Title	Exosomal microRNAs in colorectal cancer
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Publication Date	2016
Item record	http://hdl.handle.net/10379/5766

Exosomal microRNAs in colorectal cancer

A thesis submitted to the National University of Ireland, Galway as partial fulfilment of the requirements for the degree of Doctor of Medicine

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July 2015

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Acknowledgments

I am grateful to Professor Michael Kerin, Mr Myles Joyce and Dr Roisin Dwyer for affording me the opportunity to undertake this research and for their guidance and support.

The structure and framework for the academic surgery department provided by Professor Kerin is invaluable in promoting an atmosphere conducive to high quality research. Without the support provided by Mr Myles Joyce I would not have had the opportunities, both in academia and my career, which I have been given. Dr Roisin Dwyer's constant support and unwavering work ethic have ensured that all of this work came to fruition.

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Publications:

1. **C Clancy,** MR Joyce, MJ Kerin. The use of circulating microRNAs in   colorectal cancer. Cancer Biomark 2015; 15: 103-13

2. **C Clancy,** S Khan, CL Glynn, E Holian, P Dockery, P Lalor, JAL Brown, MR Joyce, MJ Kerin, RM Dwyer. Characterization and manipulation of colorectal cancer cell exosomal microRNA content in vitro. Manuscript under review

Communications (National):

1. **Secretion of exosome-encapsulated oncomiRs by colorectal cancer cells in vitro. C Clancy**, M Joyce, MJ Kerin, RM Dwyer. Sylvester O'Halloran meeting, University of Limerick, **Plenary Session,** February 28th 2014.

2. **Exosome-encapsulated microRNAs secreted by Colorectal Cancer Cells: mediators of intercellular crosstalk in the tumour micro-environment. C Clancy,** J Brown, E Holian, MR Joyce, MJ Kerin, RM Dwyer. Sir Peter Freyer Surgical Symposium, NUI Galway, **Plenary Session,** September 2014.

3. **The impact of miR-379 in colorectal cancer. C Clancy,** S Khan, C Glynn, J Brown, M Joyce, MJ Kerin, RM Dwyer. Waterford Surgical Club, **Plenary Session,** October 2014

Communications (International):

1. **Analysis of exosome-encapsulated microRNAs secreted by Mesenchymal Stem Cells in vitro. C Clancy,** C Brougham, M Joyce, MJ Kerin, RM Dwyer. Association of Surgeons in Great Britain and Ireland, Harrogate, May 1st, 2014.

2. **Investigation of exosome-encapsulated microRNA secretion in breast cancer.** C Glynn, S Khan, C Brougham, **C Clancy,** D Joyce, P Dockery, MJ Kerin, RM Dwyer. American Association of Cancer Research, San Diego, April 2014.

3. **Colorectal cancer cells actively secrete exosome-encapsulated microRNAs which are associated with epithelial-mesenchymal transition** C Clancy, J Brown, E Holian, MR Joyce, MJ Kerin, RM Dwyer. British Association of Surgical Oncology and European Society of Surgical Oncology, Liverpool, October 2014.

4. **Investigating the impact of miR-379 in colorectal cancer.** C Clancy, S Khan, C Glynn, J Brown, M Joyce, MJ Kerin, RM Dwyer. Society of Academic and Research Surgery, University of Durham, UK, January 2015.

ABSTRACT

Inroduction:

Cancer cells have been shown to release a variety of extracellular membrane vesicles including microvesicles known as exosomes. Exosomes have recently been found to contain microRNAs (miRNAs) however the full range within colorectal cancer cell secreted exosomes is unknown. Profiling exosomal miRNAs released by colorectal cancer cells may provide valuable information on the pathogenesis of cancer and novel miRNAs ideal for further investigation as biomarkers. As exosomes are immunologically inert and easily cross physiological barriers, investigating the potential for exosome mediated delivery of miRNAs may have exciting therapeutic applications.

Methods:

Exosomes were isolated from HCT-116 and HT-29 colorectal cancer cell lines. RNA was extracted from exosomes and microRNA array performed. Cells were engineered to express the breast cancer tumour suppressor miR-379(HCT-116-379) or a non-targeting control (HCT-116-NTC) and functional effects were determined. Exosomes secreted by engineered cells were transferred to recipient cells and the functional impact on recipient cells was examined.

Results:

Microvesicles 40-100nm in size secreted by both cell lines were visualised and confirmed to express exosomal protein CD63. HT29 exosomes contained 409 detectable miRNAs, HCT-116 exosomes contained 393, and 338 were common to exosomes from both cell lines. Selected targets were validated. HCT-116-379 cells showed decreased proliferation (12-15% decrease, $p < 0.001$) and decreased migration (32-86% decrease, $p < 0.001$) compared to controls. HCT-116-379 cell exosomes were found to be enriched for miR-379 compared to those secreted by wild type (WT) and HCT-116-NTC cells indicating successful engineering of miR-379 over-expressing exosomes. Confocal microscopy visualised transfer of HCT-116-379 exosomes to WT cells.

Conclusion:

The data presented show colorectal cancer cells actively and selectively secrete a large number of miRNAs within exosomes. miR-379 decreases colorectal cancer cell proliferation and migration. Exosomes can be engineered to over express miR-379 and miR-379 enriched exosomes can be transferred to recipient cells representing a potential method of gene therapy.

Chapter 1

Introduction

1.1 Colorectal Cancer

1.1.1 Introduction

Colorectal cancer is the third most common cancer worldwide accounting for over 9% of all cancer incidence [1, 2]. In Ireland it is the second most common cancer accounting for 13% of all malignant neoplasms in women and 15% in men [3]. From 2002 – 2007 the incidence of colorectal cancer increased by 4% per year. Recently Bowel Screen, the national colorectal cancer screening programme was introduced in high risk age groups (60 – 69 years currently) to aid in the early detection of tumours as survival is heavily dependent on disease stage. The American Joint Committee on Cancer defines colorectal cancer from Stage I to IV with Stage I describing a tumour confined to the mucosa of the bowel, and Stage IV describing a tumour which has spread to distant sites most commonly including the liver and lungs. Survival in early stage disease internationally is high with a 92% 5 year survival for Stage I disease. 5 year survival for Stage IV disease however is 11% [4].

1.1.2 Colorectal Cancer Screening

Bowel Screen aims to detect colorectal cancers early and is based on the principle of the adenoma-carcinoma sequence which describes a step-wise progression of colorectal cancer resulting from a series of genetic alterations [5]. Each progressive step is related to specific genetic abnormalities in oncogenes and tumour suppressor genes. Genetic mutations accumulated during tumour development cause dysregulation of critical signal transduction pathways such as Wnt and Transforming Growth Factor Beta (TGF Beta) which leads to uncontrolled cell growth and inhibits apoptosis [6]. Knowledge of the natural history of colorectal cancer and the ability to recognise histological progression allows screening and early intervention. Bowel screen currently issues a Faecal Immunohistochemical Test (FIT) to high risk age groups every 2 years to detect traces of blood in the stool. If this is positive, further investigation with a colonoscopy is performed which can identify adenomatous polyps prior to malignant transformation or early tumours. Some studies have suggested FIT do not detect cancers in the proximal bowel as accurately as those in the distal bowel [7]. Even when screening is performed, cancers and treatable neoplasms are missed, and increasing evidence suggests that important pathology is missed more often than was previously thought, highlighting a need for development of adjuncts to further improve the accuracy of diagnostic testing [8-10].

1.1.3 Colorectal Cancer Treatment

If a colorectal cancer is discovered, further radiological investigations to identify disease stage are performed. If there is no metastatic disease the gold standard treatment is radical resection of the bowel segment containing the tumour taking the blood supply of the bowel close to its origin from the aorta. The majority of draining lymph nodes in the attached mesentery are closely related to its blood supply and will be removed using this approach. This allows assessment of the draining lymph nodes and determines if chemotherapy is required following surgery. This is known as total mesocolic and total mesorectal excision for colon and rectal cancers respectively. In rectal cancers, treatment before surgery with radiotherapy and chemotherapy may be required to reduce the size of tumours which are invading surrounding structures. Chemotherapeutic agents are used following surgery if there is evidence of spread of the tumour into surrounding lymph nodes or features suggestive of this. Unfortunately, approximately 20 – 25% of patients newly diagnosed with colorectal cancer will have distant metastases at first presentation (stage IV according to Tumour Nodes Metastases (TNM) criteria) [11]. In select cases surgical resection of isolated metastases is possible but many of these patients (75 - 90%) have metastatic disease which is not amenable to resection [12]. Until recently, 5-fluorouracil (5FU) was the main basis of chemotherapy in colorectal cancer for these patients. Its activity as a single agent in the treatment of colorectal cancer is modest, with an overall tumour response rate of 10% to 15% [13]. Several biochemical modulators have been used to enhance the activity of 5FU. Among these, Folinic acid was found to improve the response rates to about 30% to 40% [13]. During the past 10 years, chemotherapeutic agents including Oxaliplatin, Irinotecan, Anti-Epidermal Growth Factor Receptor agents such as Cetuximab in select patients, Bevacizumab, Aflibercept and Regorafenib have been approved as an addition to the traditional 5-FU treatment [14-17]. These advances in chemotherapeutics, however, only account for an improvement in median overall survival from 12-22 months [18]. This highlights a need to further develop our understanding of the processes leading to advancement of metastatic disease so that therapies which halt its progression or reverse disease states may be introduced.

1.2 Intercellular communication in the tumour microenvironment

Inter-cellular communication is a complex process responsible for maintaining normal tissue homeostasis and can be altered in disease processes such as cancer. Communication in the tumour micro-environment occurs through cellular junctions, adhesions and paracrine release of soluble proteins (Figure 1) [19]. Tumours are comprised of two interactive components, parenchyma and stroma [20]. Tumour epithelial cells themselves represent the parenchyma, whereas the stroma is comprised of a mixture of non-malignant cells such as stromal cells and connective tissue elements. The relationship between tumour epithelial cells and surrounding stromal cells appears to have significant implications in tumour development and metastasis. Experimental data imply that stromal cells exposed to tumour epithelium could be "activated" and acquire an inductive potential driving the subsequent neoplastic process [21]. Cancer epithelial cells are capable of promoting pro-tumorigenic features such as angiogenesis through interaction with surrounding cells including paracrine signalling which can potentially promote metastasis [22, 23]. Further development of the understanding of intercellular communication between these two tumour components may identify factors leading to tumour growth and development of metastases. In addition to communication with surrounding cells, there is growing evidence that cancer cells secrete soluble growth factors which may prime distant tissues for engraftment creating what is called a 'pre-metastatic niche'[24-26]. In fact, media conditioned by tumour cells has been shown to specifically direct target organ preparation for pre-metastatic niche initiation [24]. Intercellular communication in the tumour micro-environment and with distant cells appears key in the progression of cancer. In addition to release of soluble proteins, multiple cell types including colorectal cancer cells have been shown to release a variety of extracellular membrane vesicles including vesicles known as exosomes. They have been recognised as vehicles capable of facilitating intercellular communication [27]. Emerging evidence indicates that exosomes may play a role in a range of biological processes including cell proliferation and migration through horizontal transfer of their contents from donor cells to recipient cells [28, 29]. Interestingly, exosomes have recently been found to contain microRNAs (miRNAs) (Figure 1.1) [30].
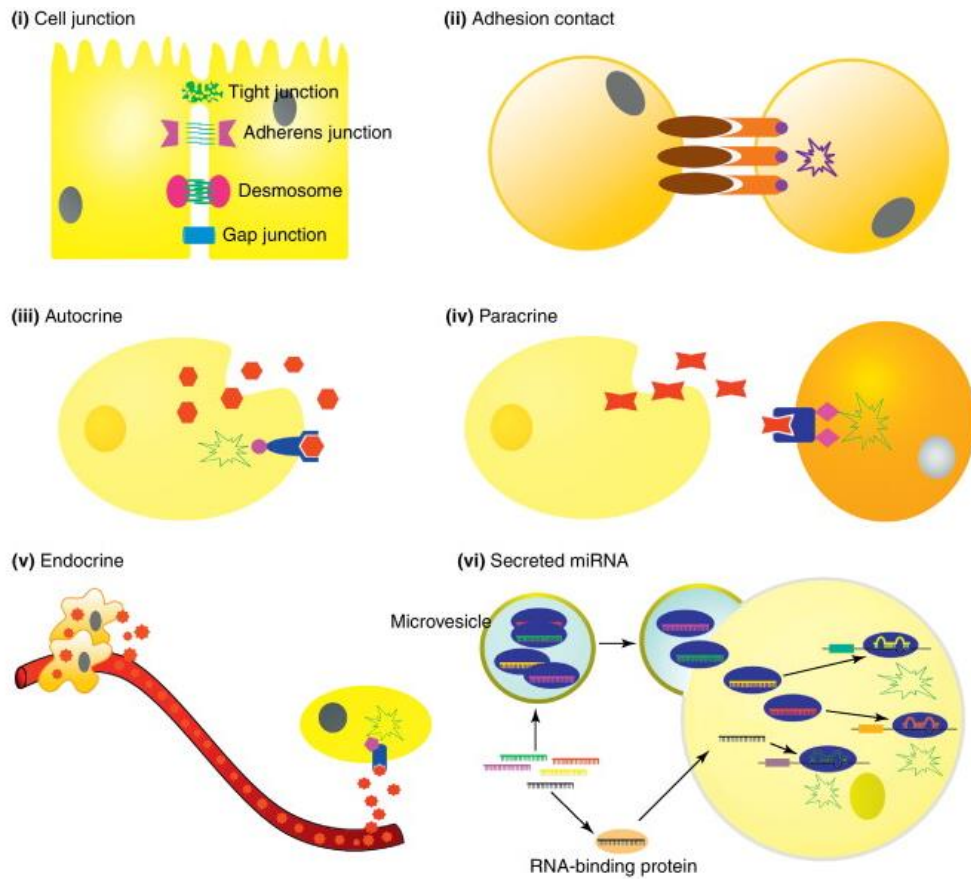
Figure 1.1: Methods of Intercellular Communication (Chen X et al. 2012) [31] i – Cell junctions allowing exchange of intra-cellular signalling molecules **, ii –** Direct adhesion between a cell surface signalling molecule on one cell and a membrane bound receptor on another, **iii –** Soluble messengers are released which act on the cell of origin (autocrine), **iv –** Soluble messengers are released which act on adjacent cells (paracrine), **v –** Communication with distant cells via soluble messengers (endocrine), **vi –** secreted miRNA mediated intercellular communication.

