analysis. Data from recurrence and non-recurrence groups was then compared to yield differential expression data.

In total, 95 miRNAs were significantly differentially expressed in stage II-R CRC stroma compared with stage II-NR stroma (figure 3.42), of which 10 were up- or down-regulated by a factor >2. In contrast, 63 epithelial miRNAs were significantly differentially expressed (figure 3.43); 6 by a factor >2.

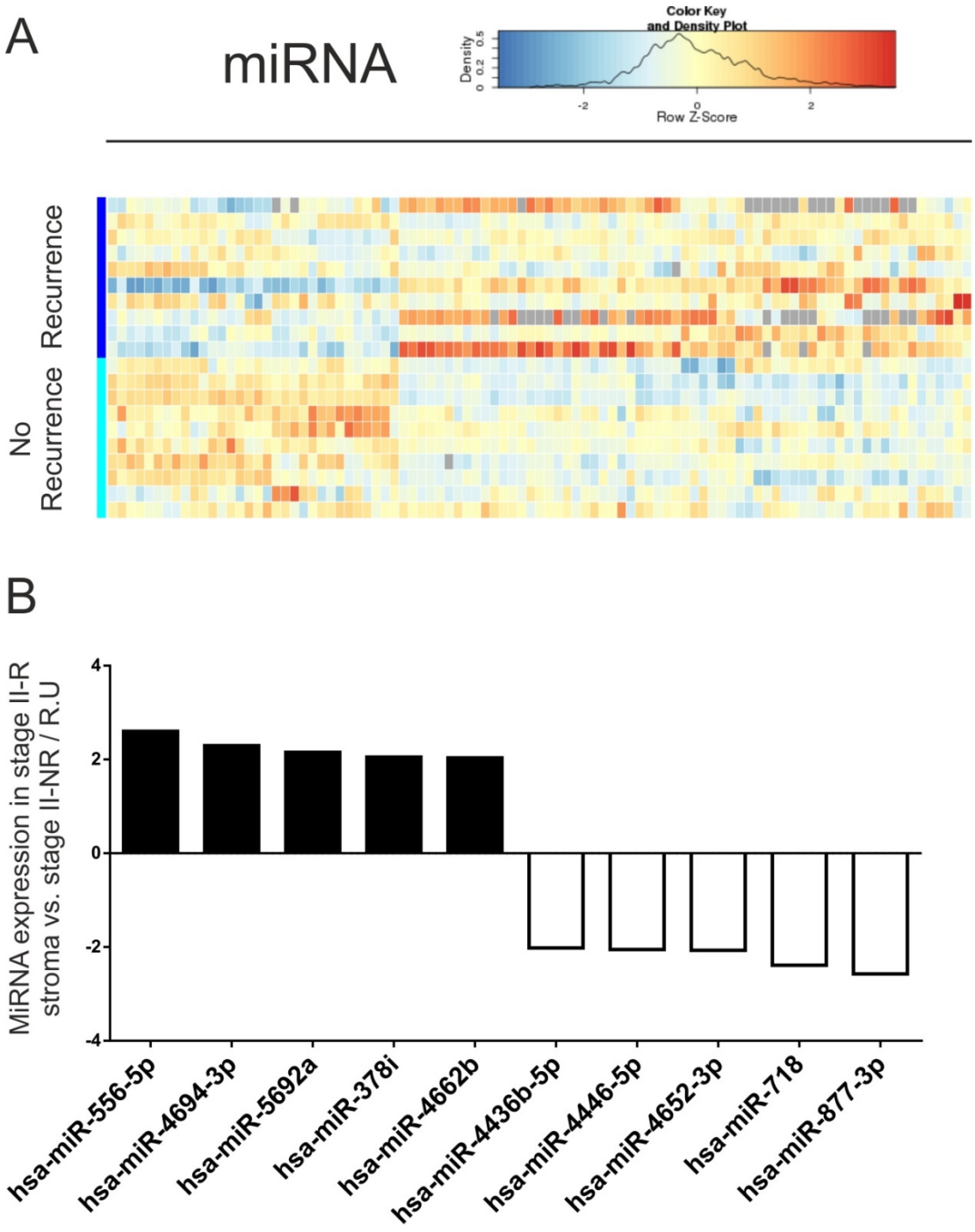


Figure 3.42 Stromal miRNA expression in stage II CRC specimens with and without recurrence at 5 years

MiRnome-wide, high through-put miRNA screening; supervised cluster analysis: (A) Heatmap of miRNAs differentially expressed (adjusted p<0.05) in LMD stroma between stage II CRC specimens with and without recurrence within 5 years (stage II-R CRC vs. stage II-NR). (B) 10 miRNAs were deregulated by a factor >2 (P<0.05).

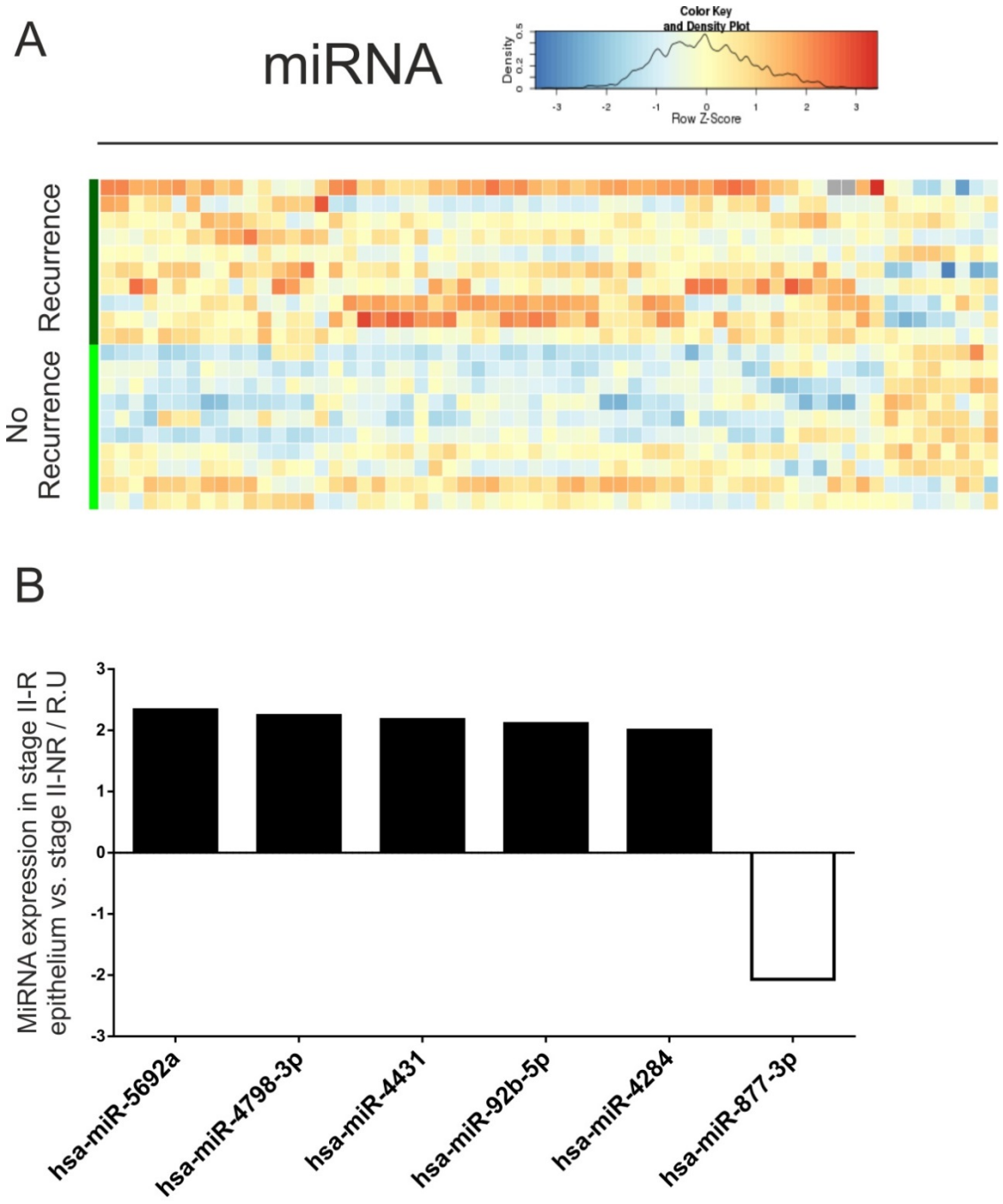


Figure 3.43 Epithelial miRNA expression in stage II CRC specimens with and without recurrence at 5 years

MiRnome-wide, high through-put miRNA screening; supervised cluster analysis: (A) Heatmap of miRNAs differentially expressed (adjusted p<0.05) in LMD epithelium between stage II CRC specimens with and without recurrence within 5 years (stage II-R vs. stage II-NR). (B) 6 miRNAs were deregulated by a factor >2 (P<0.05).

Due to constrained resources, only a selection of these candidate miRNAs were validated using the more sensitive and specific Taqman® qPCR technique, in all 50 stage II CRC specimens (figure 3.44). Epithelial miR-92b was selected for validation as it forms part of the oncogenic miR-17-92 cluster which has been heavily investigated and is implicated in cardiovascular, neurodegenerative and immune diseases as well as tumourogenesis.[499] MiR-556 was the most highly deregulated stromal miRNA in our screen and miR-718 has recently emerged as tumour suppressor gene in ovarian cancer.[500] Interestingly, miR-718 was also the most downregulated miRNA in blood samples in a recent study comparing patients with early breast cancer and matched normal control subjects.[501]

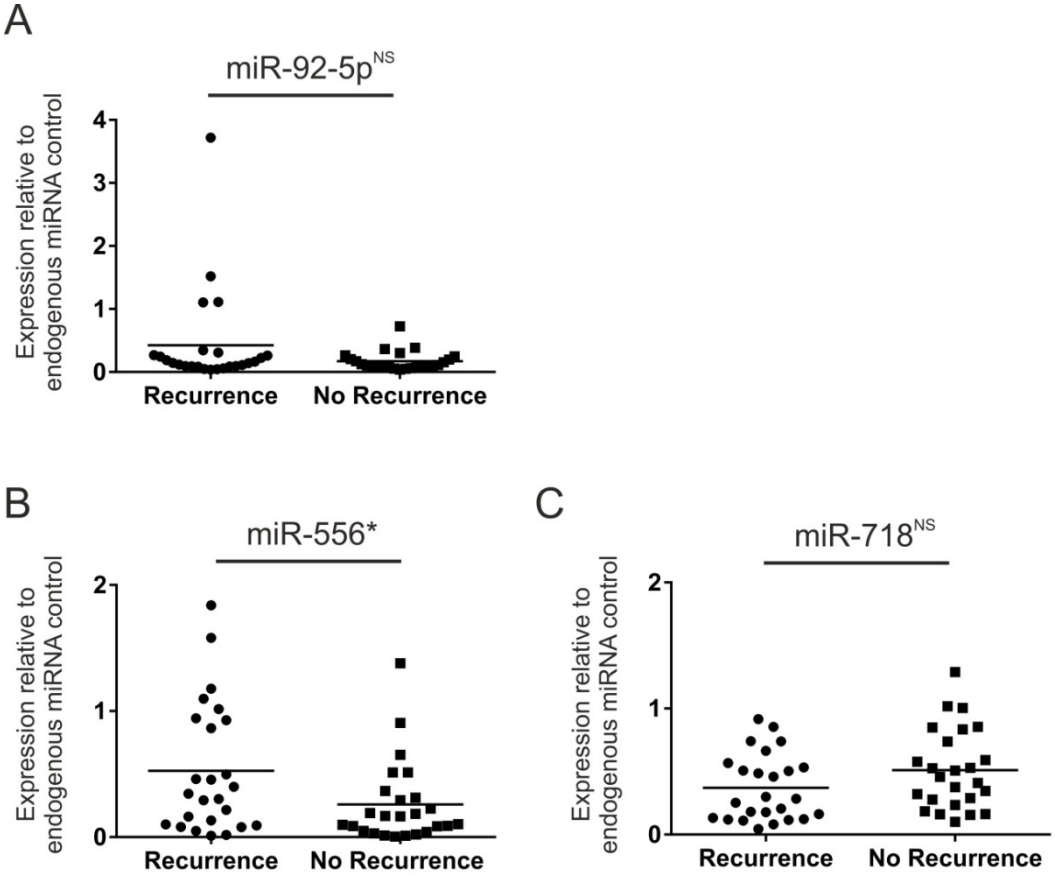


Figure 3.44 Taqman® qPCR analysis of miRNAs expressed in the epithelium and stroma of stage II CRC specimens with and without metastasis at 5 years

(A) Mean epithelial miR-92b expression was x2.5-fold greater in stage II-R vs. stage II-NR CRC specimens. (B) miR-556 and (C) miR-718 were x2.02-fold upregulated and

*x1.38-fold downregulated respectively in the stroma of stage II-R vs. stage II-NR CRC specimens. NS=Not Significant; *P<0.05*

Although differentially expressed in Stage II-R and Stage II-NR CRC specimens, the analysis of miR-92b and miR-718 by Taqman® qPCR did not attain statistical significance, raising questions about the sensitivity and specificity of high-throughput profiling of LMD tissue in our hands. Crucially however, miR-556 the most upregulated stromal miRNA in our screen was successfully validated by qPCR and was >2-fold overexpressed in CRC specimens from patients who subsequently developed metastasis compared to those who did not.