1.3 Exosomes

1.3.1 Defining Characteristics and Current Perspectives

The term exosome initially incorporated vesicles that contain a 5' nucleotidase activity, ranging in size from 40 to 1,000 nm released by a variety of cultured cells, and was described in 1981 [32]. The term was later applied to vesicles 40-100 nm in size released during reticulocyte differentiation as a consequence of multivesicular endosome fusion with the plasma membrane [27]. The current definition of exosomes dictates that they are vesicles 40-100nm in size, containing characteristic tetraspanin proteins (CD63, CD9 and CD81) originating from endosomes, and released by multivesicular endosome fusion with the plasma membrane (Table 1.1) [33]. Strict criteria to define exosomes are required as a number of vesicular bodies including microvesicles and apoptotic bodies are released by almost every cell type [33]. While apoptotic bodies are easily distinguished based on size, origin, and surface markers, there is some difficulty in the distinction between exosomes and microvesicles. Both are membrane bound vesicles but differ based on their process of biogenesis and biophysical properties [34]. In contrast to exosomes, microvesicles are produced directly through the outward budding and fission of membrane vesicles from the plasma membrane. Their surface markers largely depend on the composition of the membrane of origin. In addition, microvesicles represent a larger and more heterogeneous population of extracellular vesicles, ranging from 50 to 1000 nm in diameter (Table 1.1) [34].

Interest in exosomes has increased exponentially in recent times as a result of the discovery that exosomes are messengers that can regulate cellular processes by horizontal transfer of molecules from cells of one tissue to another. Exosomal content has been shown to be composed of different types of cytokines, growth factors, proteins, or nucleic acids. Besides messenger RNA they can also contain microRNAs (miRNAs) which are small endogenous cellular regulators of protein expression [35]. Exosomes thus have the potential to facilitate tumour progression by supplying the tumour niche locally and systemically with genetic contents and molecules that promote processes such as proliferation, invasion and metastasis, or even drug resistance [33]. As exosomes are naturally equipped for genetic transfer, are highly stable and carry contents reflective of their donor cells, they have significant potential as carriers of biomarkers and vehicles for therapeutics.

Extra-Cellular Vehicles	Size	Biogenesis	Characteristics	Content	Role
Exosomes	40-100nm	MVB fusion with plasma membrane	Homogenous in size. Markers:CD9, CD63, CD81	Protein, lipids, RNA, miRNA	Intercellular communication
Microvesicles	50-1000nm	Directly from plasma membrane	No characteristic markers	Protein, lipids, RNA, miRNA	Intercellular communication
Apoptotic bodies	50-5000nm	Breach of cell fragments	Heterogeneous in size. Markers: Annexin-V	Fragmented nuclei, cytoplasmic organelles, DNA fragments	Activated due to cellular stress/injury

Table 1.1: Characteristics of vesicular bodies (adapted from Braicu et al. 2015) [33]

1.3.2 Exosome Biogenesis

Exosomes are formed as Intra Luminal Vesicles (ILVs) by inward budding into early endosomes and Multivesicular Bodies (MVBs - characterised by the presence of vesicles in their lumen which are formed by inward budding). The mechanism of inward budding is based on 2 distinct pathways, the Endosomal Sorting Complex Required for Transport (ESCRT) dependant pathway, and the non-ESCRT dependant pathway [36-39]. The ESCRT consists of four complexes plus associated proteins responsible for cargo clustering, inducing bud formation, and vesicle release [40]. ESCRT-independent mechanisms involve lipids, tetraspanins, or heat shock proteins including CD63 and CD81 [39]. MVBs are either fused with lysosomes intra-cellularly, or fused with the plasma membrane leading to release of their contents into the extra-cellular compartment [40]. Release of exosomes from MVBs appears to be heavily governed by the RAB family of small GTPase proteins which control different steps of intracellular vesicular trafficking, such as budding, mobility through cytoskeleton interaction, and docking of vesicles to their target compartment, leading to membrane fusion (Figure 1.2) [41].
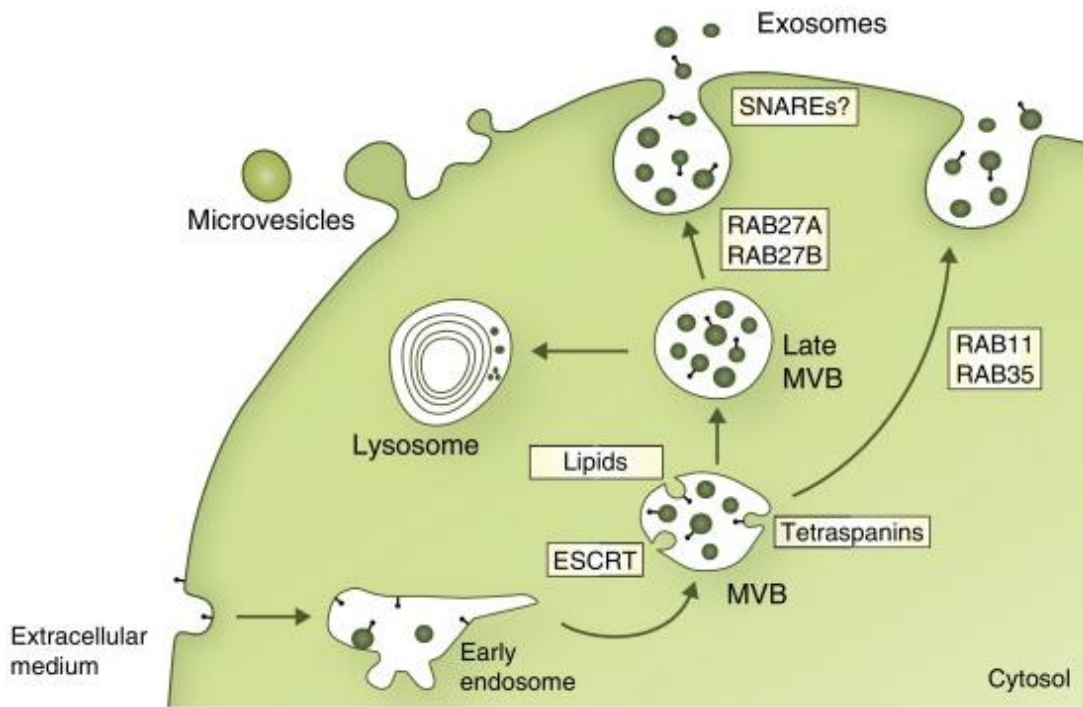
Figure 1.2: Intracellular mechanisms of exosome biogenesis and secretion (Kowal et al. 2014) [39]. Exosomes are formed as ILVs by inward budding into early endosomes and MVBs. The mechanism of inward budding is based on 2 distinct pathways, the ESCRT dependant pathway and the non ESCRT dependant pathway (lipids, tetraspanins, or heat shock proteins). MVBs are either fused with lysosomes or fused with the plasma membrane base on interaction with RAB proteins [39].

1.3.3 Current methods of exosome isolation and characterisation

As research interest in exosomes has grown due to their discovery as mediators of intercellular communication, a variety of methods have been developed to isolate them from a range of biological fluids. Once isolated, exosomes must then be characterised to differentiate them from microvesicles and apoptotic bodies. The original method of exosome isolation from culture medium involves differential centrifugation and ultra-centrifugation and remains the gold standard for in vitro isolation [42]. Differential ultracentrifugation is employed to first remove intact cells and bulky cell debris by low g force centrifugation (e.g., 500g, 2000g) followed by high g force (e.g.,

100,000g) to sediment exosomes. Microfiltration wth 0.1 μm [43] or 0.22 μm [40] filtration or inclusion of an intermediate g force centrifugation step (e.g., 60,000g) to remove shed microvesicles (500–2000 nm diameter) is also used [44] in combination with differential centrifugation. To separate protein aggregates from exosome isolates some advocate the use of a sucrose gradient for centrifugation [42]. A number of additional methods have been described recently including magnetic immune-isolation using a specific exosomal cell surface protein which can be identified that discriminates an exosome of interest from other membranous particles present in the biological matrix. This method is employed by many of the commercially available kits which allow rapid exosome isolation without the need for ultra-centrifugation. [45]. Further described methods include high-pressure liquid chromatography-gel exclusion chromatography (HPLC-GEC), solvent precipitation and field flow fractionation [46].

Exosome characterisation relies on confirming vesicle size and the presence of exosomal proteins. Their size and morphology is best visualised with Transmission Electron Microscopy (TEM). Some studies have suggested that exosomes display a cup shape morphology when visualised whole with electron microscopy but others dispute that this is due to collapsing while drying [42]. Complementary to transmission electron microscopy, nanoparticle tracking analysis with high resolution flow cytometry allows determination of the size distribution of isolated exosomes based on the Brownian motion of vesicles in suspension [47]. Further characterisation using immunoblotting to detect exosome-associated proteins such as those from the tetraspanin family (CD9, CD63, CD81) is required to fully characterise exosomes and confirm their presence [40].

1.3.4 Exosome Packaging and Contents

Exosomal content has been identified by approaches such as western blot, mass spectrometry, fluorescence-activated cell sorting and immunoelectronic microscopy. These techniques have demonstrated that exosomes contain a variety of cytokines, growth factors, lipids, a large number of proteins and thousands of RNA species including miRNAs [48-50]. Available proteomic studies define a subset of cellular proteins that are targeted specifically to exosomes and they do not contain subsets of random proteins as is the case for cellular debris [40]. In addition, studies have shown

protein content of cancer derived exosomes in various body fluids to differ from that of healthy controls furthering the evidence that exosomal content reflects its donor cell and is selective [51]. The characterisation of lipid content of exosomes has not been extensive but interestingly shows that exosomes are enriched in cholesterol, sphingomyelin, ceramide and phosphatidylserine, but not in lysobisphosphatidic acid, a lipid described in MVB ILVs suggesting even selective packaging of lipid content. Lipid content of exosomes also differs significantly from that of whole cell membranes further supporting this [52]. A wide variety of RNA species are present in exosomes including mRNA and miRNA [53]. While exosomes contain a substantial amount of small RNAs they contain little or no ribosomal RNAs compared to levels observed in donor cells. In addition miRNA profiles of exosomes do not appear to match with those of their donor cells again supporting selective packaging [35, 54]. As information on the content of exosomes is continuously being updated, a public databank of protein, RNA and lipid content of exosomes has been set up and to date has information on 11,261 protein entries, 2375 mRNA entries and 764 miRNA entries that were obtained from 134 exosomal studies [55].

Little is known of the mechanisms of selecting the content of exosomes but it is believed to be highly regulated by the group of proteins involved in their biogenesis (ESCRT and non ESRCT complexes) [56, 57]. These complexes appear to determine the relative abundance of genetic information that will be packaged and transported within exosomes. The selection of proteins packaged into exosomes is affected by the status of the donor cell and the subcellular compartment of origin [58]. The ESRCT complex and an ESRCT-independent mechanism largely dependent on the protein CD63 have been shown to sort proteins into exosomes suggesting exosome packaging is regulated at multiple levels [59, 60]. The mechanism of sorting nucleic acids including DNA, RNA, mRNA and miRNA into exosomes remains unclear.

1.3.5 Exosomes as Delivery Vehicles in Physiological and Disease States

Genetic information was previously believed to be transferred through 2 mechanisms: vertical transfer which describes gene exchange from a parent cell following division and horizontal transfer from viruses [61, 62]. The discovery that exosomes contain protein and RNA including miRNA which can be functionally delivered between cell types represents an additional method of horizontal gene transfer which appears to

play a role in a range of biological processes. Exosomes derived from a variety of immune cells impact on a number of functions including T cell activation, tolerance induction and dendritic cell maturation [63 – 66]. In the nervous system, exosomes derived from neurons transmit information in the form of proteins to facilitate neural circuit function [67, 68]. It has even been suggested that exosomes delivered to infants through breast milk which are enriched in immune and developmental-related RNAs may play a role in development of the immune system in the digestive tract [69, 70].

In addition to normal physiological processes, exosome involvement in disease states has also been identified. Several studies have found there is a significant increase in the production of extra-cellular vesicles including exosomes in disease states compared to non-disease states [71-73]. Exosomes are implicated in the propagation and spread of neurodegenerative diseases such as Alzheimer's disease through delivery of β-amyloid precursors to distant parts of the brain leading to pathological amyloid deposition [74]. Exosome secretion from eosinophils has been found to be increased in asthmatic patients and induces enhanced proliferation and chemotaxis of undifferentiated macrophages in the lungs during acute asthmatic inflammatory conditions [75, 76]. In cancer, exosomes have been found to play a significant role. Melanoma-derived exosomes promote metastasis, exosomes derived from fibroblasts encourage migration of breast cancer cells and exosomes derived from cancer cells have a pro-tumorigenic role associated with the transfer of mRNA and proangiogenic proteins [77, 78]. Exosomes derived from cancer cells can also contribute to a horizontal transfer of oncogenes, such as EGFRvIII [79]. One of the most interesting aspects of exosomes involvement in cancer is their ability to transfer miRNAs to recipient cells.

1.4 microRNAs

1.4.1 Discovery and Nomenclature

miRNAs are a class of small non-coding, naturally occuring RNA molecule 19-25 nucleotides in length [80]. They were first discovered in 1993 by Ambrose et al. [81] when they discovered a small RNA which exerted regulatory functions on a specific mRNA resulting in suppression of its action. This small RNA was subsequently discovered to be a member of an abundant family of tiny regulatory RNAs called miRNAs. The importance of miRNAs as regulatory molecules has become

increasingly obvious as more miRNAs are discovered and their regulatory targets are elucidated. Functional studies have shown miRNAs to participate in almost every cellular process including apoptosis, proliferation and differentiation [82]. In fact, single miRNAs may regulate multiple target genes acting as a master control of gene expression [83]. Although miRNAs constitute only 1-3% of the human genome, it is suggested that they regulate up to 30% of human genes [84]. Since their discovery, over 2000 miRNAs have been identified in humans and this number continues to rise [85]. As such a large number of miRNAs have been discovered in a short space of time, strict rules apply to their nomenclature and a comprehensive public database named miRBase has been established to record all validated miRNAs [86]. The first three letters specify the organism (hsa-miR-379 for example is homosapien miR-379). The numbering of miRNAs is sequential in the order they are discovered. Lettered suffixes such as miR-121a or miR-121b denote closely related miRNAs. If 2 microRNAs originate from the same precursor, the miRNA originating from the 5' arm of the precursor is called miR-149-5p and the one originating from the 3' arm is denoted miR-149-3p. Some names have been retained for historical reasons such as the let-7a miRNA. The most up to date version of miRBase (miRBase version 21) contains 24,521 miRNAs from 206 species. 2,588 of these are within humans [86].

1.4.2 miRNA Biogenesis and Action

miRNA biogenesis occurs in three phases. The first phase involves transcription of primary transcripts. RNA polymerase II or III transcribes large primary (Pri) miRNAs in the nucleus. Pri-miRNAs are several hundred or thousand nucleotides in length and contain at least one miRNA stem loop [87]. This single unit may contain up to six precursor (pre-) miRNAs, which are produced by the cleaving action of the RNase III enzyme Drosha, combined with the microprocessor complex subunit, a double stranded binding partner called the DiGeorge Syndrome Critical Region 8 (DGCR8). Pre-miRNAs are between 70 – 90 nucleotides in length. Critically, they contain a hairpin structure required for their transport to the cytoplasm from the nucleus, mediated by Exportin-5 which is the second phase of biogenesis [88]. The third phase of biogenesis occurs in the cytoplasm and results in formation of mature miRNAs. Once in the cytoplasm the hairpin structure can be cropped by the RNAase III enzyme Dicer, to produce a double-stranded structure consisting of the miRNA and its complement. This multi-step process culminates in the mature miRNA strand being

incorporated into a miRNA associated RNA-induced silencing complex (miRISC). miRISC interacts with target mRNAs to exert functional effects [89, 90] (Figure 1.3).

miRNAs exert their function on target mRNAs through 2 mechanisms, degradation of a selected mRNA or silencing of protein translation. If the target mRNA and miRISC have perfectly matched base pairing, the mRNA is cleaved and degraded by activation of an RNA mediated interference pathway. More commonly, miRNAs exert their effect by repressing protein translation which occurs when they imperfectly bind to partially complementary sequences in the 3' untranslated region (UTR) or 5' UTR of target mRNAs [90, 91]. Each miRNA may exert these effects on several mRNAs resulting in their silencing or degradation.
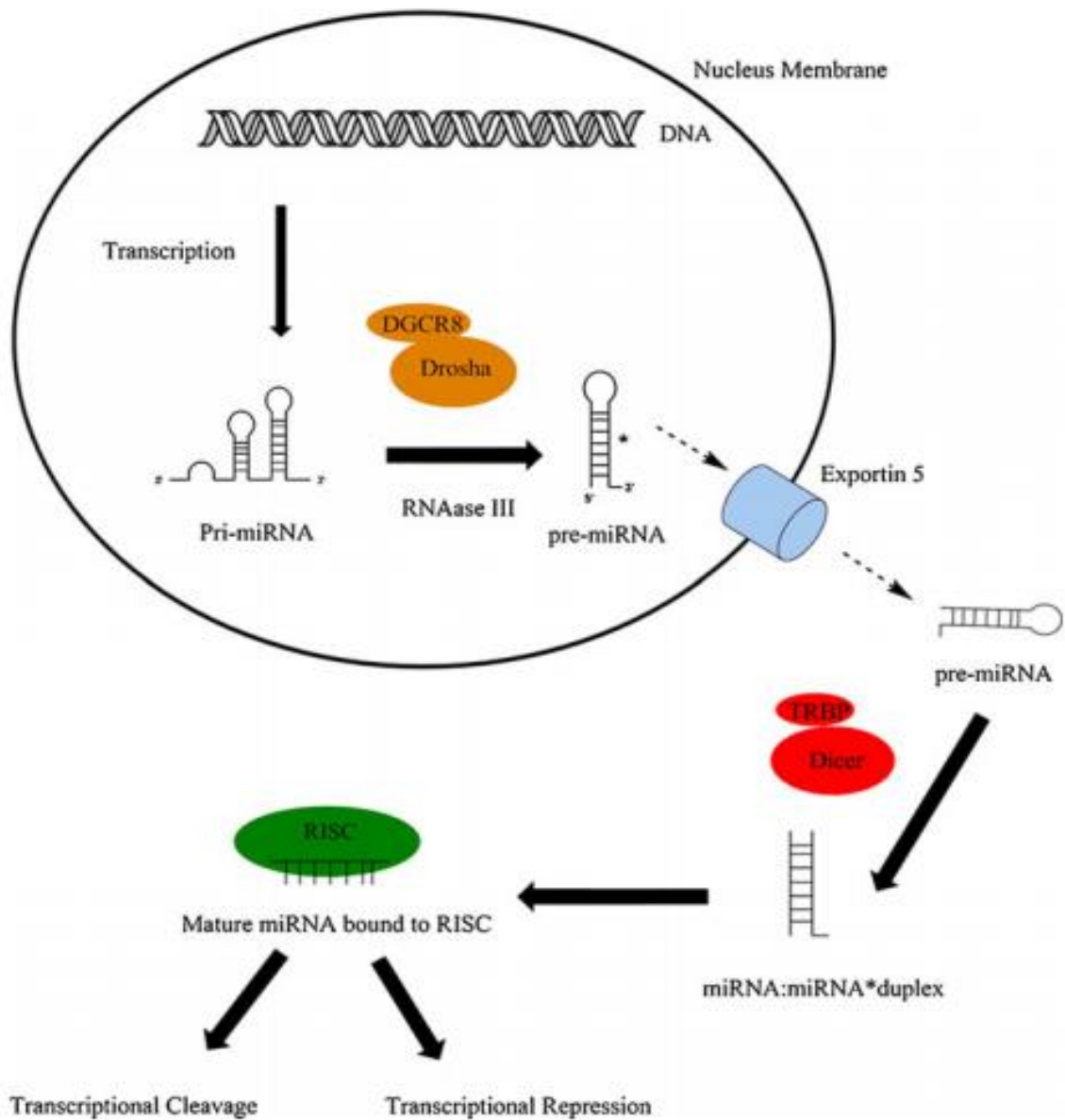
Figure 1.3: miRNA biogenesis and processing (adapted from McDermott AM et al 2011)[90]. **Phase 1 -** transcription of a large primary (pri-) miRNA. This large pri-miRNA is then cleaved by the RNase III enzyme Drosha and coupled with the microprocessor complex subunit DGCR8 to produce pre-miRNA**. Phase 2 -** Pre-miRNAs are transported to the cell cytoplasm by Exportin-5. **Phase 3 -** the hairpin structure is cropped off in the cytoplasm by the RNase III enzyme, Dicer and the mature miRNA strand is incorporated into a miRNA associated RNA-induced silencing complex (miRISC).

1.4.3 miRNAs in Normal Physiology and Homeostasis

miRNA expression has been found to be involved in numerous cellular events during animal development where it is pivotal for the timing and regulation of many key processes such as cell fate determination, proliferation, and cell death [92]. Functional roles for miRNAs have been identified in multiple biological processes, such as immune response, insulin secretion, neurotransmitter synthesis and circadian rhythm [93-95].

miRNAs are also involved in the maintenance of homeostasis in the intestine. MiR-29a modulates small bowel and colon epithelial cell permeability and regulates mucosal epithelial cell proliferation [96]. Epithelium expressed miRNAs in the colon such as miR-375 are key regulators of epithelial cell properties that are necessary for securing epithelium-immune system cross talk which maintains gut immunity and homeostasis [97]. The vast majority of studies relating to miRNAs and the intestine, however, focus on colorectal cancer.

1.4.4 miRNAs and Cancer

The first study suggesting miRNA involvement in cancer was published just over a decade ago. Calin et al. [18] identified loss of miR-15a and miR-16-1 to occur frequently in association with a deletion at chromosome 13q14, a region frequently deleted in human B-Cell Chronic Lymphocytic Leukaemia. A large number of miRNAs were subsequently found to be dysregulated in a broad spectrum of cancers. Cancer-specific expression patterns of miRNAs reflect mechanisms of cellular transformation and can provide a new insight into carcinogenesis.

The main focus of miRNA research in cancer has been in the identification of up- or down-regulated levels of miRNAs in the tissues, circulation or biological fluids of cancer patients. Reduced levels of mature miRNAs [98] often appear in tumours as a consequence of genetic loss, epigenetic silencing, and defects in their biogenesis pathway or widespread transcriptional repression [99]. Dysregulated miRNAs in tissue may act as prognostic indicators and may identify tumours more likely to respond to radiotherapy or chemotherapy. In colorectal cancer a large variety of miRNAs have been found to be up or down regulated in tumour compared to normal tissues (Table 1.2). Some of these dysregulated miRNAs in colorectal cancer tissues

have been shown to correlate with clinicopathological characteristics. MiR-21, one of the most extensively investigated miRNAs in colorectal cancer tissues, has been found to correlate with lymph node positivity and poor survival [100, 101]. A number of miRNAs such as miR-16, miR-153 and miR-590-5p have been shown to predict complete versus incomplete response to neoadjuvant chemoradiotherapy [102]. Although none have yet been incorporated into clinical practice, the identification of tumour suppressor and promoter miRNAs provides valuable information on cancer pathogenesis and can expedite the application of miRNAs in therapeutics. While prognostic information provided by tissue miRNAs is an exciting prospect, there has been an exponential increase in the number of studies looking at the potential to use circulating miRNAs as minimally invasive tools for disease diagnosis.

let 7a	miR-29a	miR-124a	miR-137	miR-181b	miR-338
miR-1	miR-29b	miR-125a	miR-142-3p	miR-182	
miR-9	miR-31	miR-126	miR-143	miR-183	
miR-17	miR-34a	miR-128a	miR-145	miR-192	
miR-17-3p	miR-92	miR-128b	miR-146a	miR-195	
miR-17-5p	miR-95	miR-133b	miR-148a	miR-200c	
miR-19a	miR-100	miR-124a	miR-137	miR-203	
miR-21	miR-101	miR-135a	miR-154	miR-328	
miR-25	miR-106a	miR-135b	miR-155	miR-335	

Table 1.2: Dysregulated miRNAs in colorectal cancer tissue; miRNAs found to be dysregulated in colorectal cancer tumour specimens compared to normal tissue (adapted from Hogan et al. 2012) [103]

1.4.5 Circulating miRNAs

Current methods of colorectal cancer screening rely on the use of faecal immunohistochemical testing which can be associated with poor uptake by the public and can fail to detect certain cancers, particularly in the proximal colon. There is significant research ongoing to identify biomarkers of colorectal cancer or advanced adenomas which may increase screening accuracy. In 2008, it was discovered that miRNAs are also present in blood, where they were detected in plasma, platelets, erythrocytes, and nucleated blood cells [104]. Plasma miRNAs are remarkably stable

even under conditions as harsh as boiling, low or high pH, long-time storage at room temperature, and multiple freeze-thaw cycles [105]. These properties make miRNAs ideal tumour markers for early detection in colorectal cancer. Circulating levels of biomarkers such as miRNAs could also be used to monitor response to treatment such as neoadjuvant chemo-radiotherapy in rectal cancer. Similar to tissue miRNAs, a large number of circulating miRNAs have been found to be dysregulated in colorectal cancer although none have yet been incorporated into clinical practice (Table 1.3) [106].

Dysregulated circulating miRNAs	Studies	No of CRC patients	No of healthy controls	Median age	TNM stage (I/II/III/IV)	Endogenous control	Source of miRNA
miR-18a (upregulated)	Luo X et al. 2013	80	144	68	22/25/26/5	miR-16	Plasma
	Vega A et al. 2013	30	26	64	0/0/30/0	miR-16 / let7a / miR-103	Serum
	Giraldez M et al. 2013	53	82	63	8/13/16/5	miR-16	Plasma
miR-21 (Upregulated)	Wang B et al. 2012	32	39	63	NA	miR-16	Plasma
	Kanaan Z et al. 2012	20	20	57	4/7/4/5	RNU6B	Plasma
	Liu G et al. 2013	200	80	51	18/96/64/22	miR-16	Plasma
	Luo X et al. 2013	80	144	68	22/25/26/5	miR-16	Plasma
	Toiyama Y et al. 2013	186	53	67	45/57/43/41	cel-miR-39	Plasma
miR-29a (Upregulated)	Huang Z et al 2010	100	59	61	27/25/38/10	miR-16	Plasma
	Vega A et al. 2013	30	26	64	0/0/30/0	miR-16 / let7a / miR-103	Serum
	Luo X et al. 2013	80	144	68	22/25/26/5	miR-16	Plasma
	Hofsli E et al. 2013	40	10	70	3/5/47/14	NA	Serum
	Giraldez M et al. 2013	53	82	63	8/13/16/5	miR-16	Plasma
miR-92a (Upregulated)	Ng E et al. 2009	90	50	71	6/34/23/27	RNU6B	Plasma
	Huang Z et al. 2010	100	59	61	27/25/38/10	miR-16	Plasma
	Liu G et al. 2013	200	80	51	18/96/64/22	miR-16	Serum
	Luo X et al. 2013	80	144	68	22/25/26/5	miR-16	Plasma
	Hofsli E et al. 2013	70	20	70	3/5/47/14	NA	Serum
miR-143 (Downregualted)	Qian X et al. 2013	41	10	NA	NA	cel-miR-39	Plasma
	Luo X et al. 2013	80	144	68	22/25/26/5	miR-16	Plasma
	Hofsli E et al. 2013	40	10	70	3/5/47/14	NA	Serum
miR-378 (Upregulated)	Hofsli E et al. 2013	40	10	70	3/5/7/15	NA	Serum
	Zanutto S et al 2014	24	19	NA	NA	miR-16	Plasma

Table 1.3: Circulating miRNAs found to be dysregulated in colorectal cancer; miRNAs found to be abnormal in the circulation of colorectal cancer patients in 2 or more studies [adapted from Clancy C et al 2015] [106-116].

The use of circulating miRNAs as biomarkers has been hindered by a number of different variables which can lead to heterogeneity among different study results. Dysregulated circulating miRNAs such as miR-18a, miR-21, miR-29a and miR-143 have been identified as promising biomarkers in colorectal cancer in several studies [106]. These results were not found to be reproducible in several other studies however [85, 117, 118]. Issues such as pre-existing patient factors including disease states, smoking and ethnicity combined with differing views on the optimum medium from which miRNA should be extracted (whole blood/serum/plasma), and problems with haemolysis secondary to collection and storage have led to heterogeneous data. Recent studies have reported a number of miRNAs such as miR-21, miR-29a and miR-92a believed to be released by colorectal tumours as circulating markers of disease, are expressed by red blood cells and may be present in the plasma and serum secondary to haemolysis due to collecting procedures or storage methods [115, 119]. Whether circulating miRNAs which appear dysregulated in cancer are secreted by the tumour itself or derived from another source is still not clear. Interestingly, in contrast to the stability of miRNAs endogenous to body fluid samples such as plasma, when synthetic miRNAs are added exogenously, they are quickly degraded by the high levels of RNase activity in plasma suggesting endogenous plasma miRNAs are protected in some manner to prevent their degradation [104]. It has been suggested that this protection is provided by vesicles including exosomes.