3.5.1.2 The role of deregulated miR-556 in the CRC stroma during the metastatic cascade – In silico analysis of putative function and molecular targets

To our knowledge, the pathological relevance of miR-556 has not previously been explored in any study to date.

To identify putative miR-556 targets, *in silico* mRNA analysis was conducted using TargetScan (http://www.targetscan.org/; Release 6.0) software. 128 mRNA transcripts with a total of 130 highly conserved and 28 poorly conserved 3'UTR target sites were identified.

The Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.7) was used to evaluate enrichment of biological processes associated with putative mRNA targets, against a background of all human genes. Clustered and non-clustered analysis showed that based on a p value cut-off of 0.001, 43 biological processes were mildly enriched for miR-556, including processes involved in metal ion binding (enrichment score 2.07), the regulation of transcription (enrichment score 1.75), and cell migration and locomotion (enrichment score 0.73).

Further analysis for miR-556 using Panther, BioCarta, KEGG and Reactome annotation tools at default settings, demonstrated minor enrichment of genes associated with G-protein signaling (1.7% enrichment P<0.05), but no other significant biological themes were identified.

Taken together, these data suggest that although miR-556 is significantly upregulated in the stroma of stage II CRCs which go on to metastasise compared with those which do not; it may not necessarily play an important biological role in the stromal response to CRC, or the regulation of metastatic progression. However, it is important to note the limitations of predictive data of this sort. *In silico* analysis is useful to prioritise the importance of miRNA candidates with putative biological function however; until formal functional studies have been concluded, the true relevance of deregulated stromal miR-556 expression during CRC progression will remain unclear.

3.5.2 Molecular profiling of the invasive tumour microenvironment in a 3- dimensional model of CRC cells and ex vivo fibroblasts

In the previous section, LMD tissue from matched metastatic and non-metastatic primary CRC specimens was used in a molecular profiling study to identify miRNAs with specific relevance during the metastatic cascade. In the following section a novel alternative approach is described which combines LMD, organotypic CRC models and high-throughput miRNA profiling.

3.5.2.1 Profiling the invasive tumour margin in CRC organotypics - A pilot study

Organotypics are a versatile platform to study interactions between tumour and stromal cells and are increasingly used to examine the impact on tumour invasion of chemical inhibitors and targeted gene alterations.[235, 423, 502] Studying the invasive tumour margin is a particularly exciting prospect. In organotypic models, established CRC cell lines typically produce a well stratified epithelial layer and in cross section cells which have acquired the capacity to invade the ECM are easily discernible. Extracting these cells using LMD and studying them in isolation, may reveal important biological insights regarding the origins of colorectal cancer metastasis and how it may be more efficiently targeted.

For this purpose, a feasibility study was designed using organotypic co-culture models comprised of an epithelial cell layer containing SW480 CRC cells, and a stromal layer comprised of MRC5 fibroblasts, Type I collagen and important ECM components (figure 3.45).

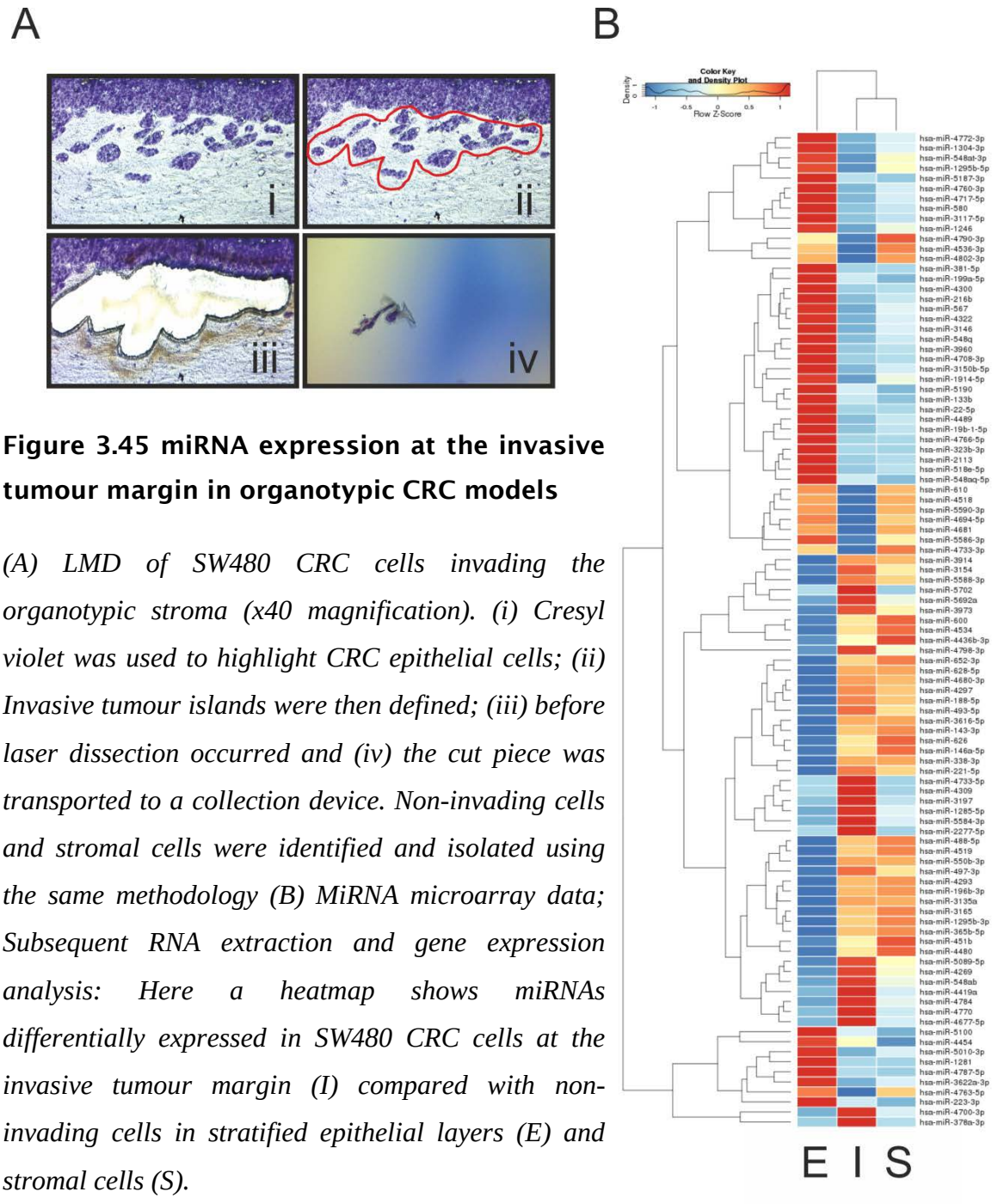


Figure 3.45 miRNA expression at the invasive tumour margin in organotypic CRC models

(A) LMD of SW480 CRC cells invading the organotypic stroma (x40 magnification). (i) Cresyl violet was used to highlight CRC epithelial cells; (ii) Invasive tumour islands were then defined; (iii) before laser dissection occurred and (iv) the cut piece was transported to a collection device. Non-invading cells and stromal cells were identified and isolated using the same methodology (B) MiRNA microarray data; Subsequent RNA extraction and gene expression analysis: Here a heatmap shows miRNAs differentially expressed in SW480 CRC cells at the invasive tumour margin (I) compared with non-invading cells in stratified epithelial layers (E) and stromal cells (S).

This preliminary comparison of differential gene expression using the MiRCURY® miRNA hybridisation array, suggests that significant differences may exist between CRC cells which have acquired the capacity to invade compared with those which have not.

3.5.2.2 Identifying conserved patterns of deregulated miRNA expression in vitro at the invasive tumour margin in different anatomical contexts within the gastrointestinal tract

As the pilot study consisted of only a single technical repeat, further experimental validation was required. However, as can be seen in figure 3.45, the only other CRC organotypic potentially suitable for this purpose was the SW620/PCF8 model, which displayed far less invasion than the equivalent SW480/PCF8 co-culture.

An alternative solution was to include organotypic models containing primary cells from other anatomical regions within the gastrointestinal tract. This approach would have the advantage of potentially uncovering candidate miRNAs consistently deregulated at the invasive tumour margin in different tumour contexts.

For this purpose, pancreatic cancer organotypics containing CAPAN1 or BXPC3 pancreatic cancer cell lines and primary pancreatic stellate cells were acquired as a kind gift from Dr Jo Tod at the University of Southampton.

As for the pilot study, LMD was conducted to separate invading cancer cells from non-invading cells and stromal cells. Identical screening analysis for miRNA expression was subsequently performed using the miRCURY™ LNA 7^{th} generation microRNA microarray platform, to identify conserved biological themes across all four tumour models (figure 3.46).

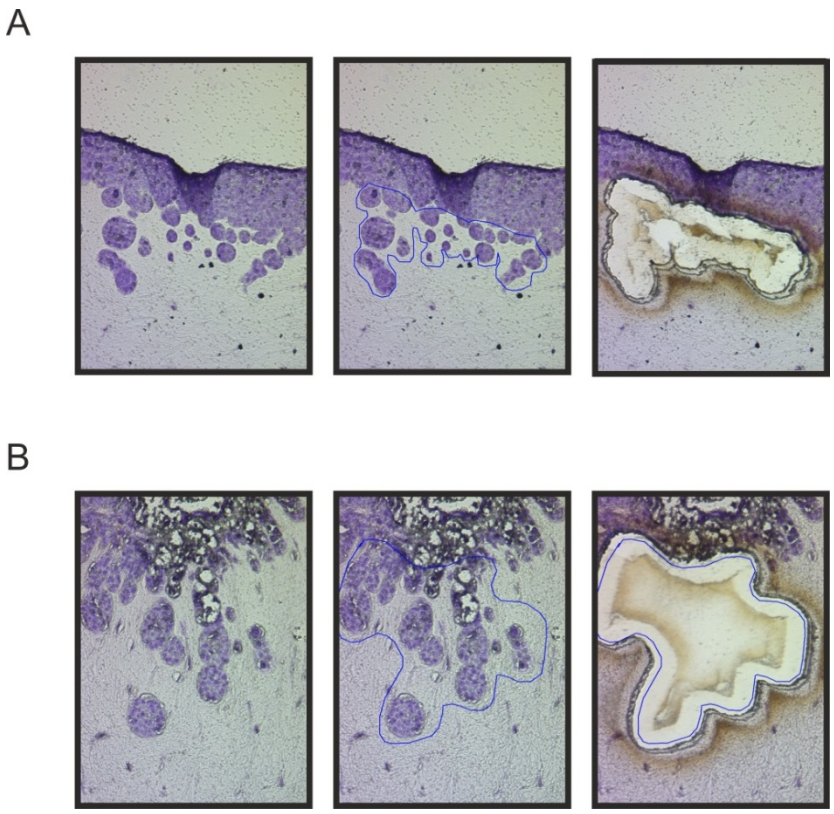


Figure 3.46 Representative images of LMD conducted with colorectal and pancreatic cancer organotypic models

(A) CRC organotypics comprised of SW480 CRC epithelial cells and PCF8 fibroblasts in the stroma and (B) pancreatic cancer organotypics comprised of BXPC3 pancreatic cancer epithelial cells and primary pancreatic stellate cells in the stroma (x10 magnification).

Analysis, summarised in figure 3.47 and Table 3.2 is focused on differential gene expression in invading vs. non-invading cancer epithelial cells. In total 19 miRNAs were significantly deregulated at the invasive tumour margin in both SW480 and SW620 CRC organotypics, compared with 7 miRNAs consistently deregulated in both pancreatic tumour models. Furthermore, 40 deregulated miRNAs common to 2 cancer scenarios were identified; 9 common to 3 cancer scenarios; and 2 common to all 4 cancer scenarios.

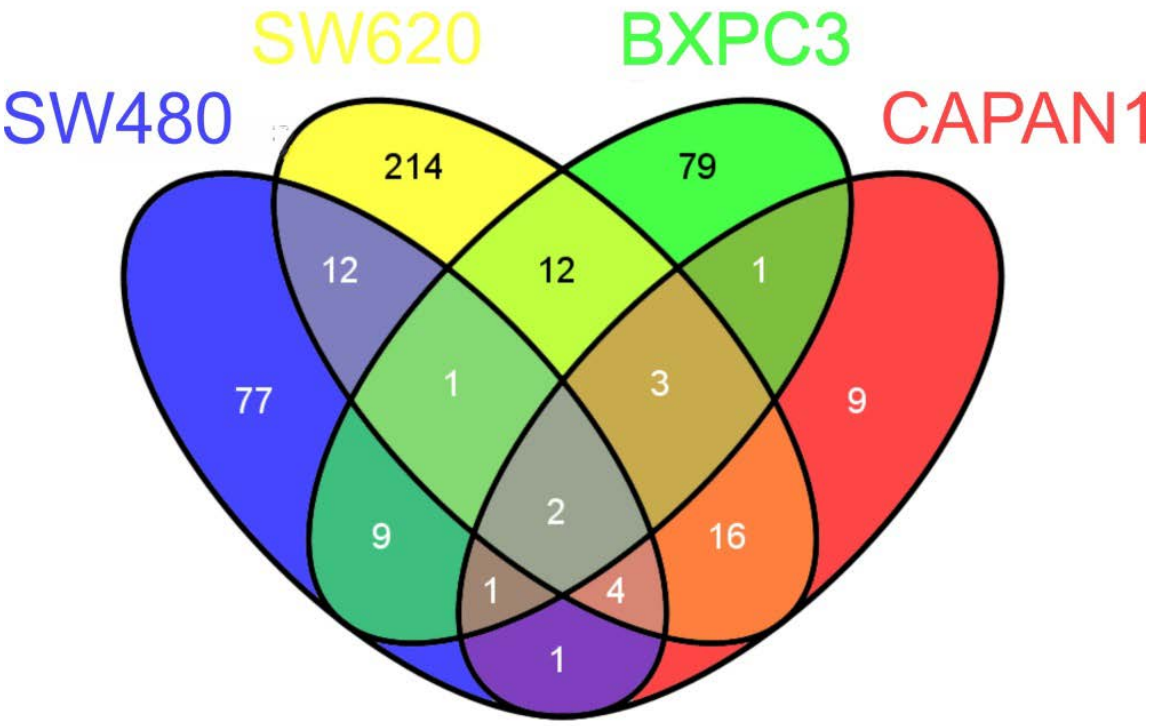


Figure 3.47 Conserved patterns of deregulated miRNA expression at the invasive tumour margin in organotypic models of colorectal and pancreatic adenocarcinoma

Venn diagram, formulated using free-to-use online bioinformatic software (Oliveros, J.C. (2007) VENNY. http://bioinfogp.cnb.csic.es/tools/venny/index.html), displays miRNA candidates consistently deregulated at the invasive tumour margin within and between tumour systems of different anatomical origin within the gastrointestinal tract.

MiRNAs deregulated at the invasive tumour margin in pancreatic cancer organotypics	MiRNAs deregulated at the invasive tumour margin in CRC organotypics	MiRNAs deregulated at the invasive tumour margin in colorectal and pancreatic cancer organotypics
hsa-miR-4454	hsa-miR-4454	hsa-miR-4454
hsa-miR-548as-3p	hsa-miR-548as-3p	hsa-miR-548as-3p
hsa-miR-659-5p	hsa-miR-382-3p	
hsa-miR-3686	hsa-miR-1260b	
hsa-miR-4787-5p	hsa-miR-4484	
hsa-miR-3613-3p	hsa-miR-4725-3p	
hsa-miR-5100	hsa-miR-589-5p	
	hsa-miR-4285	
	hsa-miR-4467	
	hsa-miR-523-3p	
	hsa-miR-5581-3p	
	hsa-miR-4510	
	hsa-miR-4311	
	hsa-miR-335-3p	
	hsa-miR-5698	
	hsa-miR-187-5p	
	hsa-miR-4714-5p	
	hsa-miR-4649-3p	
	hsa-miR-4431	

Table 3.2 Conserved patterns of deregulated miRNA expression at the invasive tumour margin in organotypic models of colorectal and pancreatic adenocarcinoma

MiRNA candidates consistently deregulated at the invasive tumour margin within and between tumour systems of different anatomical origin within the gastrointestinal tract. Results are summarised in table format.

In order to validate these findings in a sensitive and specific manner, Taqman® qPCR was used to quantitate expression of miR-4454, a miRNA apparently consistently deregulated in cancer cells at the invasive tumour margin compared with non-invading cancer cells in stratified epithelial layers. This confirmed that miR-4454 is upregulated in invading CAPAN1 pancreatic cancer cells, but analysis in other cell lines did not attain statistical significance (figure 3.48).

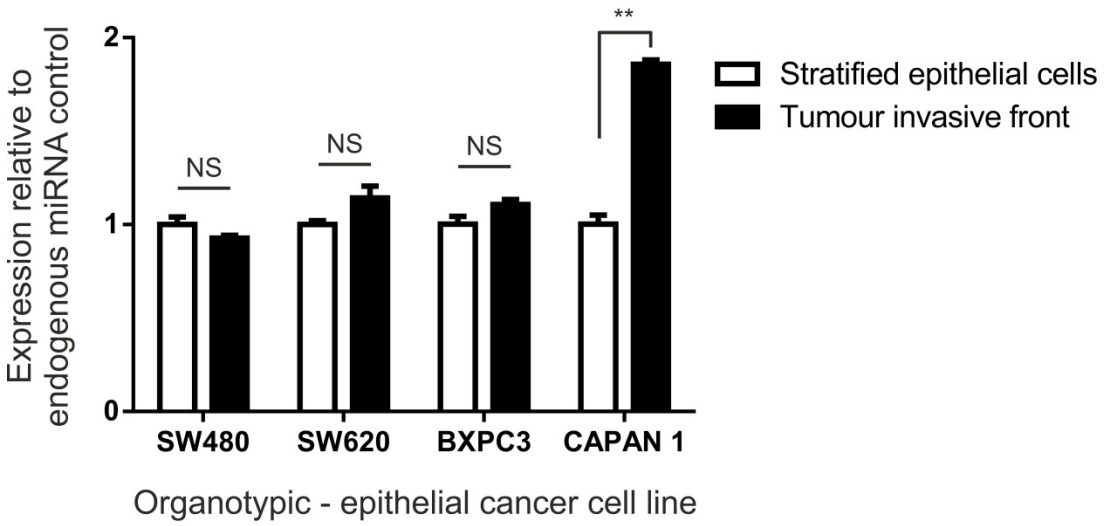


Figure 3.48 Validation study for miR-4454 expression at the invasive tumour margin in pancreatic and colorectal cancer organotypic models

*Expression of miR-4454 in LMD cells at the invasive tumour margin in organotypic models compared with identical but non-invading cancer cells in stratified epithelial layers by Taqman® qPCR. NS=Not Significant; **P<0.005.*

Once more, inconsistencies between hybridisation microarray and Taqman® qPCR data calls into question the sensitivity and specificity of high-throughput miRNA profiling in the context of LMD. However, a deeper understanding of the biology of cancer cells at the moment of metastatic departure, as they acquire the capacity to break free from their sister cells and invade surrounding stroma, would be extremely valuable. As such, methodological optimisation, as well as plans to profile the invasive tumour margin *in vivo* are underway.

3.5.3 Results discussion

Metastasis is a complex, muti-step and dynamic event. To succeed in this process in the earliest phases, cancer cells need to acquire the capacity to break free from their sister cells and invade surrounding tissues.[78] As discussed in section 1.1.5, there is increasing evidence to suggest this occurs in a non-linear manner, which is to say that metastatic progression and tumourogenesis are biologically distinct processes.[83] In section 1.3.3, the profound and multitudinous roles of miRNAs during cancer initiation were discussed and in section 1.3.5, miRNAs implicated in the hallmark processes of metastasis were also described. However, to our knowledge, no prior study has attempted to identify deregulated stromal or epithelial miRNAs during metastatic progression, or characterise their function in the context of the metastatic cascade.

Here, miRnome-wide gene expression analysis of LMD tumour specimens was used to identify miRNAs deregulated *in vivo* in stage II CRC specimens with and without subsequent metastasis; and in organotypic models at the point at which CRC cells first embark on their metastatic journey.

MiR-556 the most highly deregulated stromal miRNA identified in the current study, successfully distinguished stage II CRC specimens which subsequently metastasised within 5 years from specimens which did not. Several other miRNAs remain to be examined; however as yet no other candidate identified by high-throughput screening has been successfully validated by Taqman® qPCR. Unfortunately this throws into doubt the validity of this methodology in the context of LMD tissue.

Although, the low yield of total RNA from LMD, and subsequent use of relatively small amounts of total RNA (75-100ng) may go some way to accounting for the observed inconsistencies, it is also significant that large numbers of data-points were excluded from analysis because of widespread streaking and blemishes present on nearly all hybridisation array slides.

Furthermore, when dealing with 'big-data' analytics, attention to the statistical parameters employed is particularly important. For example, in an analysis of >1500 human miRNAs represented on a hybridisation slide, a significance threshold of $P \leq 0.05$ or 5% may, by chance alone return up to 75 falsely-positive results.

Therefore to adapt and improve our methodology, the group is considering employing once more the QuantimiR™ qPCR profiling array which works well in our hands, although it does remain a medium throughput platform with limited capacity to identify new cancer relevant genes. It would also be possible to spend more time harvesting tissue by LMD, to improve total RNA yield; or to amplify total RNA using a commercially available MessageAmp™ kit from Ambion®.

Furthermore, future profiling will be conducted using a false discovery rate (FDR) control, a method employed in multiple hypothesis testing to correct for multiple comparisons and increase statistical power.

Nevertheless, deregulated expression of miR-556, identified by miRNA screening, was successfully validated by Taqman® qPCR and is overexpressed in the stroma of stage II CRC specimens which later metastasise compared to those which do not. However, further *in silico* analysis of putative miR-556 mRNA targets, looking for enriched cancer relevant or inflammation associated biological themes, did not suggest that miR-556 is likely to play an important role in CRC progression. By comparison, identical *in silico* analysis for miR-153 in CRC identified 36 enriched biological themes, of which 55% were cancer associated including processes associated with cell motility and adhesion, with an enrichment score of 12.5.[235]

In order to provide a more definitive impression of the biological importance of miR-556 it will be necessary to identify cells of origin in the stroma by ISH, and conduct functional analysis in cells in which miR-556 has been upregulated or knocked down. As the required techniques have already been optimised during the course of the project, the analysis could be completed very rapidly.

Furthermore, as stromal miR-556 is differentially expressed in CRC specimens with and without metastasis, it may prove to be a clinically useful marker of metastatic progression in stage II disease. The prognostic utility of stromal miR-556 will be assessed in the following chapter.

In summary, high throughput molecular screening identified a novel stromal miRNA candidate (miR-556), which successfully distinguishes between stage II CRC specimens with and without subsequent metastasis at 5 years. It remains to be seen whether stromal miR-556 plays an important role within metastasis pathways in CRC, or whether

deregulated miR-556 expression is subsidiary or even inconsequential in the context of other more significant biological events.

Other than miR-556, no miRNA candidate identified in this study, in CRC stroma or epithelium, or in invading CRC cancer cells, was successfully validated by Taqman® qPCR. Although this somewhat undermines confidence in the validity of the profiling data presented, this process has allowed us to 'road-test' a novel and innovative research strategy, and has highlighted areas of planning and execution which require optimisation.