1.4.6 Therapeutic Potential of miRNAs

Sine abnormal miRNA expression appears in many disease states including cancer, it is possible that miRNAs could be used as therapeutic targets or as therapies themselves. For disease or tumour-promoting miRNAs, anti-miRNA inhibition of over-expressed miRNAs can be applied [90, 120]. Single stranded chemically modified miRNA antagonists can be delivered systemically and do not require a delivery vehicle. When delivered systemically they distribute to diverse tissue types such as the kidney, liver, lymph nodes, bone marrow and spleen [121]. Experimentally, it has been demonstrated that miRNA inhibition is possible in animal models. Inhibition of miR-122, a miRNA abundant in the liver, via systemic administration of a miR-122 antisense oligonucleotide can result in reduced levels of plasma cholesterol and decreased hepatic fatty acid and cholesterol synthesis in normal mice [122]. Therapeutic miRNA inhibition has also been demonstrated in

primates. Inhibition of miR-122 in primates, which is essential for replication of Hepatitis C virus in liver cells, resulted in an approximate 80% reduction in replicating Hepatitis C viral RNAs [123]. Data thus far shows selective inhibition of miRNAs can provide therapeutic benefits in animal models.

For miRNAs which are reduced in disease states, re-introducing or replacing them could provide a therapeutic benefit through restoration of regulation of target genes. In addition, delivery of disease or tumour-suppressors could provide therapeutic benefits. Replacement strategies and gene therapy with tumour suppressors, however, require delivery vehicles. miRNAs represent ideal molecules for use in gene therapy as they exert their function once in the cytoplasm of the targeted cell. There are several experimental examples of tumour suppressor miRNA therapeutic efficacy in animal models. Let-7 is a miRNA which exerts its effect on the RAS oncoprotein and is found to be expressed at lower levels in non-small cell lung cancer tissue compared to normal lung tissue [124, 125]. Functional studies in mouse models have shown delivery of let-7a blocks proliferation of cancer cells and reduces growth of the existing tumour [126]. In another experiment in a murine model, systemic delivery of miR-34a, a tumour suppressor miRNA found in most human cancer types and acting of the p53 pathway, led to suppression of miR34a target genes in the lung tissue and inhibited tumour growth [127]. The miR-34a mimic was delivered in a lipid-containing formulation.

Concerns over toxicity related to systemic delivery of miRNAs have been raised. The effects of accumulation of exogenous miRNAs in normal cells and tissues is not known. It has been suggested that toxic effects could occur due to overloading of cellular machinery with exogenous miRNAs resulting in competition with endogenous miRNAs which are essential for normal cellular functions and homeostasis. In addition, miRNAs target multiple mRNAs and certain miRNAs with desired effects on one target gene may produce a deleterious effect on a multitude of other genes. While there is theoretical basis for concerns, studies in murine models of systemically delivered miRNAs have failed to demonstrate any serious adverse effects thus far [124, 125]. Overall, miRNA therapeutics appears a highly promising new method of gene therapy. The most significant challenge, however, lies in the area of in vivo delivery. A vehicle to deliver miRNAs to recipient cells is required. Exosomes thus

represent an excellent potential delivery vehicle for therapeutic miRNAs as they easily cross biological barriers, are naturally taken up by cells and contain miRNAs.

1.4.7 miR-379

miR-379 has been identified as a tumour suppressor in breast cancer through previous work in our laboratory [128]. miR-379 is located on chromosome 14q32, 31 and is involved in regulation of interleukin-11 production in breast cancer cell lines [129]. Work in our laboratory found in an examination of over 100 breast cancer tissue specimens compared to a control group of over 30 normal tissue specimens miR-379 levels were decreased in breast tumours. In addition, with increasing tumour stage, the level of miR-379 expression further decreased. Over-expression of miR-379 reduced breast cancer cell proliferation likely due to negative regulation of Cyclin-B1, a key initiator of mitosis in breast cancer [128]. Nothing is known of the effect of miR-379 in colorectal cancer. Given the effect of miR-379 on Cyclin-B1 and evidence that Cyclin-B1 regulated genes such as E-Cadherin which play a pivotal role in control colorectal cancer cell metastasis, further investigation of the effect of miR-379 in colorectal cancer was undertaken [130].

1.5 Exosome Encapsulated miRNAs

1.5.1 Discovery and Current Perspectives

In 2007 Valadi et al. [35] published a landmark study demonstrating that exosomes contained miRNA which could be delivered to another cell and be functional in its target cell representing an entirely new and previously unexplored method of inter-cellular communication. It has been further demonstrated that miRNAs are selectively packaged into exosomes rather than randomly inserted [131]. The exact mechanism of sorting miRNAs into exosomes has not been fully elucidated. From both deep sequencing and relative quantification polymerase chain reaction (RQ-PCR) reactions it appears that miRNA content in exosomes is not a reflection of cellular RNA. Despite the fact that relative amounts of miRNA released in exosomes is low comparative to cellular content, miRNAs can exert significant effects in target cells as they may regulate multiple genes [132].

The discovery that miRNAs are selectively packaged into exosomes and exert functional effects on recipient cells has fascinating implications for miRNA research particularly in cancer. It is hypothesised that tumour cells secrete a large number of exosomes and a higher number of exosomes in circulation is associated with an unfavourable prognosis [133, 134]. It has also been demonstrated in some studies that circulating levels of certain miRNAs in the serum or plasma correlate with poor prognosis or advanced disease stage such as miR-25a in oesophageal cancer and miR-205 in cervical cancer [135, 136]. A functional role for circulating miRNAs contained within exosomes has become apparent which may partly explain certain circulating miRNAs correlation with prognosis or advanced stage. Proof that circulating exosome encapsulated miRNAs can exert function on recipient cells has been shown in a small number of studies. Zhang et al. [137] found in human blood cells and a cell line derived from a leukaemia patient (THP-1 cells) that miR-150 was selectively packaged into exosomes and secreted. It was then delivered into a human epithelial cell line (HMEC-1) resulting in reduced expression of c-Myb and subsequent increased cell migration. Another study by Rana et al. [138] has shown exosomal tumour miRNAs can modulate selected host tissues in distant organs toward a pro-metastatic phenotype forming a pre-metastitic niche. They found that poorly metastatic ASML-CD44 cells in rats regained their metastatic potential after conditioning the rats with exosomes. The exosomes were taken up in vivo and effects on target cells were strongly mediated by

exosomal miRNAs [138]. These studies raise exciting possibilities regarding the potential for exosome encapsulated miRNAs as circulating biomarkers and new methods of gene therapy.

1.5.2 Exosome Encapsulated miRNAs as Biomarkers in Colorectal Cancer

Conflicting reports exist regarding the diagnostic utility of circulating miRNAs in serum or plasma in colorectal cancer [85, 139] As previously discussed, some miRNAs believed to be released by colorectal tumours as circulating markers of disease are expressed by red blood cells and may be present in the plasma and serum secondary to haemolysis due to collecting procedures or storage methods [115, 119]. Tumour derived exosomal miRNAs may provide a more specific miRNA signature. A small number of studies have identified individual miRNAs present in colorectal cancer cell-secreted exosomes (let-7a, miR-21, miR-34a, miR-143, miR-192, miR-1246, miR-215, miR-221), however the full range remains unknown and may include multiple novel targets and previously unexplored miRNAs suitable for further investigation as biomarkers of disease [140, 141]. Different cancer subtypes including those with greater metastatic potential could potentially secrete their own exosome-encapsulated miRNA signature which could advance the possibility of individualised treatment. In prostate cancer, exosomal miR-34a has been found to predict drug resistant, castration resistant prostate cancer and could potentially be used to avoid the significant morbidities associated with surgery [142]. Similar exosomal miRNAs could be used in a variety of cancers to avoid unnecessary chemoradiotherapy and the associated morbidity.

1.5.3 Exosome-Encapsulated miRNAs as Therapeutic Tools

miRNA based therapeutics, particularly delivery of tumour suppressor miRNAs or what is called miRNA replacement therapy, has been somewhat hindered by the requirement for a delivery vehicle to transfer miRNAs to diseased or cancerous tissue. Growing evidence demonstrates, however, that exosomes can be used to efficiently deliver anti-tumour miRNAs to cancer tissues in vivo. Recent studies have shown exosomes are capable of delivering chemotherapeutic agents in vitro and can deliver anti-tumour miRNAs to breast cancer cells when injected systemically in vivo [143]. Ohno et al. [143] have shown intravenously injected exosomes can deliver let-7a miRNA to EGFR-expressing xenograft breast cancer tissue in mice. This subsequently

resulted in inhibition of tumour development in vivo. Further, direct intratumoral injection of miRNA enriched exosomes suppresses target genes in an in vivo model of prostate cancer [143]. Iguchi et al. [144] found exosomal miR-16 was transferred into prostate cancer PC-3M cells subcutaneously xenografted in nude mice following intra-tumoral injection, resulting in suppression of its target gene. Despite significant interest in exosomal miRNAs as therapeutic gene delivery systems in cancer, there are no studies examining the potential for this in colorectal cancer.

1.6 Hypothesis and Aims

In order to develop our knowledge of the effect of exosomal miRNAs in colorectal cancer molecular profiling of exosomal miRNA signatures is paramount and will firstly advance our knowledge of intercellular communication in the tumour microenvironment but also facilitate biomarker discovery and progression of miRNA-based therapies.

The hypothesis of this study is that exosomal miRNAs are robust diagnostic biomarkers in colorectal cancer and can act as delivery vehicles for targeted gene therapy. The study aimed to identify the full range of miRNAs packaged into exosomes and secreted by two distinct colorectal cancer cell lines in vitro. In addition, the study aimed to evaluate the therapeutic potential for exosomal miRNAs by investigating the transfer of miRNA enriched exosomes from one cell line to another and determining the subsequent functional effects on recipient cells

Chapter 2

Materials and Methods

2.1 Cell culture

2.1.1 Cell Culture Principles

Cell culture involves the growth of cells in an artificial environment. Glass or plastic containers with medium enriched in nutrients tailored to specific cell types are used for this. Protocols to minimise the risk of infection and cross-contamination are essential to maintain an aseptic environment for culture of cell lines. Established commercially available immortalised cell lines representing models of colorectal cancer in vivo were cultured aseptically in a designated cell line laminar air flow (LAF) hood and incubated in a specific cell line incubator. All cell culture work was performed in a designated cell culture room in which no other experimental work is performed.

2.1.2 Equipment and asepsis

The LAF hood required was turned on 15 minutes prior to usage to allow circulation of air. Laboratory coats specific to the cell culture room and gloves were worn at all times to avoid exposure of skin within the LAF hood. All containers, flasks and gloves entering the LAF hood were sprayed with 70% Industrial Methylated Spirits (IMS). All clean equipment such as pipettes and flasks were kept on the right side of the LAF hood, waste flasks and used pipettes were transferred to the left. LAF hoods were thoroughly sprayed down with 70% IMS and remained on for 15 minutes following usage. LAF hoods were emptied and cleaned extensively on a weekly basis according to a cleaning rota to ensure minimal risk of contamination. Cells were incubated in High Efficiency Particulate Air (HEPA) filtered Thermo incubators at 37º C and 5% CO_2. A water bath containing de-ionised water which had been sterilised by auto-clave was used to maintain humidity within the incubators. The water was autoclaved and replaced on a weekly basis according to a cleaning rota and the water baths were thoroughly cleaned with IMS.

2.1.3 Cell line culture and maintenance

Colorectal cancer cell lines which demonstrated distinct growth patterns and displayed different invasive characteristics were cultured. HCT116 cells are an immortalised adherent epithelial colorectal cancer cell line which has partially undergone Epithelial to Mesenchymal Transition (EMT). The cells were originally isolated from an adult male and are a suitable transfection host. HT29 cells are an immortalised adherent

epithelial colorectal cancer cell line which grow in pillars and have not undergone EMT. The cells were originally isolated from an adult female and are a suitable transfection host. Both colorectal cancer cell lines were cultured in T-175 cm^2 flasks in McCoy's 5A medium supplemented with 50 mL of 10% Fetal Bovine Serum (FBS) and 5 mL of 100IU/mL Penicillin/100µg/mL Streptomycin (complete media). Cells were inspected at the beginning of every weekday. Firstly, media colour and turbidity was inspected without the need for a microscope. Media colour becomes pink with decreasing pH and turbidity was inspected to ensure cells were not in suspension. Cells were then inspected under a microscope to assess confluence and the need for passage. All cell types received a change of medium on alternate weekdays to remove waste products and replace nutrients required for healthy growth. Complete media was heated in a water bath until it reached 37° C. Spent medium was aspirated directly from flasks and decanted into waste containers inside a LAF hood and new pre-heated medium was added to the flasks by pipetting down the ceiling of the flask to avoid disturbing adherent cells. For a T-175 cm^2 flask, 25 mL of complete media was added.

2.1.4 Cell passaging

Cells were re-passaged into new T-175 cm^2 flasks when they reached over 80% confluence or if they were required for further experiments. Firstly, new flasks were appropriately labelled with cell type, passage number, date and initials. Medium from flasks containing cells to be passaged was decanted directly into waste containers from a height to avoid contamination by splash back. 5 mL of phosphate buffer solution (PBS) which had been pre-heated to 37° C was pipetted onto the ceiling of the flask and the cell monolayer was rinsed to ensure removal of FBS. This was required as serum can interfere with the action of trypsin, a serine protease which is used to cleave bonds allowing cells to adhere to the flask. 5 mL of trypsin was then added and cells were incubated at room temperature in trypsin for 30 seconds to 1 minute depending on cell line sensitivity to the action of trypsin. Excess trypsin was removed and cells were then placed in the appropriate incubator for 3 minutes to allow loosening of cells from the surface of the flask. Following inspection to ensure adequate dissociation, complete media was added to the flask directly onto cells and the flask was manoeuvred to ensure the majority of cells were in suspension. A 100 µL aliquot of cell suspension was taken for cell counting to determine the appropriate volume

required for re-seeding into the pre-prepared flasks. Once determined, the adequate volume to achieve the appropriate amount of cells required was pipetted directly into a pre-prepared, labelled flask.

2.1.5 Cell Counting

Cell counts were performed using a Nucleocounter® (Chemometic) according to manufacturer's instructions (Figure 2.1). Propidium Iodide, an intercalating agent, was used to stain the DNA within nuclei of non-viable cells in this process allowing a count of non-viable cells. Total number of cells can be counted following cell membrane lysis with a lysis agent. To determine the number of viable cells/mL of a given cell suspension, the number of non-viable cells was subtracted from the total number of cells/mL of suspension. Firstly, to determine the total number of cells/ml in suspension, a 50 µL volume of cell suspension was added to an appropriately sized tube. 50 µL of Lysis Buffer was then added to the suspension and vortexed thoroughly. Following this, 50 µL of Stabilising Buffer was added and the suspension was vortexed thoroughly again. The 1:3 dilution of cell suspension was then loaded into a nucleocassette containing propidium iodide. The number of lysed cells was then read with the Nucleocounter ® using fluorescence microscopy (Figure 2.1). The number of cells counted was multiplied by 3 as the cell suspension was diluted to 1:3. This figure represented the total number of cells/ml in suspension as all cells were lysed. Propidium iodide cannot enter cells with an intact membrane, so to determine the number of non-viable cells a 50 µL volume of cell suspension was again added to an appropriately sized tube. The suspension was then loaded into a nucleocassette and read with a Nucleocounter ®. As the suspension was not exposed to Lysis Buffer only existing non-viable (permeable) cells will be counted following staining by propidium iodide. The non-viable count was subtracted from the total count to determine the number of viable cells/mL of suspension.

Figure 2.1: Cell Counting Protocol

2.1.6 Cryopreservation and recovery of cells

To maintain an adequate stock of all cell types, cells were frozen as required. A standard of 1.5 x 10^6 cells in 1.6 mL of media were frozen in a cryovial containing 80 µL of cryoprotective agent Dimethylsulfoxide (DMSO). To achieve this concentration of cells in suspension, cells were trypsinised and counted as previously described. DMSO is used to minimise intracellular ice crystal formation and resulting membrane damage as it rapidly penetrates and dehydrates cells during freezing. DMSO is highly toxic to cells at room temperature so cryovials containing 80 µL of DMSO were placed in an ice-bucket prior to the addition of the cell suspension. Once the cell suspension containing an appropriate amount of cells was added to the cryovials containing DMSO, the cryovials were transferred to an isopropanolol bath and placed into a -80 ^{o}C freezer facilitating gradual freezing of cryovials at -1°C/minute. The cryovials were allowed to gradually freeze overnight and subsequently transferred into a liquid nitrogen container (-196 ^{o}C) where they were stored until required for recovery. Recovery of cells required rapid defrosting of cryovials containing cells through immersion in a pre-heated 37° C water bath. Once partially defrosted the contents of the cryovial were emptied directly into a flask containing the appropriate and pre-warmed complete medium. As DMSO is toxic to cells at 37 ^{o}C the media was changed 24 hours following recovery after confirming the cells were adherent by visualisation under a microscope.

2.2 microRNA expression analysis

2.2.1 Bio-bank: ethics and consent process

The Discipline of Surgery NUI Galway maintains a bio-bank of tissue, blood and extracted RNA related to ongoing breast cancer and colorectal cancer research. The biobank has received approval from the Galway University Hospitals Clinical Research Ethics Committee (Appendix 1). Patients and members of the public provide informed consent prior to donation of samples to the bio-bank (Appendix 2). Patient details related to samples are anonymised and recorded in the Shire laboratory information management system. A unique code and storage location is generated for each sample and all samples are labelled with these anonymous codes prior to storage.

2.2.2 Tissue specimens

Tissue samples are routinely collected by surgeons at the time of resection or diagnostic biopsy and are not placed in Formalin. Samples are then transported to the histopathology laboratory in Galway University Hospital where a pathologist examines the samples to ensure usage of part of the sample for research purposes will not interfere with diagnosis. A tissue sample is then removed from the tumour and a corresponding sample removed from adjacent normal tissue within 2cm of the tumour edge (tumour associated normal tissue – TAN). Patient tissue samples are susceptible to degradation by RNase contamination so are immediately placed in stabilisation solution (RNAlater®, Qiagen, Netherlands). Samples are then transferred to the laboratory where the solution is allowed to permeate the tissue for 24 hours prior to its removal. Tissue samples are then stored in the bio-bank at -80 ^{o}C. RNA is extracted from tissues and stored in the biobank.

2.2.3 RNA extraction

RNase contamination of samples can cause poor quality RQ-PCR results and must be avoided where possible. To maintain the highest standard of RNA integrity possible several precautions were taken. A designated RNA extraction hood with designated equipment such as pipettes, tubes etc. was used for all RNA extractions. An ultraviolet light was turned on in the extraction hood for 15 minutes prior to usage to inactivate any RNases present. Disposable, non-latex gloves and laboratory coats were worn at all times. Following usage, the RNA extraction hood was carefully cleaned with 70% IMS and the ultraviolet light was left on for 15 minutes.

RNA and miRNA were extracted using different methods from the following sample types
  - Cell pellets
  - Conditioned medium
  - Exosomes

Different commercially available RNA extraction kits are available and some sample types are more suited to specific kits. Both the RNeasy® (cell pellets) and *mir*Vana™ kits (conditioned medium and exosomes) were used to extract RNA and miRNA from relevant samples.

2.2.3.1 RNA extraction from cell pellets

The RNeasy® mini kit provides purification of high-quality RNA from cells and tissues using silica-membrane RNeasy spin columns with a binding capacity of 100 µg RNA. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer (Trizol®), which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in water.

Cell pellets were stored at -80 °C and required defrosting at room temperature prior to homogenisation. To homogenise pellets, 700 µL of Trizol® Lysis Reagent was added to the pellet and it was thoroughly vortexed. The solution was then passed through a needle tip using a syringe to ensure all cell membranes were lysed. The homogenate was then allowed to rest at room temperature for 5 minutes. 140 µL of chloroform was added to the homogenate which was vigorously vortexed. The sample was again incubated at room temperature for 5 minutes and then centrifuged for 15 minutes at 15,300 x g at 4 °C. As a result of centrifugation the sample separated into 3 phases: an upper aqueous phase, a white interphase and a lower red phase. The upper aqueous phase containing RNA was transferred to a new collection tube taking great care to avoid disturbing the interphase. 1.5 volumes of 100% ethanol was added to the aqueous phase and mixed by pipetting up and down. 700 µL of the sample was then transferred to an RNeasy® Mini column placed in an appropriate collection tube. The collection tube containing the column was then centrifuged at 15,300 x g for 15 seconds at room temperature. The filtrate was discarded and the remainder of the ethanol/aqueous phase sample was passed through the column in a similar fashion. When all the ethanol/aqueous phase sample had been passed through the column, 350 µl of RW1 wash buffer was pipetted onto the column and it was centrifuged at 15,300 x g for 20 seconds and the filtrate discarded. A DNase digest was then performed to remove residual contaminating DNA by pipetting 80 µL of DNase/RDD buffer mix onto the column and allowing it to rest at room temperature for 15 minutes. A repeat wash with 350 µL of RW1 wash buffer was then performed. Following this, 500 µL of RPE buffer was pipetted onto the column and centrifuged for 20 seconds. The filtrate was discarded and this step was repeated. To dry the membrane the column

was centrifuged at 15,300 x g for 1 minute. The column was then placed in a new tapered collection tube and 50 µL of chilled RNase-free water was pipetted onto the column membrane ensuring it is wet in all areas to maximise the RNA yield. The column then stood for 1 minute at room temperature and was centrifuged at maximum speed for 1 minute to collect an eluate at the bottom of the tapered tube. This process was then repeated with 10 µL of RNase-free water to ensure all areas of the membrane were wet and all RNA was eluted from the membrane. The extracted RNA was stored at -80 °C in an appropriately labelled tube.

2.2.3.2 miRNA extraction from conditioned medium

Conditioned medium was collected following exosome isolation which will be discussed further in a later section. Following aspiration of conditioned medium from the exosome pellet, it was stored at -20 oC and miRNA extracted as required. This method of microRNA extraction from conditioned media was optimised by another member of the research group (CG) [145]. The mirVana™ miRNA Isolation Kit was used with some modifications to the described protocol in the manual. The mirVana™ miRNA Isolation Kit uses organic extraction followed by purification on a glass fibre filter under specialized binding and wash conditions.

Conditioned media was stored at -80 °C and thawed at room temperature prior to extraction. 700 µL of Trizol® was added to 1 mL of conditioned media and thoroughly vortexed. The sample was then incubated at room temperature for 5 minutes. 140 µL of chloroform was then added to the sample and it was thoroughly vortexed and inverted to adequately mix it. Samples were then allowed to rest for 5 minutes at room temperature and following this were centrifuged at 20,800 x g, 4 °C for 15 minutes. The sample separated into 3 phases and the upper aqueous phase was transferred to a new tube. 1/3 the volume of 100% ethanol was added to the aqueous phase and mixed by pipetting. From this point onwards the *mir*Vana™ miRNA Isolation Kit was used following the manufacturers protocol. A filter was placed into a tube and 700 µL of the ethanol/aqueous phase mixture added. The lysate was then centrifuged for 15 seconds at 10,600 x g at 4 °C. The filtrate was collected into a new tube and this was repeated until all the lysate was passed through the column. For microRNA extraction 2/3 the volume of 100% ethanol was then added to the filtrate. A new filter was placed

into a new collection tube and 700 µL of the ethanol/filtrate mixture added. This was centrifuged for 15 seconds at 10,600 x g at 4 °C. The flow through was discarded and this was repeated until all the sample had passed through the filter. 700 µL of wash solution 1 (provided in the kit) was then added to the filter and this was centrifuged at 10,600 x g for 10 seconds at 4 °C. The filtrate was discarded and 500 µl of wash solution 2/3 was added to the filter and centrifuged for 10 seconds. The flow through was discarded and this step was repeated. The filter was then centrifuged at maximum speed for 1 minute at 4 °C to dry. The filter was transferred to a new tapered collection tube and 50 µL of pre-heated elution solution (provided in the kit) was added to the filter ensuring the entire filter was wetted. The filter was then centrifuged at maximum speed for 1 minute. This step was repeated with an additional 10 µL of elution solution. The eluent was collected in an appropriately labelled tube and stored at -80 °C.

2.2.3.3 Nanodrop Spectrophotometery

The NanoDrop ND-1000 Spectrophotometer® (NanoDrop technologies) was used to assess the concentration and purity of RNA and microRNA following RNA extractions (Figure 2.2). Prior to usage, it was necessary to clean the optic fibres and measure a control sample which contained no RNA. To measure total RNA concentration the 'RNA-40' setting for sample type was selected. To measure microRNA the 'RNA-33' setting for sample type was selected. 1 µL of sample was pipetted onto the end of a fibre-optic cable on the apparatus pedestal. A second fibre-optic cable on the instrument arm was then brought into contact with the liquid sample causing the liquid to bridge the gap between both fibre-optic cables. A pulsed xenon flash lamp provided a light source and a spectrophotometer analysed the light after it passed through the sample. RNA concentration was automatically calculated using the formula

RNA concentration (ng/µL) = (A260×e)/b

A260 = Absorbance at 260 nm, e = extinction coefficient (ng-cm/mL), b =
pathlength (cm)

In addition to RNA concentration, the protein contamination could be measured (A260/A280 ratio target reading 2-2.1) and the contamination with organic or buffer components could be measured (A260/A230 ratio target > 2).
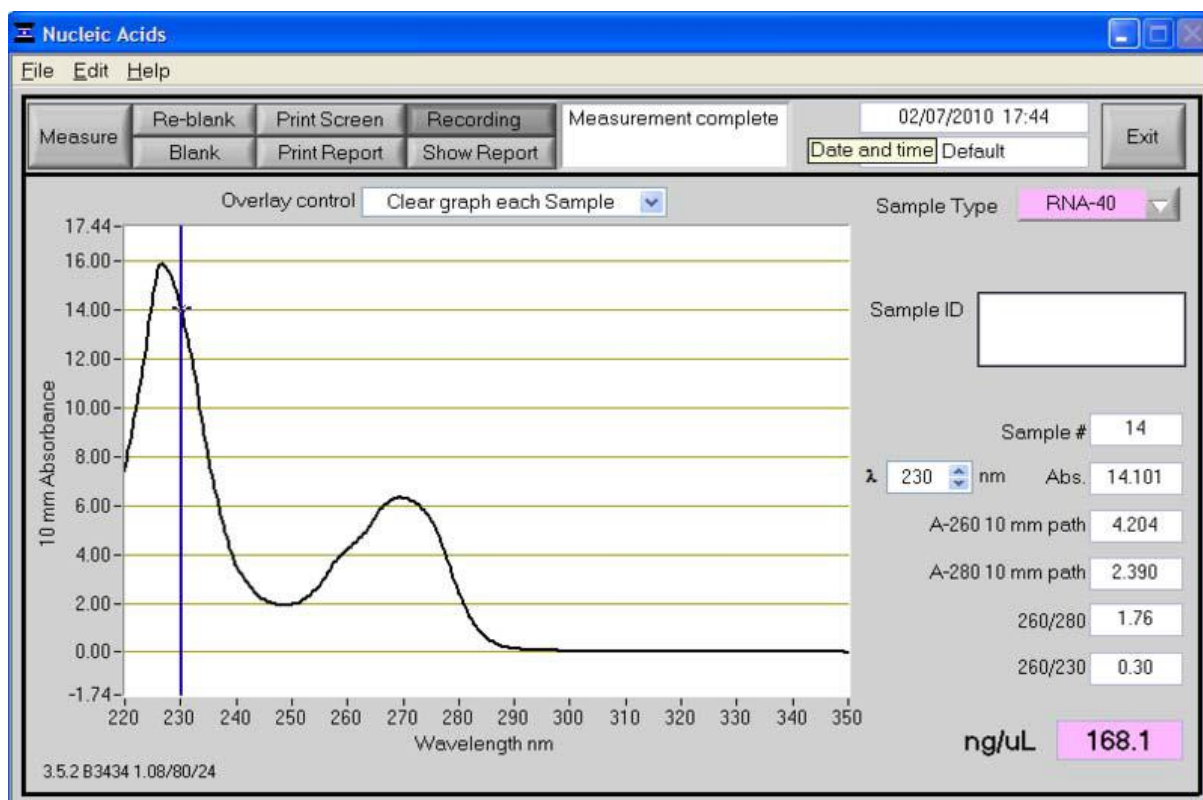
Figure 2.2: Nanodrop software concentration and purity reading.

2.2.4 Reverse transcription of miRNA to complementary DNA (cDNA) for miRNA expression analysis.

Reverse transcription is the process in which single stranded RNA is converted to double stranded cDNA which can subsequently be amplified to allow relative quantification. Small RNA was reverse transcribed to cDNA using the TaqMan® MicroRNA Reverse Transcription Kit (applied Biosystems).