3.6 The utility of microRNA expression profiling as a prognostic tool in stage II colorectal cancer

The identity and function of deregulated stromal and epithelial miRNAs in CRC is increasingly well described. Understanding the molecular events underlying tumourogenesis and the metastatic cascade is important not only for the development of novel, targeted drugs, but also because categorising heterogeneous tumours based on their molecular characteristics may lead to enhanced prognostication and better tailored treatment.

This is particularly relevant for patients with stage II (node and visceral metastasis negative) CRC. Disease recurrence remains a substantial problem in this group as 20-25% of patients following surgery with curative intent go on to develop metastases within 5 years.[2] Although this clearly represents a significant minority of patients, the blanket use of adjuvant treatment is not supported by evidence in this group.[326] Thus a molecular staging approach enabling early identification of patients at high risk of tumour recurrence would create the opportunity both to provide targeted interventions where necessary, and avoid overtreatment in other cases.

To address this issue, the prognostic utility of deregulated stromal and epithelial miRNAs identified in this study, was examined in a cohort of 50 CRC patients with stage II disease. Only patients for whom formalin-fixed archived tumour tissue was available alongside adequate histopathological, pre, intra and post-operative clinical information, were included (Demographic data in Material and Methods; Table 2.3).

Specifically, Taqman® qPCR was used to compare miRNA expression in LMD CRC epithelium and stroma from patients with no histological or clinical features of biologically aggressive disease but who subsequently developed metastases within 5 years, with carefully matched patients who remained metastasis free (n=25 vs. 25).

Median follow-up for the group was 6.08 years (95% CI:4.98-7.19).

3.6.1 The prognostic utility of stromal microRNA candidates in stage II CRC

In stroma, mean miR-21 expression is X1.84-fold greater in stage II tumours from patients who developed metastatic recurrence (stage II-R) compared with patients who did not (stage II-NR) ($p<0.05$) (figure 3.49A).

Stratifying patients into high and low expression groups relative to mean miR-21 expression for the group as a whole (mean = 0.857; 95% CI: 0.607-1.107) revealed that 13 out of 25 patients with stage II-R disease expressed high levels of stromal miR-21 compared with 6 out of 25 patients with stage II-NR disease ($p<0.05$).

Patients expressing high levels of stromal miR-21 had significantly shorter disease free survival (DFS) (HR = 2.68, 95% CI: 1.21-5.93, p=0.015) and overall survival (OS) (HR= 2.47, 95% CI: 1.19-5.55, p=0.029) than patients expressing low levels of stromal miR-21 (figure 3.49 B and C). 13 out of 19 patients with high stromal miR-21 expression developed distant metastases during the period of follow-up compared with 12 out of 31 patients in the low stromal miR-21 expression group ($p<0.05$).

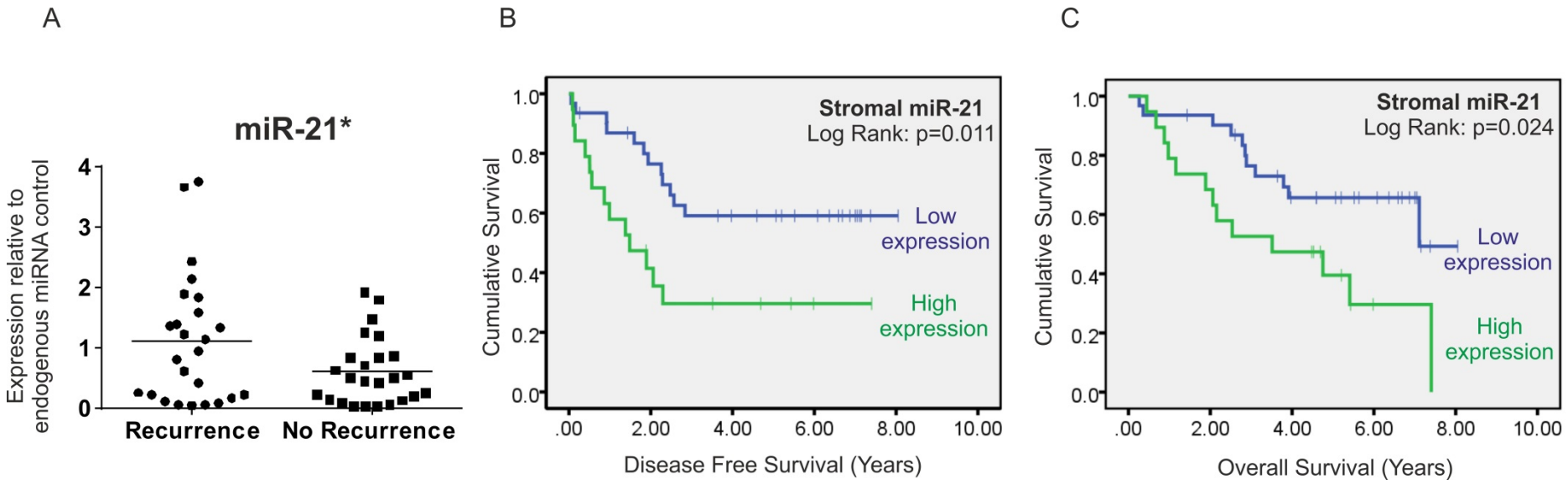


Figure 3.49 The prognostic utility of deregulated stromal miR-21 expression in stage II CRC

*(A) Expression of stromal miR-21 by Taqman® qPCR in stage II CRC with (n=25) and without (n=25) recurrence at 5 years. Mean miR-21 expression is represented by a horizontal bar *P<0.05.*

Kaplan-Meier curves of (B) disease free survival and (C) overall survival in stage II disease. Patients were stratified into high or low expression groups based around mean stromal miR-21 expression for the group as a whole by Taqman® qPCR.

As noted in chapter 3.5.1.1, mean miR-556 expression is also increased X2.02-fold in the stroma of stage II CRC specimens which go on to metastasise compared with those which do not ($p<0.05$) (figure 3.50A). When stratified into high and low expression groups relative to mean expression (mean = 0.393; 95% CI: 0.266-0.519), patients expressing high levels of stromal miR-556 had significantly shorter DFS (HR = 2.60, 95% CI: 1.18-5.73, $p=0.018$), but not significantly shorter OS than patients expressing low levels of stromal miR-556 (figure 3.50 B and C). 12 out of 17 patients with high stromal miR-556 expression developed distant metastases during the period of follow-up compared with 13 out of 33 patients in the low stromal miR-556 expression group ($p<0.05$).

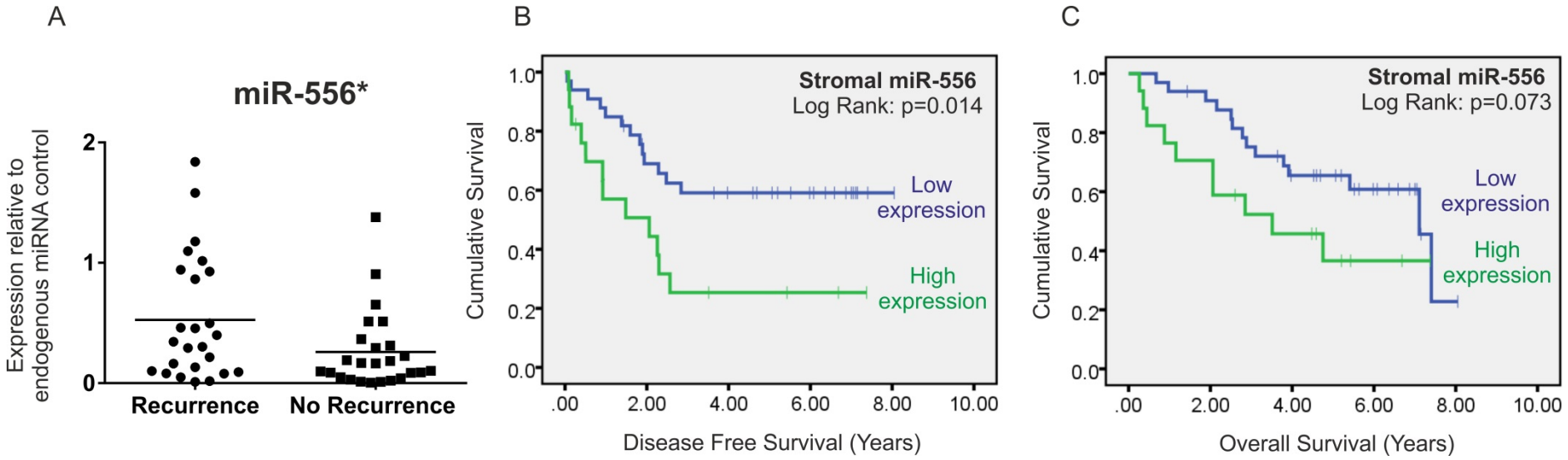


Figure 3.50 The prognostic utility of deregulated stromal miR-556 expression in stage II CRC

*Expression of stromal miR-556 by Taqman® qPCR in stage II CRC with (n=25) and without (n=25) recurrence at 5 years. Mean miR-556 expression is represented by a horizontal bar *P<0.05.*

Kaplan-Meier curves of (B) disease free survival and (C) overall survival in stage II disease. Patients were stratified into high or low expression groups based around mean stromal miR-556 expression for the group as a whole by Taqman® qPCR.

Next, expression of miR-215 was examined as it was the most downregulated stromal miRNA identified in the initial QuantimiR™ array (preliminary data; section 1.7). Although Taqman® qPCR did confirm a 3.3-fold reduction in miR-215 expression in CRC stroma compared with paired normal colonic stroma, in stage II disease, stromal miR-215 was ubiquitously expressed and so was not included in survival analysis (figure 3.51).

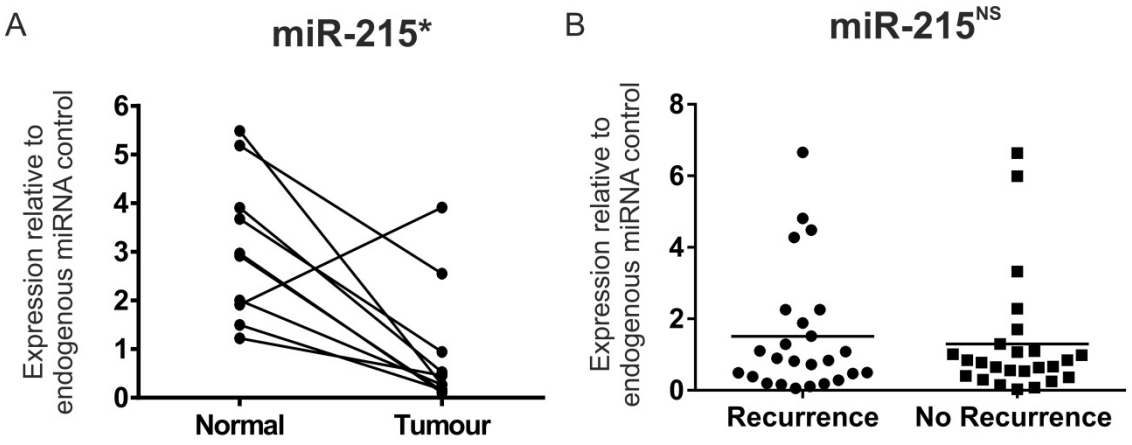


Figure 3.51 miR-215 expression in CRC stroma

*Stromal miR-215 expression by Taqman® qPCR in (A) CRC vs. paired normal tissue and (B) stage II CRC with and without metastasis at 5 years. NS = Not Significant; *P<0.05*

3.6.2 The prognostic utility of epithelial microRNA candidates in stage II CRC

MiR-224 and miR-106a were the highest scoring miRNA candidates identified in the QuantimiR™ comparison of CRC epithelium with paired normal colonic epithelium.

In stage II disease miR-224, was not significantly different in LMD epithelium from tumour specimens with and without subsequent metastasis at 5 years however, mean expression of miR-106a, was upregulated X1.88 fold in the recurrence group ($p<0.05$) (figure 3.52 A and B).

When patients were stratified according to high or low miR-106a expression (mean = 0.372; 95% CI: 0.265-0.478), patients expressing high levels of epithelial miR-106a had significantly shorter DFS (HR = 2.91, 95% CI: 1.32-6.42, p=0.008) and OS (HR = 2.25, 95% CI: 1.00-5.04, p=0.049) than patients expressing low levels of epithelial miR-106a figure 3.52 C and D). 13 out of 18 patients with high epithelial miR-106a expression developed distant metastases during the period of follow-up compared with 12 out of 32 patients in the low epithelial miR-106a expression group ($p<0.05$).