All procedures were carried out in designated reverse transcription/PCR vented hoods. Prior to and after usage, a UV light was turned on inside the hood for 15 minutes. The hood was also thoroughly cleaned with 70% IMS before and after use. All instruments inside the hood were designated specifically for that hood and were not removed. Gloves and lab coats were worn at all times.

2.2.4.1 Reverse Transcription Protocol

Each reaction was primed using a specific stem loop primer as microRNA requires target-specific reverse transcription. miR-16 and miR-10b primers were obtained from MWG biotech®. All other primers were obtained from Applied Biosystems® (miR-379, miR-149-5p, miR-143). RNA was removed from storage at -80 °C prior to

commencing. A concentration of RNA from between 5-100 ng was required. RNA samples at concentrations above this required dilution with nuclease free water (NFW). An RT pre-mix was created using a specific set of components listed (Table 2.1).

Component of pre-mix	Volume
RNA (5 – 100ng)	5 µl
dNTP mix (100mM)	0.17 µl
10XRT Buffer	1.65 µl
Multiscribe (50U/ µl)	1.1 µl
RNase Inhibitor (20U/ µl)	0.21 µl
Stem Loop Primer (50nM)	3.1 µl

Table 2.1: Required components for preparation of master mix for Reverse Transcription

All components were added to tubes in a cooling tray. The total volume of the pre-mix and RNA dilution was 15 µl. An RT-negative control was included for each set of reactions. This contained pre-mix without any RNA present to allow identification of any potential contamination. Tubes containing the pre-mix/RNA solution and negative control were centrifuged and then placed in the thermal cycler (Gene Amp PCR System 9700 Thermal Cycler®, Applied Biosystems®). Samples were incubated at 16 °C for 30 minutes, 42 °C for 30 minutes, 85 °C for 5 minutes and then cooled down to 4 °C. All samples were centrifuged, transferred to appropriately labelled pre-sterilised tubes and stored at -20 °C until needed.

2.2.5 Real Time Quantitative Polymerase Chain Reaction (RQ-PCR)

cDNA synthesised for particular microRNA targets can be amplified and quantified by real time quantitative polymerase chain reaction. The reaction relies on thermal cycling for DNA melting and enzymatic replication of DNA. Individual microRNA primers along with a DNA Polymerase allow selective and repeated amplification of target regions. 'Real Time' describes the process by which targets are quantified based on the time point during the reaction that they are first detected. Detection of the

amount of microRNA amplified is made possible by fluorescently labelled microRNA specific probes present in the reaction. The RQ-PCR reaction consists of an exponential phase and a plateau phase. The amount of amplified product doubles during each cycle of denaturation, primer annealing and template extension in the exponential phase. Reduced reagents limit the reaction during the plateau phase. The point in the reaction at which enough amplified product has been produced to cause a detectable fluorescent signal is called the Cycle Threshold (C_T). If there is a large amount of a target microRNA present at the start of the reaction, the C_T value will be low as it is detected early, where as if there is a small amount of a target microRNA the C_T value will be high as it is detected late in the reaction.

2.2.5.1 RQ-PCR protocol

Pre-prepared target specific cDNA samples were removed from storage at -20 °C and allowed to thaw prior to commencing. A pre-mix for both ABI (Table 2.2) and MWG (Table 2.3) product based reactions consisting of a specific number of components were required.

ABI miRNA pre-mix	Volume
TaqMan Fast Mastermix	5 µl
Nuclease Free Water	3.8 µl
ABI miRNA PDAR	0.5 µl
cDNA	0.7 µl

Table 2.2: Required components for preparation of pre-mix for RQ-PCR with ABI probes

MWG miRNA pre-mix	Volume
TaqMan Fast Mastermix	5 µl
Nuclease Free Water	1.6 µl
Probe	0.5 µl
Forward Primer	1.5 µl
Reverse Primer	0.7 µl
cDNA	0.7 µl

Table 2.3: Required components for preparation of pre-mix for RQ-PCR with MWG probes

The list of components outlined was required for 1 reaction with a final volume of 10 µl which was pipetted into individual wells on a 96 well plate. All reactions were carried out in triplicate and only results with a standard deviation of < 0.3 were accepted. The 96 well plate was held on a cooling tray to maintain a low temperature. For each microRNA target, a Non Targeting Control (NTC) sample was included. This was composed of the MWG or ABI pre-mix without any cDNA. This NTC sample, in conjunction with the RT negative control sample, was used if required to determine at which stage of the process contamination may have occurred. Once all samples required were pipetted onto a 96 well plate, RQ-PCR reactions were carried out using an AB7900HT (Applied Biosystems®) fast real-time PCR system. Standard 'fast' thermal cycling conditions were applied consisting of 40 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds.

2.2.5.2 Relative Quantification and Data Analysis

Relative quantification was used for all studies. This involves expression of the miRNA of interest relative to an endogenous control miRNA. Endogenous controls are selected based on their stable expression levels despite any disease process or other abnormal state. For every sample, reactions were performed in triplicate for the miRNA of interest and the endogenous control miRNA. The average C_T value was obtained from sample triplicates. Following this, the average C_T value of the

endogenous control microRNA was subtracted from the average C_T value of the miRNA of interest, resulting in a ΔCT value [146].

ΔCt = average Ct (target microRNA) – average Ct (endogenous control).

A calibrator sample was then chosen. The calibrator sample was the sample with most significant difference between the target miRNA and endogenous control i.e the highest ΔCT value (lowest expresser in the group). This was then subtracted from all ΔCT values and termed ΔΔCT.

ΔΔCT = ΔCT (sample) - ΔCT (calibrator sample)

The ΔΔCT values were subsequently converted to a linear form using the formula

$$RQ = 2^{-\Delta\Delta CT}$$

The relative quantification is the fold change in expression compared to the calibrator. Finally, all results were expressed in Log_{10} form for analysis. Statistical analysis was performed using the statistical software programme Minitab Version 16.0. Two-sample t-tests were used to assess significance where appropriate and results with a p-value of less than 0.05 were considered to be statistically significant.

2.3 Over expression of miRNA-379 in vitro

2.3.1 Lentiviral vector

A license for contained use of genetically modified organisms from the Environmental Protection Agency was obtained by the Department of Surgery for all work with lentiviral vectors. HCT116 and HT29 colorectal cancer cell lines were transduced with a lentivirus vector forcing strong and stable over-expression of microRNA-379 (miR-379). For comparison, another identical lentivirus that does not over-express miR-379 but contains a non-targeting control (NTC) was used to transduce additional HCT116 and HT29 cells. The lentivirus used for this purpose was ThermoScientific SMARTchoice shMIMIC lentiviral microRNAs®. The lentiviral vectors used contain the human Cytomegalovirus (hCMV) promoter which forces strong expression of both miR-379 and the NTC components. The vectors used also contain a red fluorescence

protein which assists in estimating transduction efficiency and a Puromycin resistant gene which confers resistance of the transduced cell to the antibiotic Puromycin (Figure 2.3). Puromycin, an aminonucleoside antibiotic, which inhibits protein synthesis by disrupting peptide transfer on ribosomes causing premature chain termination during translation, is used to select out and kill any non-transduced cells.
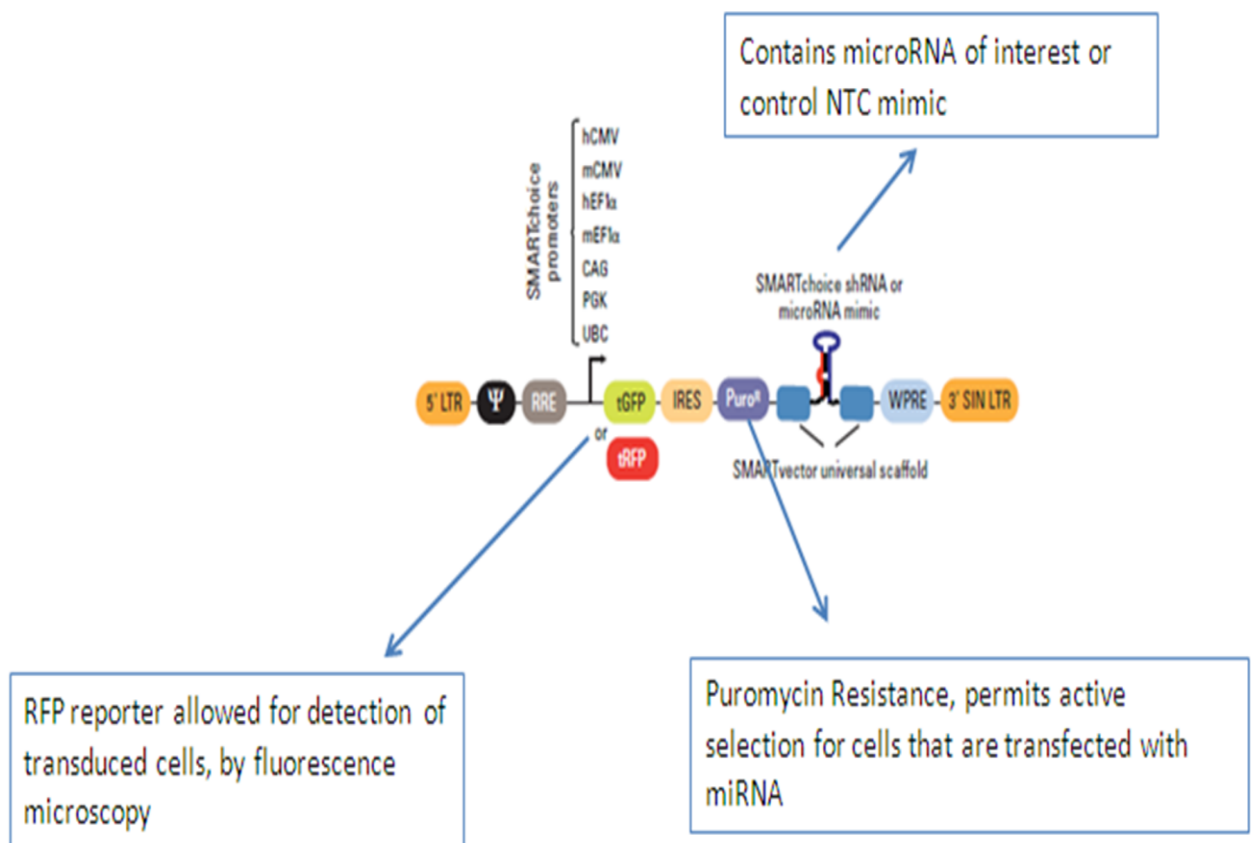
Figure 2.3: Individual components of the SMARTvector Lentiviral microRNA vector [147]

2.3.2 Transduction

To achieve successful transduction of cell lines an appropriate number of cells must be incubated in virus containing media in the presence of Polybrene (Hexadimethrine bromide) for an adequate length of time. Polybrene increases the efficiency of virus mediated gene transfer and is believed to act by neutralizing repulsive forces between viral particles and cell surface components. Two T75 cm^2 flasks were seeded with 3.4 x 10^5 HT29 cells (for transduction with miR-379 and NTC) and 2 T75 cm^2 flasks were seeded with 3.4 x 10^5 HCT116 cells (for transduction with miR-379 and NTC) and these were incubated overnight in complete media. The following day, media containing an optimal concentration of Polybrene was prepared. The concentration of Polybrene required was previously optimised by another member of the research group (SK) [148]. Firstly Polybrene was added to PBS to achieve an 8 µg/mL solution. Following this 4.5 µL of the lentivirus (MOI 4.5 µL/1000 cells) vector is added to the basal media, swirled and allowed to sit for 2 minutes. The spent media was then removed from the cells and then virus-containing media (miR-379 over-expressing or NTC) was added. Cells were incubated with virus containing media for 6 hours after which it was removed and fresh complete media added again (Figure 2.4). This entire process was also performed in an identical fashion with chamber slides seeded with 1 x 10^4 cells for each transduced cell line to allow visual confirmation of transduction with fluorescence microscopy (Olympus BX60, analaSIS® software - Ridom GmbH, *Münster*, *Germany*) prior to and after Puromycin selection. Puromycin selection was initiated 48 hours after transduction by adding Puromycin to complete medium to achieve a final concentration of 8 µg/mL. This medium was then used to feed cells for a week to ensure adequate selection of transduced cells.
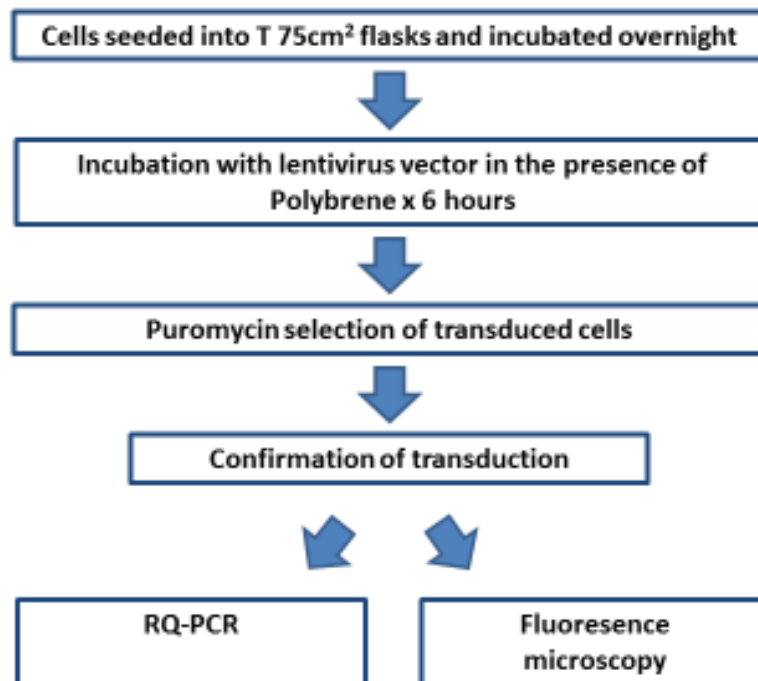
Figure 2.4: Cell transduction flow chart

2.3.2.1 Confirmation of Transduction

As previously described, the lentiviral vectors contain a red fluorescence protein (RFP) which can be visualised using fluorescence microscopy. Chamber slides containing miR-379 over-expressing HCT116 (HCT-116-379), Non Targeting Control HCT-116 (HCT-116-NTC) and virus free HCT116 (HCT-116-WT) cells were prepared as previously described and labelled with pencil. An identical set-up was used for HT29 cells. Cell nuclei could then be counterstained with 4',6-diamidino-2-phenylindole (DAPI) to identify the cell nuclei of all cells which could be compared to the cells displaying red fluorescence to visually establish transduction efficiency. To do this the slides were washed twice with PBS. Cells were then fixed with 4% paraformaldehyde for 20 minutes at room temperature. The chamber walls were then removed and the slides washed 3 times for 5 minutes in PBS. The fixed cells were then immersed in 1 µg/mL DAPI solution for 5 minutes at room temperature and protected from the light using tinfoil. The slides were then washed again 3 times for 5 minutes in PBS. Dehydration with a series of graded alcohol washes at 3 minute

intervals was then performed using 75% ethanol, 95% ethanol, 100% ethanol and 2 xylene washes while protecting the slides from light. DPX mounting medium was gently pipetted along one side of the slide and a cover slip slowly but firmly lowered at an angle to maximise the spread of mounting medium over the slide. Slides were left to dry overnight, protected from the light by tinfoil and then viewed using fluorescence microscopy (Olympus BX60, analaSIS® software) using the appropriate filters to detect DAPI and RFP. The excitation/emission wavelengths for RFP are 553 nm/574 nm. The excitation/emission wavelengths for DAPI are 345 nm/ 455 nm.