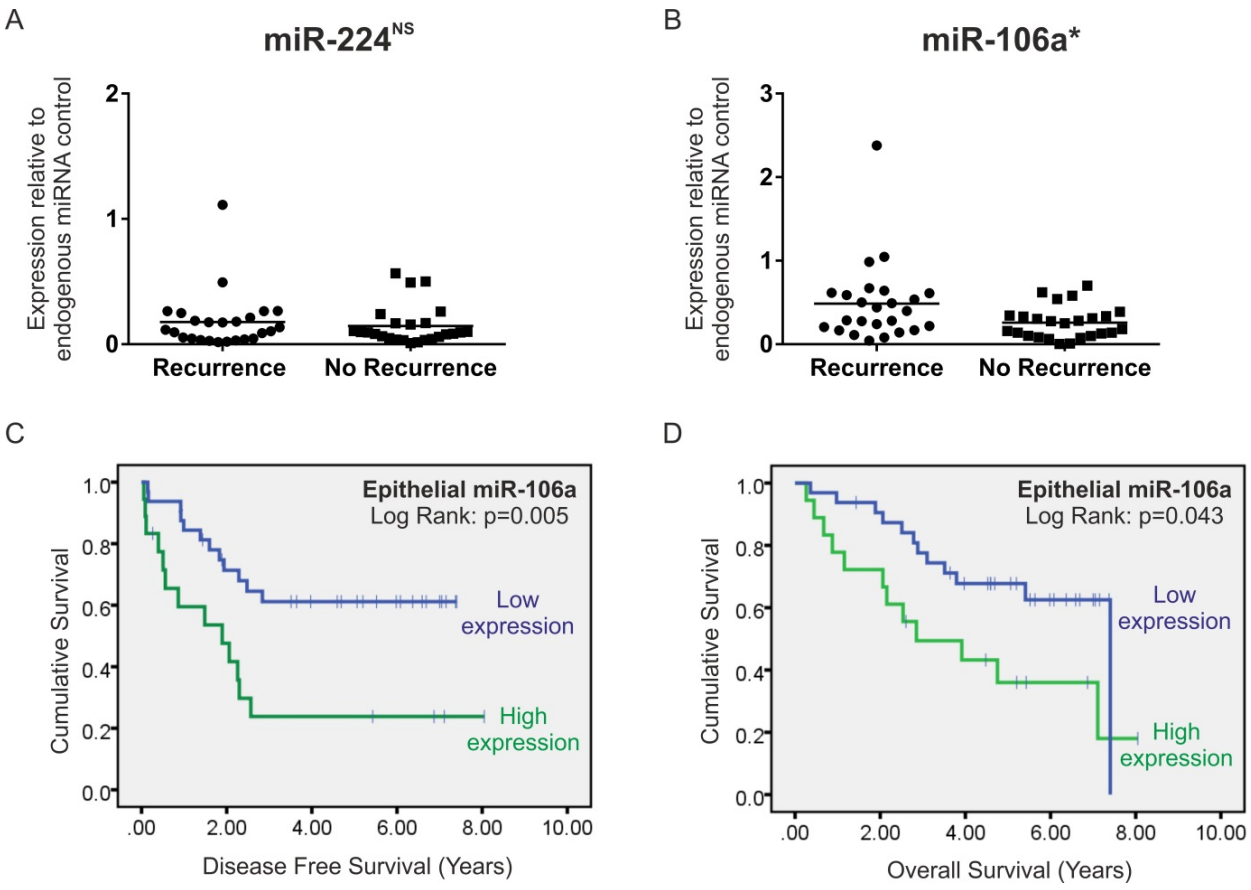


Figure 3.52 The prognostic utility of deregulated epithelial miRNA candidates in stage II CRC

*Expression of (A) epithelial miR-224 and (B) epithelial miR-106a by Taqman® qPCR in stage II CRC with (n=25) and without (n=25) recurrence at 5 years. Mean miRNA expression is represented by a horizontal bar. NS = Not Significant; *P<0.05.*

Kaplan-Meier curves of (C) disease free survival and (D) overall survival for epithelial miR-106a. Patients were stratified into high or low expression groups based around mean epithelial miR-106a expression for the group as a whole by Taqman® qPCR.

3.6.3 Survival analysis based on combined expression of epithelial and stromal microRNA candidates in stage II CRC

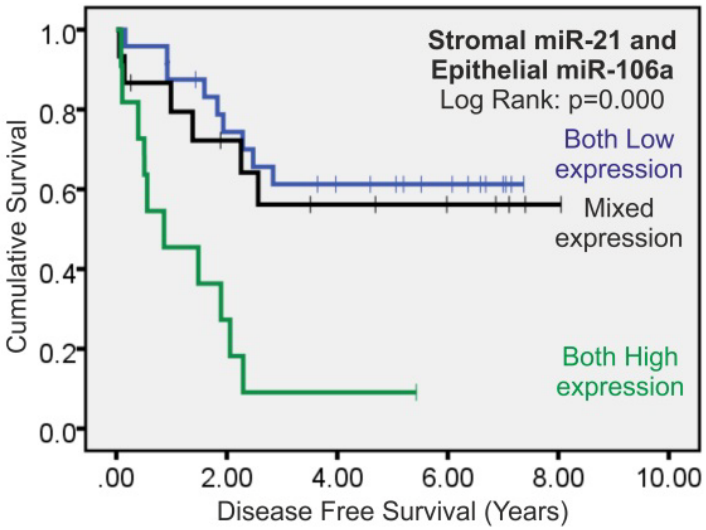
Next we wanted to examine whether panels of deregulated miRNA expression provided better prognostic discrimination than miRNA candidates examined in isolation.

When considered in combination, high expression of stromal miR-21 and epithelial miR-106a were associated with poor DFS (Both High vs. Both Low: HR = 5.09, 95% CI: 2.02-12.85, p=0.001) (figure 3.53A) and OS (Both High vs. Both Low: HR = 4.13, 95% CI: 1.48-11.52, p=0.007) (figure 3.53C).

When all three potentially prognostically relevant miRNA candidates were considered (stromal miR-21 and miR-556; and epithelial miR-106a), metastases were identified in 7 out of 8 patients expressing high levels of all three candidates, compared with 12 out of 23 patients expressing high levels of two or one candidate; and 6 out of 19 patients expressing none of the candidates at high levels ($p<0.05$). Furthermore, those expressing high levels of all three miRNAs had significantly shorter DFS (All High vs. All Low: HR = 5.83, 95% CI: 1.92-17.57; p=0.002) (figure 3.53B).

This novel data, which combines for the first time prognostic markers of stromal and epithelial origin, suggests that panels of deregulated miRNA expression may provide practical prognostic information for clinicians treating patients with CRC.

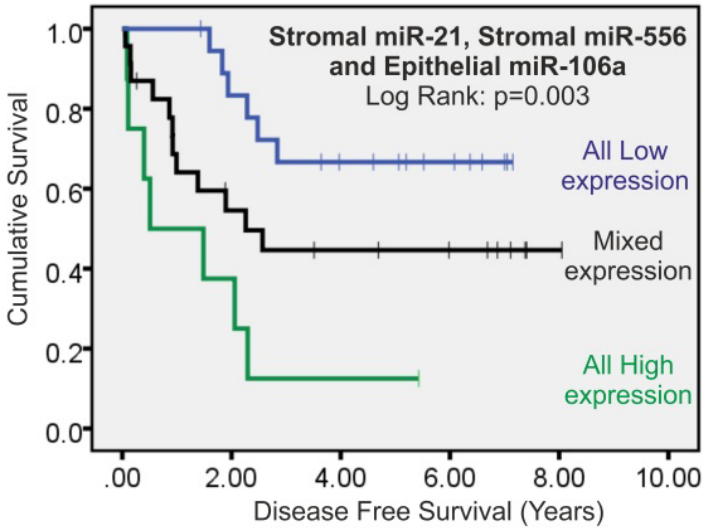
A



Patients at risk

Years post-operation	0	2	4	6	8	10
All Patients	50	29	19	12	1	0
MiRNA - Both Low expression	24	17	12	8	0	0
MiRNA- Mixed expression	15	9	6	4	1	0
MiRNA- Both High expression	11	3	1	0	0	0

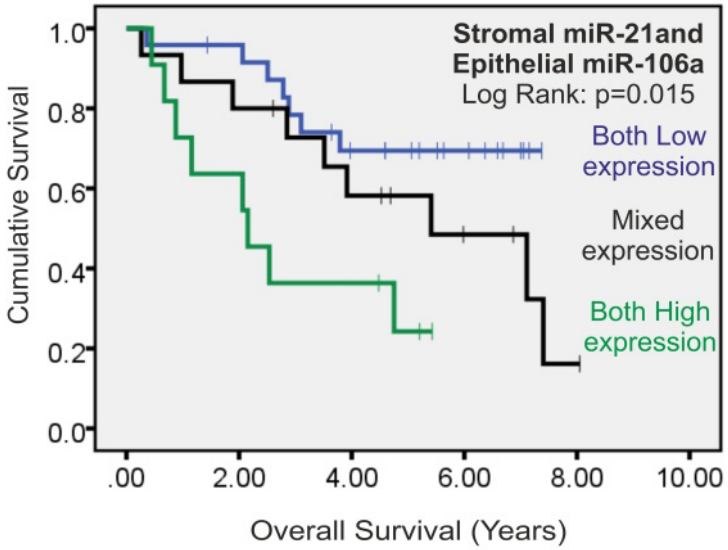
B



Patients at risk

Years post-operation	0	2	4	6	8	10
All Patients	50	29	19	12	1	0
MiRNA- All Low expression	19	15	10	6	0	0
MiRNA- Mixed expression	23	11	8	6	1	0
MiRNA- All High expression	8	3	1	0	0	0

C



Patients at risk

Years post-operation	0	2	4	6	8	10
All Patients	50	41	34	12	1	0
MiRNA- Both Low expression	24	22	14	8	0	0
MiRNA- Mixed expression	15	12	8	4	1	0
MiRNA- Both High expression	11	7	4	0	0	0

Figure 3.53 Survival analysis based on combined stromal and epithelial miRNA expression in stage II CRC

Kaplan-Meier curves of disease-free survival and overall survival in patients with stage II disease based on significantly differentially expressed stromal and epithelial miRNAs. Patients were stratified into high or low expression groups based around group mean expression of stromal miR-21 and epithelial miR-106a for analysis of disease free survival (A) and overall survival (C). As stromal miR-556 expression was only discriminatory in the context of disease free survival, it was included in combined analysis of disease free survival (B), but not overall survival (C).

3.6.4 Survival analysis based on expression of microRNA candidates and their putative molecular targets in vivo

Next, we wanted to test whether the relationship between miRNAs and their molecular targets *in vivo* provided additional prognostic information in stage II disease. For this purpose, a human tissue microarray (TMA) was generated using triplicate cores from the CRC specimens used in comparative miRNA gene expression analysis, from patients with stage II disease with and without metastasis at 5 years (n=25 vs. 25) (Demographic data in Material and Methods; Table 2.3).

Subsequently, the TMA was immunostained for known miR-21 targets RECK and TIMP3, and scored by an independent investigator blinded to the clinical data. Each core was scored semi-quantitatively on a scale of 1-7 corresponding to absent, low, medium or high intensity staining (0-3) with <25% positivity, 25-50% positivity, 50-75% positivity and >75% positivity (1-4).

Unfortunately staining for RECK was absent despite pre-optimisation of the antibody and the use of tonsillar tissue as a positive control. However, staining for TIMP3 was successful and revealed a pattern of downregulated expression in advancing tumour stage (figure 3.54).