To further confirm successful and stable transduction, over-expression of miR-379 was confirmed by extracting RNA from HCT-116-379 and HCT-116-NTC cells. Reverse transcription and relative quantification PCR was performed as previously described to confirm consistent and stable over-expression of miR-379 at 1 and 2 weeks.

2.3.3 Transduced cell culture principles
All culture of transduced cells was performed in a dedicated LAF hood and adhered to guidelines for working with genetically modified organisms according to the Environmental Protection Agency. Asepsis was maintained as previously described at all times. Waste flasks contained bleach and any pipettes or flasks exposed to virus infected cells were thoroughly cleaned with bleach prior to autoclaving and incineration. Routine cleaning of the LAF hoods and HEPA filtered Thermo incubators was performed as previously described. Transduced cells were frozen, recovered, maintained and passaged in a similar fashion to non-transduced cells.

2.4 Cell proliferation assay
The functional effect of miR-379 on HCT116 cell proliferation was measured using a CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega). The proliferation assay used is a colorimetric method to determine the metabolic index of cells. The solution used is a combination of a novel tetrazolium compound known as MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron coupling reagent known as PMS (phenazine methosulfate). Together these compounds form a stable solution which is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The

conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product is measured by a spectrophotometer analysing the absorbance at 490nm. The absorbance at 490nm is directly proportional to the number of living cells in culture.

To identify any difference in proliferation in HCT-116-379, HCT-116-NTC, and HCT-116-WT cells they were seeded into a 96-well flat bottom plate at a density of 1 x 10^3 in 100 µL of basal media. Each cell type was seeded vertically on the 96 well plate in replicates of 8. Wells containing basal media only were also filled in replicates of 8 to act as a control for background detection. Separate plates were seeded to be read at 48 hours and 72 hours to assess proliferation over different time points. At the required time point 20 µL of 5mg/mL MTS solution was added to each well. The plates were returned to the incubator at 37 °C. Following 3 hours incubation plates were read in a Bio-Rad microplate reader at 490 nm. Readings from replicates of 8 were averaged and assays were repeated in triplicate. Data were expressed as Mean +/- SEM of 3 experiments and were plotted against time to form a growth curve showing proliferation at different time points. Statistical significance was assessed with one way ANOVA and a p value < 0.05 was considered significant.

2.5 Cell migration assay

Migration assays were performed using Corning® (New York, USA) Transwell ® porous membranes with a 6.5 mm diameter and a 0.8µm pore size (Figure 2.5). Cells seeded above the membrane can adhere to it or migrate through the membrane in response to a chemoattractant in the well below. Once cells adherent to the membrane have been washed off it is possible to count the number of cells which have migrated through the pores in response to the chemoattractant. Chemokinesis, or the random movement of cells, is accounted for using a control in which conditions above and below the membrane are identical.
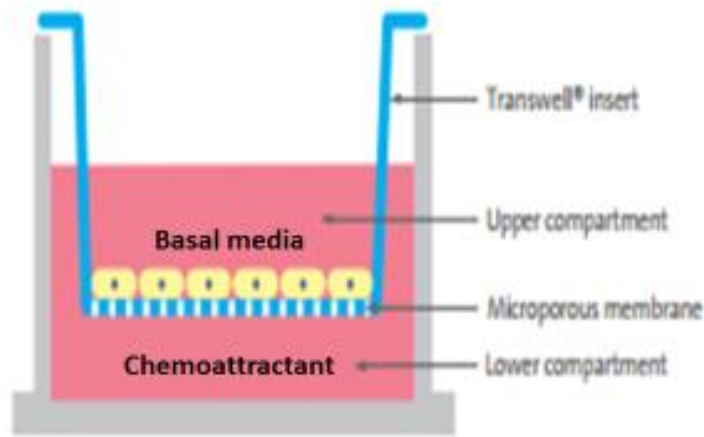
Figure 2.5: Transwell ® porous membranes set-up [149]

To improve cell attachment wells and inserts with membranes were rehydrated with basal medium for 1 hour prior to use. The lower compartments of the wells were filled with 600 µL of basal media with 2% FBS as a chemoattractant to encourage cell migration across membranes. To account for chemokinesis duplicate inserts for each cell type were set up using basal media in the upper and lower compartment. To assess any difference in migration between HCT-116-379, HCT-116-NTC and HCT-116-WT cells each cell type was seeded onto separate membranes with 7.5×10^4 cells in 100 µL of basal media. The membranes contained within inserts in a 24 well plate were then returned to the incubator and migration was allowed to occur overnight (18 hours). Following 18 hours incubation any cells which had not migrated through the porous membrane were scrubbed off with sterile cotton swabs. The scrubbed inserts were then transferred to a 24 well plate containing ice-cold methanol for fixation for 15 minutes. The inserts were then transferred to a new 24 well-plate containing Haematoxylin for 3 minutes at room temperature to stain cell nuclei. Serial washes in 2 separate beakers of dH20 were performed to remove excess Haematoxylin and prevent non-specific staining of membrane pores. Inserts were then inverted on tissue and left to dry inside a sterile laminar air flow hood. The membranes were then cut away from the inserts using a scalpel and mounted on a slide on top of a drop of

immersion oil. Another drop of immersion oil was then placed on top of the membrane followed by a coverslip. Migrated cells were then counted in 5 fields of view per membrane using a Leica Light Microscope (Wetzlar, Germany) at a magnification of 40X.

2.6 Exosome isolation

2.6.1 Preparation of Exosome-Free Fetal Bovine Serum

To isolate exosomes from cell lines, exosome depleted culture medium is required. FBS is the only component of culture medium containing exosomes. To ensure removal of exosomes from FBS it was firstly passed through a 0.2 µm sterile filter. Approximately 33 mL of FBS was then transferred to a Quick-Seal®, Polypropylene, 33 mL Beckman Coulter ultracentrifugation tube taking care to avoid any bubble formation. To remove exosomes from the FBS, it underwent ultracentrifugation for 16 hours at 100,000 x g, 4 $^{\circ}$C. A Sorvall 100SE® ultracentrifuge was used with a Ti70 fixed angle rotor. The following settings were used on the ultracentrifuge: Spinning time was set for 16 hours, temperature was set at 4 $^{\circ}$C, acceleration was set at 9 and deceleration at 1, speed was set at 100,000 x g. Following 16 hours, the tubes are removed from the ultracentrifuge and the exosome free FBS is carefully pipetted from the tubes, taking care to avoid the exosome pellet formed. The FBS can then be frozen at -20 $^{\circ}$C and used as required.

2.6.2 Isolation of exosomes secreted from cells

Isolation of exosomes from cell culture medium is performed by a process of differential centrifugation, microfiltration and ultra-centrifugation (figure 2.6). To isolate exosomes secreted by cells, cells were cultured in the appropriate volume of exosome depleted complete media for 48 hours. Following 48 hours incubation the media containing exosomes secreted by cells was harvested in a labelled 50 mL falcon. The cells remaining in the T-175 cm^2 flask were then trypsinised and counted as previously described. Once counted, a cell pellet was formed by spinning the suspension in a 15 mL falcon at 1000 x g for 4 minutes at 4 ^{o}C. This was then stored at -20 ^{o}C. The media containing exosomes then underwent a process of differential centrifugation, microfiltration and ultracentrifugation. The 50 mL falcon containing cell conditioned media was placed into a centrifuge and spun at 300 x g for 10 minutes at 4 ^{o}C. The media was then carefully collected from the falcon using a pipette leaving 1-2 mL remaining at the bottom which contains cellular debris. Collected media was then transferred to a new 50 mL falcon. This underwent centrifugation at 2000 x g for 10 minutes at 4 ^{o}C. It was carefully collected from the falcon using a pipette leaving 1-2 mL remaining at the bottom, passed through a 0.2 µm sterile filter and collected in a new 50 mL falcon. A primary fixative was added at this point prior to ultracentrifugation to maintain exosome morphology if exosomes were being isolated for visualisation with Transmission Electron Microscopy (TEM). The media containing exosomes was then transferred to an ultracentrifugation tube taking care to avoid any bubble formation. A circle was drawn around the area that the exosome pellet will form. A Sorvall 100SE ultracentrifuge was used with a Ti70 fixed angle rotor. The following settings are used on the ultracentrifuge: spinning time was set for 1 hour and 10 minutes, temperature was set at 4 ^{o}C, acceleration was set at 9, deceleration at 1 and speed was set at 100,000 x g. The ultracentrifugation tubes were carefully removed from the rotor following the spin and the supernatant collected from the tubes using a disposable pipette. The supernatant represents the cell conditioned media from which exosomes have been removed. This was stored in a 15 mL falcon at -20 ^{o}C. The exosome pellet was collected by pipetting 60 µL of PBS up and down the area marked where the exosome pellet forms in the ultracentrifugation tube. Alternatively if exosomes were harvested for Western Blot analysis, protein lysis buffer was used to re-suspend the exosome pellet. This is then stored in an appropriately labelled tapered tube at -80°C.
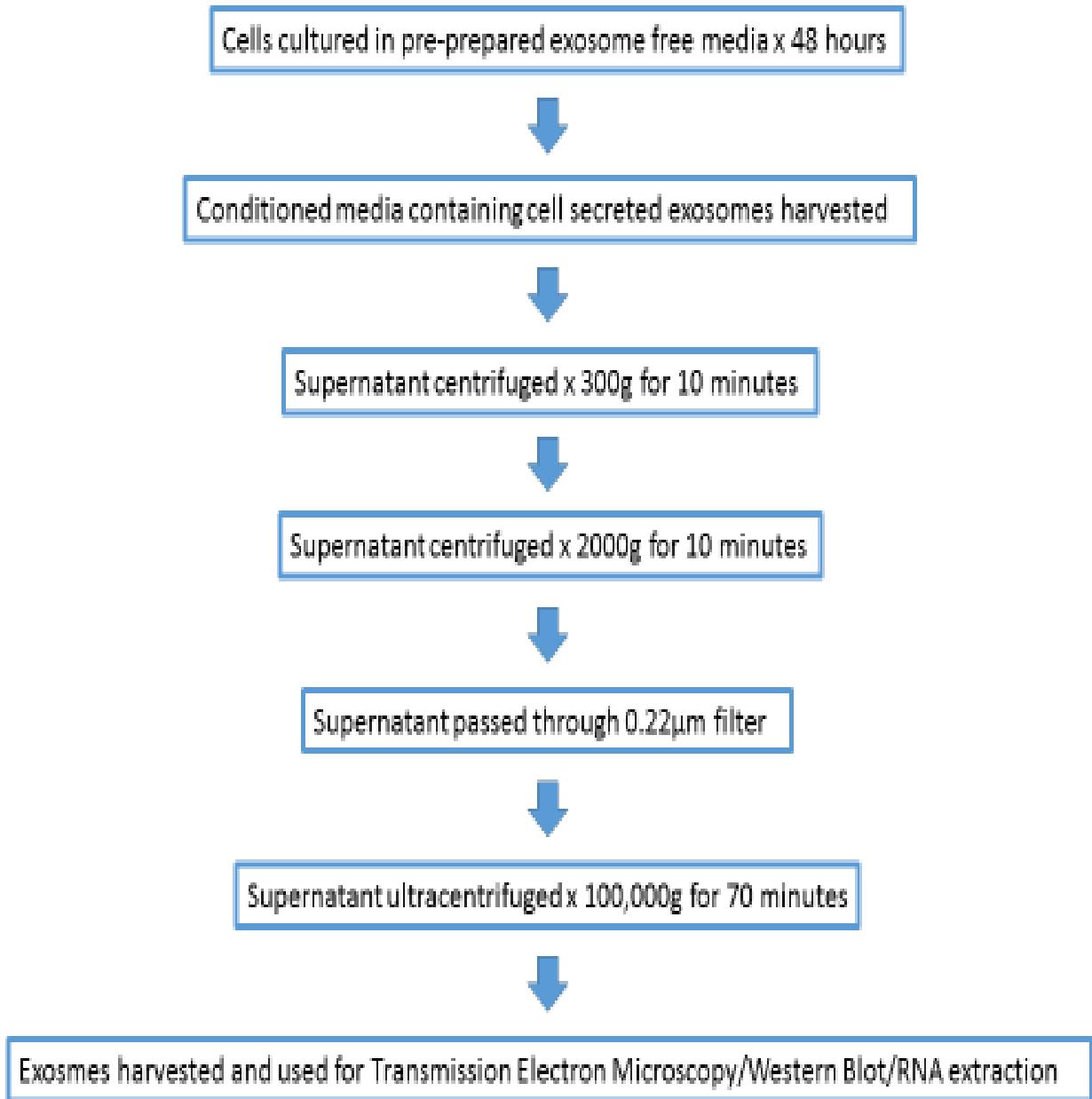
Figure 2.6: Exosome harvest protocol

2.7 Exosome characterisation

Exosomes are characterised based on their size, morphology and the presence of exosomal protein markers. To confirm the presence of exosomes in samples, visualisation of isolated exosomes was performed using Transmission Electron Microscopy (TEM). To detect the exosomal protein marker CD63 the protein content

of exosomes was quantified with a protein assay and the presence of CD63 was assessed with Western Blot.