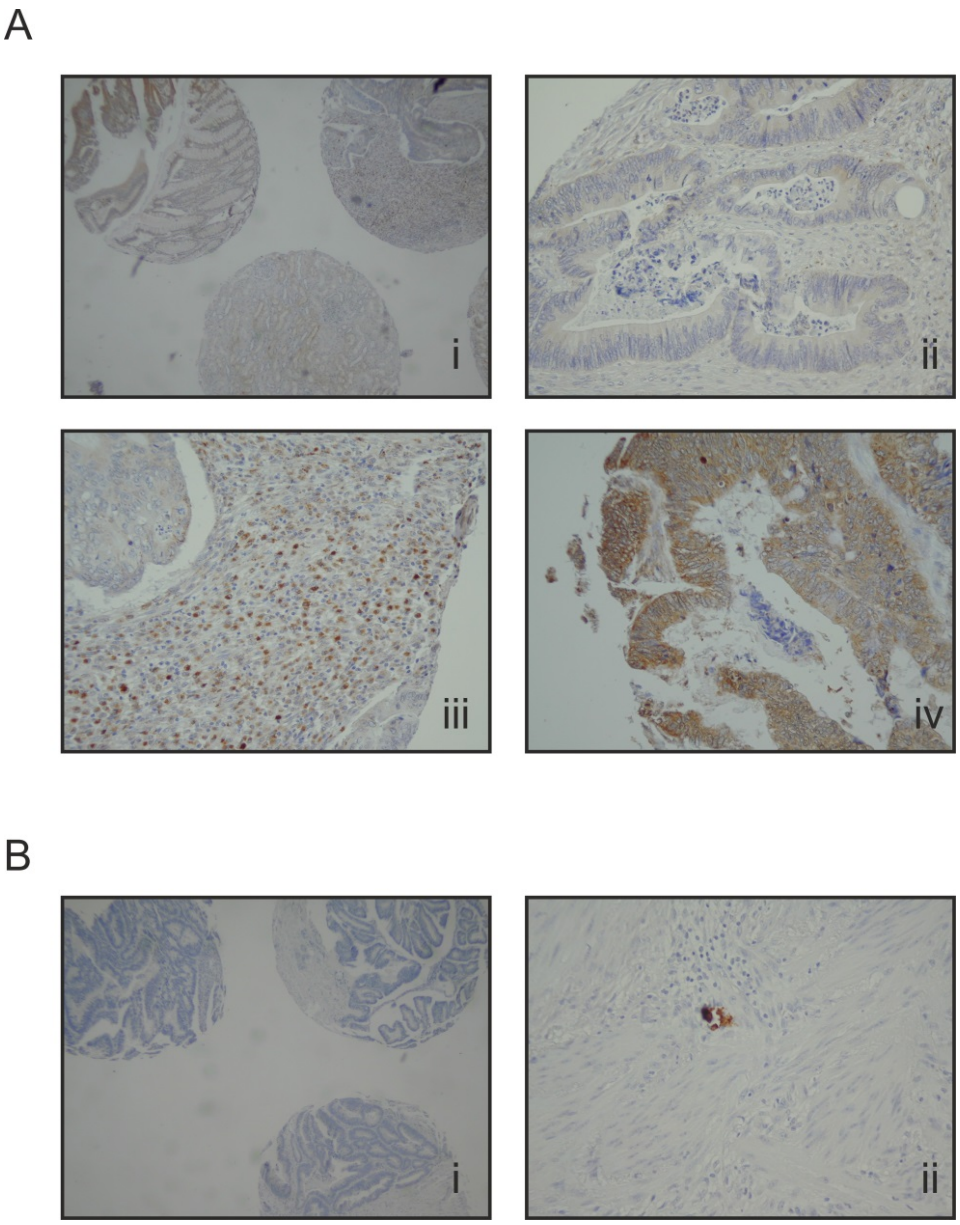


Figure 3.54 Immunohistochemistry for the purpose of (A) TIMP3 and (B) RECK quantitation in a TMA comprised of stage II CRC specimens with and without recurrence at 5 years

(Ai) Tissue microarray sections immunostained with primary TIMP3 antibody; staining was observed both in stromal and epithelial tumour compartments (x4 magnification). TIMP3 expression was measured semi-quantitatively on a scale of 1-7 corresponding to staining intensity and percentage of cells with positive staining. (Aii) represents low/negative staining: score 0-3; (Aiii) represents moderate staining: score 4-5 and; (Aiv) represents strong staining: score 6-7 (x40 magnification).

(Bi) Tissue microarray sections immunostained with primary RECK antibody (x4 magnification). (Bii) Staining was absent except in non-specific patchy areas (x40 magnification).

The mean TIMP3 expression score in stage II tumours with recurrence at 5 years was 2.50 (95% CI: 1.97-3.03) compared with 3.18 (95% CI: 2.71-3.67) in tumours which did not metastasise (figure 3.55 A).

This is consistent with the tumour suppressor properties of TIMP3 identified in CRC in a previous study.[471] Here however, TIMP3 expression alone was not an independent predictor of outcome in stage II disease (High vs. low TIMP3 expression based around group mean expression: DFS; HR = 0.564; 95%CI = 0.247- 1.292; P=0.17) (figure 3.55 B).

In the current study, miR-21 dependent TIMP3 downregulation was observed in cultured fibroblasts but not *ex vivo* colonic fibroblasts. In previous studies, luciferase reporter assays have suggested that direct regulation of TIMP3 does not occur, despite the presence of conserved binding sites within the TIMP3 mRNA 3'UTR.[284]

To inform this debate, analysis of matched tumours for expression of miR-21 and TIMP3 was conducted. This revealed a weak but significant inverse correlation in CRC specimens *in vivo* (R=-0.339; P=0.017) (figure 3.55 C). Further analysis of progression free survival suggested that patients expressing high miR-21 but low TIMP3 had the worse outcome of all in stage II disease, but in the presence of high TIMP3, prognosis was considerably improved. Conversely, in the presence of low miR-21, TIMP3 expression whether high or low, made little difference in terms of clinical outcome (figure 3.55 D).

Taken together, these results support the notion that *in vivo* miR-21 may regulate TIMP3 expression but not through direct suppression of mRNA transcription.

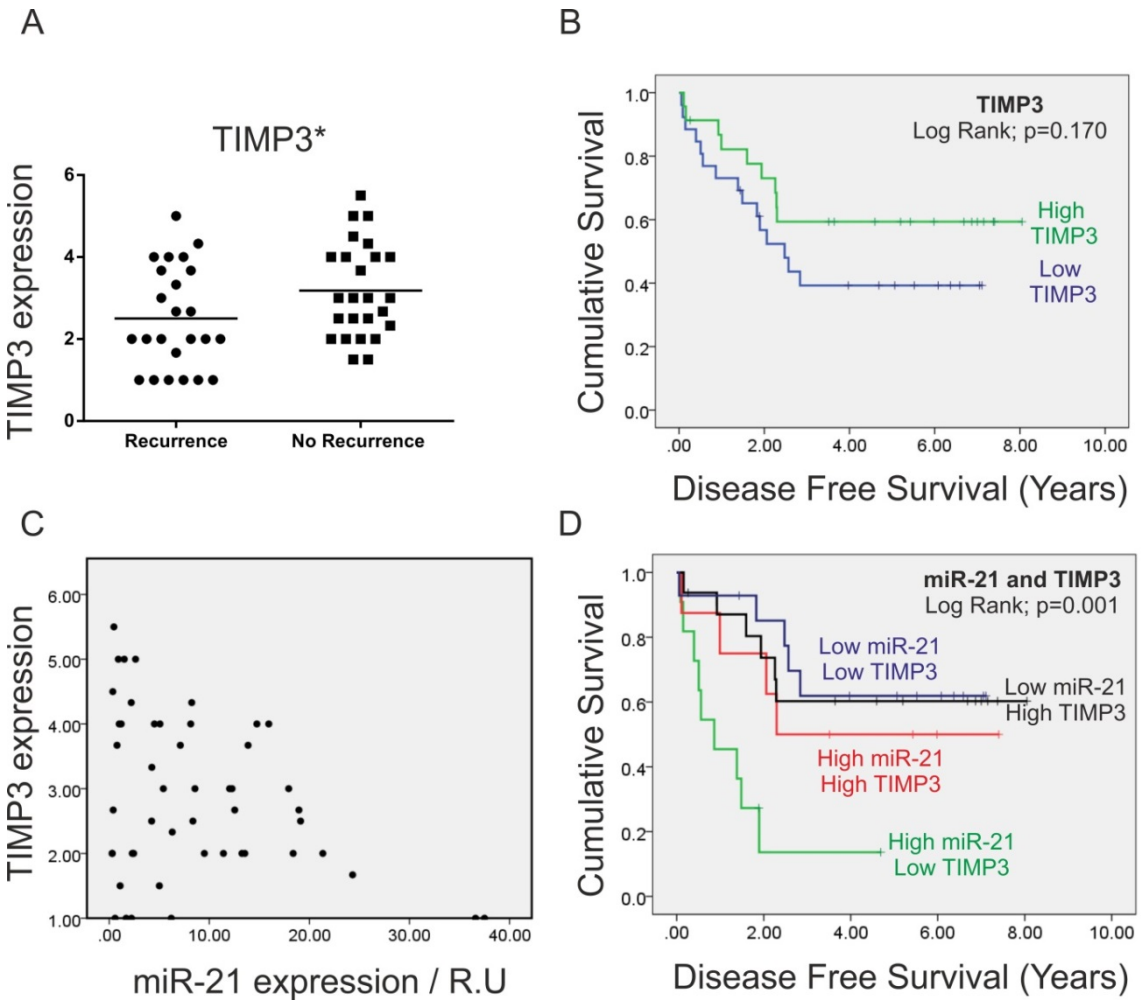


Figure 3.55 The significance of TIMP3 and miR-21 expression in stage II CRC

*(A) TIMP3 expression by immunohistochemistry on a tissue microarray showing higher mean expression in non-recurrent tumours compared with tumours which subsequently metastasised *P=0.05. (B) Disease free survival for patients with CRC according to high or low expression of TIMP3 (Kaplan-Meier). (C) Correlation of miR-21 and TIMP3 expression in human CRC specimens. (D) Disease free survival for patients with CRC according to combined tissue expression of miR-21 and TIMP3.*

3.6.5 Results discussion

As discussed in chapter 1.3.7, non-coding molecules such as miRNAs have promising clinical applications in cancer. [380, 381] The analysis of miR-21 in plasma and stool [312, 313, 503] for example, has been identified as a potential diagnostic/screening tool in CRC and high miR-21 expression in tumour [311] and serum [314] predicts poor post-operative outcomes.

MiRNAs also play important roles during tumour initiation and progression, and an important theme in recent years is the emergence of pro-metastatic events in the tumour microenvironment regulated by miRNAs [300].

In the present study, miR-21 was the most highly upregulated stromal miRNA candidate in CRC tissue compared with paired 'normal' stroma by Taqman® qRT-PCR. In a separate analysis of 50 patients with stage II disease, stromal miR-21 was also significantly overexpressed in patients who developed metastases within 5 years compared with those that remained metastasis free. These data contribute to a growing body of evidence which suggests deregulation of the key oncogene miR-21 is a stromal phenomenon in CRC, and not just a feature of cancer cells. [298, 299, 423]

Prompted by the notion that miRNA expression signatures may be used to identify patients at high risk of disease recurrence in stage II CRC, the most upregulated epithelial candidates from our QuantimiR™ screen were also examined.

MiR-106a expression was significantly increased in epithelial tumour tissue from patients who subsequently developed metastases compared to those who did not. Intriguingly, both miR-21 and miR-106a were identified in a previous study which compared miRNA expression in CRC and paired normal colonic tissue. However, in contrast to Schetter et al.,[311] who used whole tumour tissue sections, here an initial LMD step prior to profiling with high-throughput microarrays. This approach, which was designed to avoid masking important biological differences between cancer cells and their supportive microenvironment, also allowed potentially relevant molecular events to be mapped to the appropriate tumour compartment.

Crucially, it emerged that both elevated stromal miR-21 and epithelial miR-106a are associated with significantly reduced DFS and OS in patients with stage II CRC, which encouraged further interrogation of our QuantimiR™ screen:

MiR-215 was downregulated more than X2-fold in CRC stroma compared with paired normal stroma by Taqman® qPCR. Downregulated expression of miR-215 has previously been identified in nephroblastoma and is associated with upregulated activin receptor type 2B (ACVR2B) expression in the TGFβ pathway.[504] MiR-215 suppression has also been demonstrated in CRC, in a study which paradoxically linked declining miR-215 expression with improved overall survival.[353] However, as patterns of stromal miRNA expression are potentially made less distinct by the presence of extraneous epithelial tissue, this paradox may be explained by the use of bulk tumour specimens rather than LMD tissue.

It is reassuring that many of the observations presented here mirror a complementary study by Nishida et al., namely that miR-21 and members of the miR-17-92a cluster are strongly upregulated; and miR-215 downregulated in CRC stroma. However, having identified miRNA candidates deregulated in CRC stroma, the authors did not ascertain their prognostic significance, nor did they examine differentially expressed miRNAs in tumour vs. normal epithelium.[296]

Here, the utility of deregulated miRNAs such as miR-21 in CRC stroma and miR-106a in CRC epithelium, were scrutinised in stage II disease, in a 'real-world' clinical scenario which presents significant prognostic and therapeutic challenges. In contrast, stromal miR-215 and epithelial miR-224 were ubiquitously expressed in all stage II CRC specimens regardless of metastatic status at 5 years and hence they were not included in survival analysis. Nevertheless, miR-215 and miR-224 may still be biologically relevant in CRC and this raises an interesting point; that the same miRNAs deregulated in cancer are not necessarily deregulated during metastatic progression. Crucially, this observation supports the view that molecular mechanisms underpinning the metastatic cascade are distinct from those which drive carcinogenesis.[83]

To broaden and enrich the search for clinically relevant miRNAs, a miRnome-wide profiling approach was adopted to specifically compare miRNA expression in stage II CRC specimens with and without recurrence at 5 years. In this comparison of 20

patients (10 stage IIR vs.10 stage IINR) 95 consistently deregulated miRNAs were identified in stroma compared with 63 miRNAs in tumour epithelium (figure 3.42 and 3.43).

Most cancer research is focused on tumour cells, but because stromal cells are less likely to acquire de-novo mutations, evade anti-cancer immunity, or develop drug resistance than malignant epithelial cells, the concept of stroma targeted therapy has become increasingly attractive. [152, 153] Another important theme in recent years is the development of therapies which target or exploit non-coding RNAs.[380, 381, 503] In this context, the characterisation of miRNAs differentially expressed in metastatic vs. non-metastatic tumour groups may reveal opportunities to develop novel pharmacological interventions both in malignant tissue and the supportive tumour microenvironment.

MiR-556 was the most upregulated stromal miRNA identified in stage IIR CRC specimens, a finding which was validated by Taqman® qPCR in all 50 stage II CRC specimens.

Although important biological functions have not as yet been ascribed to miR-556, in the current study high stromal miR-556 expression was associated with significantly decreased DFS, but perhaps more interestingly, when combined with stromal miR-21 and epithelial miR-106a, stromal miR-556 contributed to a distinct molecular signature of disease recurrence. At 3 years following surgery with curative intent, 3/19 (16%) patients expressing low levels of all three miRNAs had developed metastases compared with 10/23 (43%) patients expressing high levels of one or two miRNAs and 7/8 (88%) patients expressing high levels of all three miRNAs.

MiR-21 expression was also examined in relation to its putative target, the tumour suppressor gene TIMP3. In an earlier study which characterised the prognostic potential of several members of the TIMP and MMP families, stromal TIMP3 expression was the only biomarker significantly associated with 5-year survival in a cohort of 350 patients with stage I-IV CRC.[471] In a further study by Curran and colleagues, loss of TIMP3 contributed to an increasingly aggressive CRC phenotype, in homogenous cohort of stage III CRC specimens.[505]

Here, TIMP3 expression was supressed in stage II tumours which later metastasised compared with those that did not, but TIMP3 expression alone was insufficiently robust to act as a biomarker of tumour recurrence.

The TMA approach did however permit examination of TIMP3/miR-21 co-expression *in vivo,* and identified a weak but significant inverse correlation. However, in several tumour specimens, miR-21 and TIMP3 were found to both be highly expressed or suppressed concurrently, which chimes with published mechanistic data suggesting that although TIMP3 is responsive to miR-21, regulation is unlikely to occur through a direct inhibitory mechanism.[284] Alternatively, these findings may reflect a deeper regulatory complexity for TIMP3 *in vivo*. In-fact a recent publication has identified other miRNA based mechanism which regulate TIMP3, such as miR-191 a miRNA overexpressed in CRC which appears to inhibit TIMP3 expression directly by targeting mRNA translation.[506]

In summary, the data presented in this chapter demonstrate that stromal and epithelial miRNA profiles may be used to identify patients at high risk of CRC recurrence and reduced OS. This supports the notion that miRNAs are important actors during the metastatic cascade, and that miRNA profiling may be a valuable aid to therapeutic decision making.

Furthermore, by using FFPE tissue, a widely available clinical resource, the translational appeal of this research has been highlighted. However, in order to fully evaluate these findings, additional validation in a larger and independent cohort will be required.

4 Conclusions and future directions

4.1 Study objectives

The objective of this study was to identify deregulated miRNAs in the epithelial and stromal compartment of CRC specimens and develop a deeper understanding of their impact on disease progression. The broad aims were as follows:

1. Identify and validate candidate miRNAs differentially expressed in the stroma and epithelium of different stage clinically relevant CRC samples, with emphasis placed on the comparison of stage II CRC specimens with and without subsequent metastatic progression.
2. Determine biological consequences of deregulated miRNAs using *in vitro* functional cell assays for disease progression and a 3-D CRC organotypic model to facilitate better understanding of the complex tumour-stroma cross-talk.
3. Validate the functional impact of candidate miRNAs using a novel *in vivo* model of CRC progression
4. Identify potential gene targets of candidate miRNAs in order to map the molecular pathways which mediate their biological effects.
5. Establish the utility of miRNA expression profiling as a clinical prognostication tool in CRC.

4.2 Summary of findings

The first objective of this study was to identify and validate candidate miRNAs differentially expressed in the stroma and epithelium at different clinically relevant stages of CRC. Preliminary profiling work comparing miRNA expression in paired SW480 and SW620 CRC cells and LMD CRC specimens with paired normal tissue identified a number of candidates of which three were selected for further analysis (epithelial miR-153 and miR-224; and stromal miR-21).

Further profiling with high-throughput miRNA hybridisation arrays, was designed to identify additional miRNAs specifically relevant during CRC progression, rather than tumour initiation. Candidate miRNA validation is ongoing, but Taqman® qPCR analysis has confirmed that the novel miRNA miR-556 is upregulated by a factor >2 in the stroma of stage II CRC specimens which metastasised within 5 years compared with carefully matched specimens which did not.

The second objective of this study was to determine the biological consequences of deregulated stromal and epithelial miRNAs in CRC.

In functional screening studies, miR-224 did not appear to impact on CRC invasion or proliferation, but miR-153 did produce a more invasive cellular phenotype. This effect was particularly notable in organotypic models, where miR-153 transfected cells were seen to cluster at the tumour-invasive front.

Organotypic models are synthetic tumour constructs which mimic *in vivo* conditions by juxtaposing multi-layered tumour epithelial cells with a stromal layer consisting of cellular and molecular ECM components. Although organotypics are increasingly widely used to examine tumour invasion in 3-dimentions, this study presents the first example of this highly versatile experimental platform used in conjunction with miRNA-manipulated epithelial and/or stromal cells.

The novel finding that miR-153 upregulation leads to enhanced tumour invasion is interesting, as a pathogenic role in CRC and metastasis has not previously been described.

However, the main focus of this research has been miR-21, an oncomiR with well documented regulatory roles in numerous cancer-relevant biological pathways. In CRC epithelial cells, miR-21 has been shown to promote metastatic progression by targeting tumour suppressor PDCD4. [230] Interestingly, profiling data in this study identified miR-21 as one of the highest scoring stromal miRNA candidates, and high sensitivity Taqman® qRT-PCR confirmed a 4-fold mean increase in relative miR-21 expression in the stroma of CRC specimens compared with paired normal tissue. To pinpoint the cellular location of miR-21 in CRC, ISH with highly specific LNA probes was used. By this method, it was possible to demonstrate that miR-21 overexpression occurred exclusively in fibroblast like cells in the stroma and that expression was virtually absent within the malignant epithelial layer. This finding is potentially extremely relevant, as studies which previously established miR-21's credentials as a 'master-regulator' of metastatic progression adopted an exclusively epithelium-centred approach.[409] Hence, the discovery that miR-21 over-expression in CRC actually occurs in stromal fibroblasts, presents a paradigm shift, and suggests the pathogenic influence of miR-21 may be part-mediated indirectly by cancer-associated stroma.

Prompted by these results, analysis of the functional impact of upregulated miR-21 expression in the stroma became an important priority. Furthermore, both miRNAs and the stroma are promising therapeutic targets and the synthesis of these two research themes has the potential to produce valuable preliminary data for the development of future stroma/miR-21 targeted therapeutic strategies.

The most striking effect of miR-21 upregulation in cultured fibroblasts was the adoption of an elongated and enlarged morphology, accompanied by increased αSMA expression. Although suggestive of myofibroblast differentiation status, in the absence of characteristic stress-fibre formation it was necessary to conclude that miR-21 over-expression alone is insufficient to drive fibroblast-to-myofibroblast transdifferentiation. Instead miR-21 appeared to prime fibroblasts to transdifferentiation signals in a manner consistent with earlier reports.[299]

In the current study miR-21 over-expressing fibroblasts were shown to be phenotypically similar to myofibroblasts despite their biochemical differences, which is an entirely novel finding. CM extracted from miR-21 overexpressing fibroblasts was associated with increased CRC invasion, proliferation and chemoresistance, all of

which promote tumour progression. Only one other study has identified a deregulated stromal miRNA which impacts upon the phenotype of the tumour microenvironment (miR-320; Bronisz et al., 2012);[300] and the only study to examine miR-21 in the context of fibroblast differentiation, identified a mechanism through which myofibroblast markers are regulated, but not mechanisms involved in cell function or pathobiology.[299]

Crucially, the metastasis promoting influence of deregulated stromal miR-21 was confirmed here using an entirely novel orthotopic fibroblast/cancer cell co-implantation model of CRC in mice. This data satisfies the third objective of the current study, and opens a promising new line of research which will potentially allow us to examine to therapeutic utility of stroma or epithelium-targeted antagomiRs *in vivo*.

In line with the fourth objective of this study and in order to explore how miR-21 overexpression in stromal fibroblasts drives CRC invasion, organotypic models were immunostained for known miR-21 targets, the MMP inhibitors RECK and TIMP3, both of which were found to be downregulated compared with miR-SCC control transfected fibroblasts.

Western blotting, Taqman® qPCR and immunofluorescence confirmed that RECK and TIMP3 are directly or indirectly targeted by miR-21 in *ex vivo* and immortalised fibroblasts. Ectopic miR-21 expression also resulted in elevated MMP2 activity and by demonstrating that excess invasion associated with elevated stromal miR-21 expression is MMP2 dependent, this study revealed a novel stromal miRNA mediated pro-metastatic mechanism in CRC.

Using an antibody based protein array, the impact of excess miR-21 expression on the fibroblasts secretome was also assessed. A number of pro-inflammatory cytokines and chemokines were sensitive to miR-21 expression suggesting that significant corruption of the tumour microenvironment may occur in response to stromal miRNA deregulation in cancer. ELISA assays confirmed that one of the most promising candidates ENA-78/CXCL5, a chemokine implicated in anti-tumour surveillance and angiogenesis, was upregulated in CM from *ex vivo* and cultured fibroblasts transfected with miR-21.

As well as conventional *in vitro* techniques, an *in silico* approach was used to address objective 4 with respect to the potentially biological relevance of the putative

metastamiR miR-556. This reflects a diversity of skills acquired during the course of this research, which includes but is not limited to: high through-put miRNA profiling; *in vitro* and *in vivo* tumour modelling; protein, mRNA and miRNA quantitation techniques; immunohistochemistry and immunofluorescence; functional assays of cellular phenotype and transient and stable miRNA manipulation.

Computational gene target prediction and analysis for enriched biological themes, did not suggest that miR-556 plays a substantial role in molecular pathways which govern metastatic progression. Although this is yet to be validated *in vitro* and *in vivo*, stromal miR-556 did nevertheless have prognostic utility in stage II disease. Crucially stromal miR-556 expression along with expression of other miRNAs identified in this study (miR-21 and miR106a) were capable of stratifying recurrence risk in early stage CRC, suggesting for the first time that panels of deregulated miRNAs may be used in the clinical setting to guide therapeutic decision making. On this point, the final study objective is addressed.

4.3 Conclusions

The objectives of the current study have been met in an efficient and timely manner. The findings presented in this document support the hypothesis that:

- *Stromal and epithelial miRNA expression patterns (individually or communally) have important consequences for CRC progression and metastasis*

MiRNAs such as miR-21 in stromal fibroblasts have profound biological consequences for CRC progression by promoting a pro-metastatic tumour microenvironment *in vitro* and *in vivo*, which facilitates tumour invasion, promotes CRC cell proliferation and protects CRC cells from chemotherapy induced apoptosis. MiRNAs such as miR-153 deregulated in colorectal epithelial cells during malignant transformation also promote malignant invasion, as demonstrated in near-physiological conditions using a novel, purpose built organotypic CRC model.

These miRNAs, which are differentially expressed in tumour compared with normal colonic tissue, which profoundly affect CRC cell phenotype and which are potentially targetable by drugs, could in future form the basis of a novel drug strategy capable of selectively impacting on malignant tissue whilst simultaneously minimising any potential side-effects from therapy.

Crucially a number of miRNAs identified in this study (miR-21, miR-556 and miR-106a specifically) were also shown to provide valuable prognostic information in CRC. By identifying patients at high risk of future recurrence in early stage disease, these miRNAs, both alone and in combination may have utility in the clinical setting by helping to distinguish patients who might benefit from adjuvant therapy from those in which adjuvant therapy is unnecessary and potentially detrimental.

In summary, the following specific conclusions may be drawn from the current study:

1. MiR-224 and miR-153 are upregulated in the epithelium of CRC specimens compared with paired normal tissue.
    a. MiR-153 is associated with upregulated CRC cell invasion
2. MiR-21 is upregulated in the stroma of CRC specimens compared with paired normal tissue
    a. MiR-21 localises exclusively to fibroblast-like cells in the CRC stroma

3. MiR-21 upregulation in stromal fibroblasts promotes myofibroblast-like properties
    a. Stromal miR-21 upregulation indirectly promotes CRC cell proliferation
    b. Stromal miR-21 upregulation indirectly promotes CRC cell chemoresistance
    c. Stromal miR-21 upregulation indirectly promotes CRC cell invasion through downregulated RECK and TIMP3 expression and reciprocally increased MMP2 activity
4. Excess stromal miR-21 dependent CRC cell invasion is MMP2 dependent
5. Ectopic miR-21 expression in stromal fibroblasts may substantially alter the composition of the tumour microenvironment
    a. ENA-78/CXCL4 a pro-inflammatory chemokine is upregulated by ectopic miR-21 expression in *ex vivo* and cultured fibroblasts cell lines
6. Excess stromal miR-21 expression in fibroblasts supports increased CRC metastasis *in vitro* and *in vivo*
    a. This study is the first to manipulate stromal and epithelial miRNAs in the context of 3-D CRC organotypic models.
    b. This study is the first to employ a novel cancer-cell/fibroblast co-implantation technique in orthotopic murine models of CRC progression
7. MiRNAs deregulated during CRC initiation are not necessarily deregulated during metastasis which supports a 'non-linear' model of tumour progression
    a. MiR-556 was the most deregulated stromal miRNA in stage II CRC specimens which later metastasised compared with matched specimens which did not.
8. MiRNAs deregulated in the CRC stroma and epithelium during CRC progression may have prognostic utility in stage II disease
    a. Stromal miR-556: Disease free survival (HR = 2.60, 95% CI: 1.18-5.73, p=0.018).
    b. Stromal miR-21: Disease free survival (HR = 2.68, 95% CI: 1.21-5.93, p=0.015) and overall survival (HR= 2.47, 95% CI: 1.19-5.55, p=0.029)
    c. Epithelial mir-106a: Disease free survival (HR = 2.91, 95% CI: 1.32-6.42, p=0.008) and overall survival (HR = 2.25, 95% CI: 1.00-5.04, p=0.049)

9. Panels of deregulated epithelial and stromal miRNAs offer better prognostic discrimination compared with individual miRNAs in stage II CRC.

a. Stromal miR-21 and epithelial miR-106a: Both High vs. Both Low; Disease free survival (HR = 5.09, 95% CI: 2.02-12.85, p=0.001); and overall survival (HR = 4.13 (95% CI: 1.48-11.52, p=0.007)

b. Stromal miR-21, stromal miR-556 and epithelial miR-106a: All High vs. All Low; Disease free survival (HR = 5.83 (95% CI: 1.92-17.57; p=0.002)

4.4 Future directions

One of the strongest themes to emerge from the current project is the potential clinical utility of stromal and epithelial miRNAs, both as prognostic markers of disease progression and as potential targets for novel pharmacological intervention in CRC.
The following section outlines the next phase of functional and mechanistic experimentation, required to advance this research towards the clinical setting.

4.4.1 The therapeutic utility of LNA based miR-21 antagomiR

The most advanced RNA based therapy uses antisense oligonucleotides to supress hepatic miR-122 expression, thus reducing Hepatitis C viraemia (Hepatitis C Virus; HCV), and HCV induced liver pathology in primates.[380] Clinical trials are currently underway to investigate their use in the treatment of chronic HCV infection in humans.[507]

AntagomiRs have also increasingly been utilised in *in vivo* murine models to demonstrate the potential therapeutic benefits of oncomiR inhibition in breast cancer.[375, 508]

Crucially, in both scenarios, gene silencing is achieved using antisense LNA constructs which are highly stable and resistant to degradation. [498]

In the current study, the powerful impact of deregulated stromal miR-21 on metastasis was demonstrated *in vivo* using a novel orthotopic murine CRC model. Furthermore, inhibition of CRC invasion was achieved *in vitro* by transfecting fibroblasts with commercial miR-21 antagomiR.

The next phase of research, will utilise antimiR-21 LNA constructs, synthesised by our collaborators Dr Ali Tavassoli and Dr Jon Watts (Department of Chemistry; University of Southampton), to optimise conditions for miR-21 antagonism *in vivo.*

Conclusions and future directions

Initially, the proxy effects of antimiR-21 LNA transfection in fibroblasts will be examined in *in vitro* assays of CRC function including invasion, proliferation and resistance to apoptosis.

If significant biological consequences are observed, study will transfer to our orthotopic murine model of CRC progression. It is anticipated that stable miR-21 and control miR-SCC transfected fibroblasts and non-transfected fibroblasts will be co-injected with SW620 CRC cells into the caecum of nude mice. AntagomiR-21 or control miR-SCC treatment will start 2 days after tumour implantation through twice weekly tail vein injection at a concentration of 50 mg/kg, in accordance with published protocols.[375]

To mirror the current study, mice will be euthanized at 5 weeks. CRC tumours will be removed, weighed, fixed in formalin; sectioned and stained to assess for stromal, muscular and vascular invasion. Liver and lung metastases will be examined and counted under a dissecting microscope.

4.4.2 Large scale retrospective examination of the prognostic utility of stromal miR-21, stromal miR-556 and epithelial miR-106a expression *in vivo* in stage II CRC.

The finding that deregulated stromal and epithelial miRNAs have prognostic utility in early stage CRC raises the possibility that they may be used in the clinical setting to more accurately predict cancer outcomes and guide decisions around adjuvant therapy.

In the current study the expression of stromal and epithelial miRNAs was examined by qPCR in LMD material from 50 patients with stage II CRC.

However, LMD and subsequent RNA extraction are labour intensive and time consuming processes, and although the 'gold standard' technique, qPCR may not be the preferred method to process large numbers of specimens in a 'scaled-up' study designed for the clinical setting.

Crucially, our collaborators in the commercial sector (Bioneer; Horsholm: Denmark) have developed a highly sensitive and specific ISH technique using high affinity LNA probes against miR-21. Imaging analysis is used to rapidly produce an objective semi-quantitative score based on ISH staining intensity, and in a study of stromal miR-21 expression in 120 patients with CRC this method also predicted short disease free survival in stage II disease (HR = 1.28, 95% CI: 1.06-1.55, p = 0.004).[298]

LNA probes against miR-21 have been optimised however; this is not yet the case for the other miRNAs of interest: stromal miR-556 and epithelial miR-106a. To address this, the author has successfully secured funding by means of a supplementary MRC award and in the coming months antimiR-556 and antimiR-106a LNA probes will undergo optimisation by Bioneer for use with FFPE CRC tissue.

Furthermore, to build on the modest data-set presented in this document, optimised LNA probes against miR-21, miR-556 and miR-106a will be used to examine miRNA expression in CRC on larger scale.

As part of the Follow-up After Colorectal Surgery (FACS) trial (ISRCTN: 41458548), administered through the University of Southampton Clinical Trails unit, we have access to specimens from >1000 patients who underwent surgery for CRC at various

UK centres between January 2003 and August 2009. This extensive tissue archive is linked to a database containing high quality patient demographic, histological and outcome data.[509, 510]

Building on experience with tissue microarrays presented in this study, a TMA containing CRC specimens from several hundred of these patients is under construction. Local ethics approval has been granted for this study and once the TMA is completed, expression of all candidate miRNAs will be examined by ISH.

Crucially, this is not the first time this approach has been used. The expression of various miRNA candidates has been examined in several different tumour contexts previously.[297, 511-513]

If successful, further prospective analysis is planned.

4.4.3 Examine the biological impact of chemokine ENA-78/CXCL5 in CRC stroma.

In the current study expression of the chemokine ENA-78/CXCL5 was upregulated in stromal fibroblasts in the presence of ectopic miR-21. ENA-78/CXCL5 has been shown to act as a potent chemoattractant of pro-tumoural neutrophils in oesophageal cancer,[514] it promotes malignant invasion in cholangiocarcinoma,[515] and it is upregulated in pancreatic malignancies compared with normal pancreatic tissue.[516] Furthermore, ENA-78/CXCL5 activity is strongly induced in murine models of peritonitis, via a mechanism potentiated by MMP2 .[517]

ENA-78/CXCL5 therefore, merits further study because it may provide a link between deregulated stromal miRNA expression, the stromal inflammatory response and CRC initiation and progression; which would be extremely novel.

Initially, a number of simple experiments are proposed. These include: staining CRC TMAs for ENA-78/CXCL5 to correlate expression *in vivo* with patient outcome and with endogenous miR-21; and conducting assays of CRC function in the presence of fibroblasts in which ENA-78/CXCL5 expression has been suppressed (with siRNA) with and without miR-21 rescue.

Depending on the findings of this preliminary work, further detailed mechanistic and functional analysis would be considered to determine the up and down-stream regulatory biology of ENA-78/CXCL5 and better define the relationship with miR-21 *in vivo* within the context of CRC stroma.

4.4.4 Long non-coding RNAs: Emerging players in CRC

Interest in the pathobiology of miRNAs has recently led to the discovery of an entirely novel class of regulatory non-coding RNA. Long non-coding RNAs (lncRNAs) exceed 200 nucleotides in length and act concurrently with DNA-binding proteins to epigenetically regulate DNA transcription.[518-520] Emerging data suggests that lncRNAs participate in key biological processes in both physiology and disease, and that deregulated lncRNA expression may have important consequences during malignant transformation.[521-523] Within the last 18 months, studies have shown that lncRNAs provide clinically relevant prognostic information in a number of tumour contexts.[524-527] Furthermore, George Calin and colleagues have described a single nucleotide polymorphism within the *Colon cancer associated transcript 2 (CCAT2)* lncRNA gene associated with elevated risk of CRC development.[528]

However, the functional significance of lncRNAs, and patterns of deregulated stromal and epithelial lncRNA expression are yet to be fully determined.

In Collaboration with Professor Calin, lncRNA expression in LMD stroma and epithelium from different stage CRC specimens will be examined, as well as paired normal and colon cancer-associated fibroblasts and normal colonic fibroblasts with and without exposure to TGFβ.

Only patients for whom blood, formalin-fixed and frozen tumour tissue alongside adequate histopathological, pre-, intra-, and post-operative clinical information are available will included in this study. CRC stroma and epithelium will be separated using LMD and subsequently profiled with custom-made lncRNA microarrays which have been constructed and pre-optimised in the host laboratory. LncRNA candidates will be selected according to the magnitude of differential expression between groups. The highest scoring lncRNAs will be validated by Taqman®™ qRT-PCR and assessed for biological function.

Identifying and characterising deregulated lncRNAs in the tumour microenvironment represents an opportunity to better understand the biology of CRC metastasis. Furthermore, working in this novel field with significant translational promise, would represent a new paradigm for non-coding RNA research at the University of Southampton.

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