2.7.1 Transmission Electron Microscopy

Exosomes cannot be visualised with a normal microscope so Transmission Electron Microscopy is required to characterise them. In order to maintain the morphology and architecture of exosomes for visualisation so that their size could be accurately determined, a primary fixative was added to cell-conditioned media before the final ultra-centrifugation step of exosome isolation (Figure 2.7). The primary fixative was composed of 2% glutaraldehyde, 2% paraformaldehyde in a 0.1M sodium cacodylate/HCL buffer at a pH of 7.2.
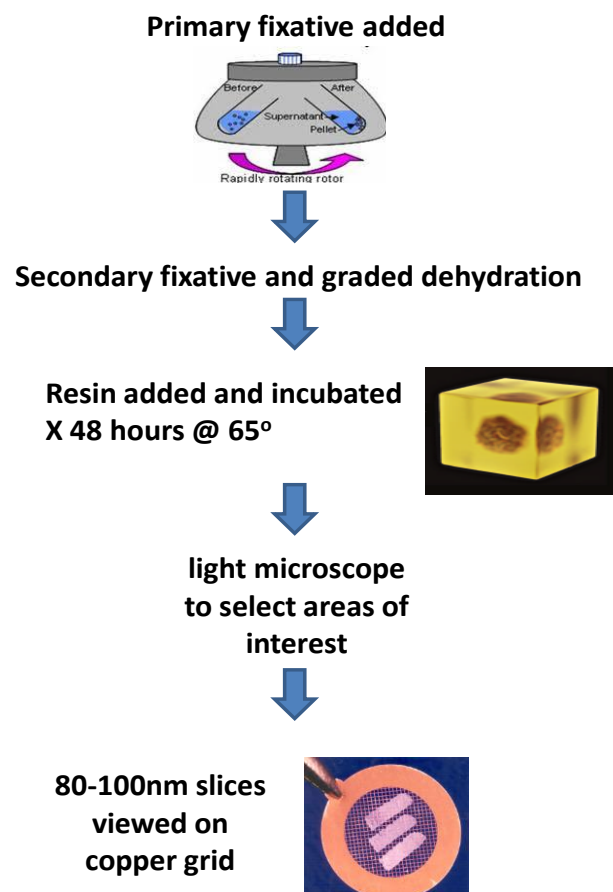
Figure 2.7: Protocol for analysis of exosome morphology

After the ultracentrifugation step, the supernatant was removed and the exosome pellet was re-suspended as previously described in 50 µL of sterile PBS. The re-suspended isolated exosomes were then treated with a secondary fixative composed of 1% osmium tetroxide in 0.1M sodium cacodylate/HCL buffer at a pH of 7.2. 2 mL of the secondary fixative was combined with an equal volume of sterile PBS and 150 µL was added to the isolated exosomes. Samples were centrifuged for 30 seconds to mix. Following this, the samples stood at room temperature for 2 hours allowing a density gradient to form until a black/brown pellet was observed at the bottom of the tube. The osmium supernatant was then aspirated to waste in a 2% solution of ascorbic acid, ensuring the black/brown pellet was not disturbed. The pellet was then dehydrated with graded alcohol (50%, 70%, 90%, 95% and pure ethanol). Each dehydration step was performed for 15 minutes twice. The pure ethanol was then aspirated to waste and propylene oxide added for 20 minutes twice. A 50:50 mixture of resin and propylene oxide was then pipetted onto the sample and allowed rest for 2 hours. After 2 hours, the resin was replaced with a 75:25 mixture of resin and propylene oxide for 4 hours. The resin/propylene mixture was then replaced with pure resin overnight. The following morning the pure resin was replaced at least twice again with a period of 2 hours incubation between each resin change. The sample was then placed into a 65 ºC oven and incubated for 48 hours to allow the sample to polymerise. After polymerisation, the resin block was trimmed by hand under microscopic vision to expose the embedded exosomes. Sections were cut to 1 µm thickness, loaded onto glass slides, stained with 1% toluidine blue and viewed using a light microscope to identify areas of interest. Areas of interest selected were then trimmed using an ultramicrotome (Reichert-Jung Ultracut E). Ultrathin sections between 80 -100 nm were loaded onto copper grids. The grids were stained with 1.5% aqueous uranyl acetate for 30 minutes followed by lead citrate for 10 minutes. The sections were allowed to dry and finally viewed with a Hitachi H7000 Transmission Electron Microscope.

2.7.2 Protein Assay and Western Blot Analysis

To further confirm the presence of exosomes in samples isolated, Western Blot targeting the exosome associated protein CD63 was performed. Prior to performing a Western Blot, determining the protein content of isolated exosomes was required using the Micro BCA™ Protein Assay. This assay is suitable for determining the protein

concentration of dilute samples accurately measuring concentrations as low as 0.5 µg/mL. 5 µL of isolated exosomes was added to 145 µL of lysis buffer. The 150 µL samples and standards provided (0.5-20 µg/mL) were pipetted into wells on a 96 well plate. 150 µL of Working Reagent provided in the kit was added to each well and the plate was placed on an orbital shaker for 30 seconds to allow mixing. Following this the plate was incubated at 37 °C for 2 hours. The absorbance at 560 nm was measured using a Bio-Rad microplate reader (Genesis software). The average absorbance reading of the blank standards was subtracted from the individual samples. Using Genesis software a standard curve was then formulated and the protein concentration of each sample was calculated. It was then possible to calculate the appropriate concentration of exosomal protein for use in the Western Blot. The first step in preparation for Western Blotting was to denature protein samples. Samples were prepared by adding 2.5 µL of 4X sample buffer at room temperature, 1 µL of 10X reducing agent and 6.5 µL of exosomal protein sample. The samples were placed in the PCR sprint machine at 70 °C for 10 minutes to denature them. The second step was electrophoresis (Figure 2.8). Samples were loaded into wells in a gel cassette. The gels used were BioRad Mini-Protean® TGX™ Precast Gels (4-15% gradient) with a 10 well comb. These gels do not contain SDS so for standard denaturing electrophoresis the buffers used must contain SDS. The gel cassette was clamped in place in a Mini Trans-Blot® cell and the cell chamber was filled with a running buffer composed of 2.5mM Tris, 19.2mM Glycine and 0.1% SDS page/H_2O. Each well was loaded with 10 µL of protein sample mixture and the first and last well were loaded with a MagicMark™ XP Western Protein Standard (Initrogen) to allow estimation of the molecular weight of any detected protein. The cell was then connected to a voltage source and run at 100V for 1 hour. The third step in Western Blotting was the transfer from gel to a nitrocellulose membrane (Figure 2.8). The gel cassette was taken apart and a corner of the gel was cut for orientation. Fibre pads, filter paper and a nitrocellulose membrane were pre-soaked in 1X Transfer Buffer composed of 2.5mM Tris, 19.2mM glycine, 20% methanol and H_2O. The gel was placed on a nitrocellulose membrane and a sandwich prepared in the following order: fibre pad, filter paper, gel, nitrocellulose membrane, filter paper, fibre pad. The direction of the transfer is from the black cathode to the red anode so the nitrocellulose membrane must be on the anode side of the gel. The sandwich was placed in cell with an ice block to avoid overheating and a current was passed through at 100V for 30 minutes.
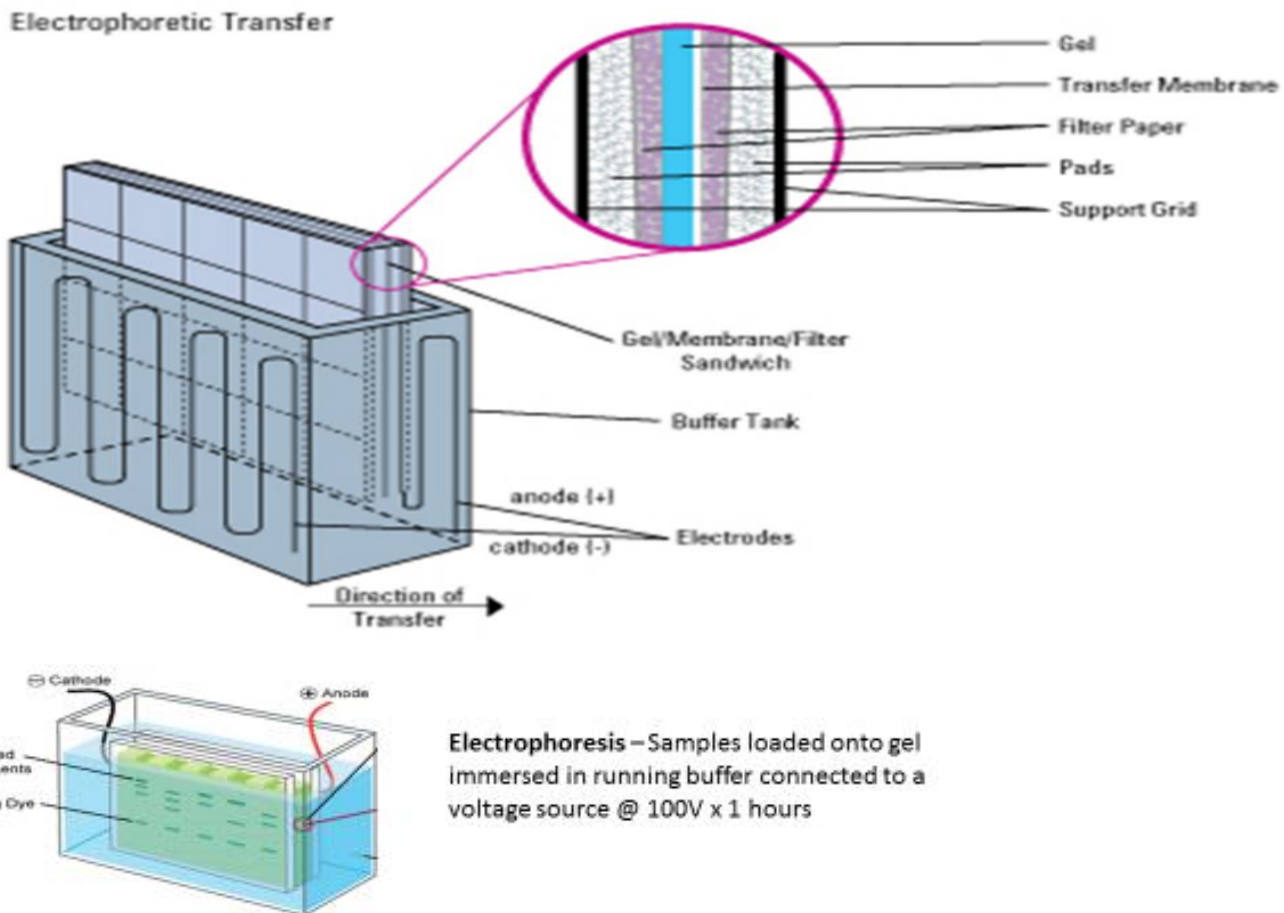
Figure 2.8: Electrophoresis and transfer steps of Western Blotting (adapted from www.lifetechnologies.com) [150]

The fourth step involved blocking and washing. The nitrocellulose membrane was removed from the sandwich and mixed at 120 RPM in 5% milk solution for 1 hour. The membrane was removed from the milk solution and washed at 120 RPM for 15

minutes in a washing buffer composed of 0.5M Tris, 1.5 M NaCl, 0.05% tween and H_20. The membrane then underwent 2 further washes for 5 minutes at 120 RPM. The membrane was then placed in a 0.1% milk solution containing a 1:1,000 dilution of primary anti CD63 antibody (Abcam rabbit monoclonal to CD63: 1mg/ml) overnight at 4 °C. The container was covered in cling-film to avoid evaporation overnight. The following morning the membrane was placed in washing buffer once for 15 minutes at 120 RPM and twice for 5 minutes at 120 RPM. It was then placed in a 0.1% milk solution with a 1:3000 dilution of the secondary antibody (Abcam goat anti-rabbit IgG HRP: 2mg/mL) and incubated on an orbital shaker at 120 RPM for 90 minutes. The following morning the membrane was placed in washing buffer once for 15 minutes at 120 RPM and four times for 5 minutes at 120 RPM. The fifth and final step of the Western Blot involved detection of a protein band. The membrane was removed from the wash solution and placed face up on cling film. Supersignal™ West Pico Chemiluminescent Substrate was prepared combining equal parts of the two provided reagents according to the manufacturer's instructions and added to the surface of the membrane. The membrane was incubated for 5 minutes at room temperature in the presence of the chemiluminescent substrate. Excess reagent was then drained from the membrane by blotting the corner on filter paper and the membrane was covered with cling film avoiding any bubbles or wrinkles. Images were captured using a Syngene G-Box with Genesnap software. Serial images were taken at 5 minute intervals for 30 minutes.

2.8 microRNA extraction from exosomes

Exosomes were isolated as previously described and suspended in 60 µL of PBS. Prior to RNA extraction exosome suspensions were taken from storage at -80 °C and allowed to thaw. The *mir*Vana™ miRNA Isolation Kit was used to extract microRNA from exosomes. 300 µL of lysis binding solution was added to the re-suspended exosome pellet and vortexed for 30 seconds. 36 µL of miRNA homogenate additive was added to the lysate. This was thoroughly mixed by vortexing and inverting and then left on ice for 10 minutes. 330 µL of acid phenol:chloroform was added to the sample which was subsequently centrifuged for 15 minutes at 4°C at 20,800 x g. Following this the same protocol for isolation of miRNA from conditioned medium was followed as previously described.

2.9 miRNA Array

miRNA was extracted from exosomes secreted by HCT116 and HT29 colorectal cancer cells as previous described. The Nanodrop Spectrophotometer was used to assess the quantity and quality of RNA present prior to delivery of samples containing 20 ng/ µL to Exiqon Services, Denmark.

Exiqon, Denmark performed the subsequent microRNA profiling. The quality of RNA was verified by Exiqon using an Agilent 2100 Bioanalyser. All RNA from sample and artificial reference was labeled with Hy3™ and Hy5™ fluorescent label, respectively, using the miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ (Exiqon, Denmark) following the procedure described by the manufacturer. The Hy3™-labeled samples and a Hy5™-labeled reference RNA sample were mixed pair-wise and hybridized to the miRCURY LNA™ microRNA Array 7th Gen (Exiqon, Denmark), which contains capture probes targeting all microRNAs for human, mouse or rat registered in miRBASE 18.0 [151]. The hybridization was performed according to the miRCURY LNA™ microRNA Array Instruction manual using a Tecan HS4800™ hybridization station (Tecan, Austria). After hybridization the microarray slides were scanned and stored in an ozone free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. Array slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and image analysis was carried out using the ImaGene 9.0 software (BioDiscovery, Inc., USA). The quantified signals were background corrected (Normexp with offset value 10) and normalized using the quantile normalization method [152]. An unsupervised data analysis was then performed using Prinicipal Component Analysis (PCA) and a heat map with hierarchical clustering was generated. PCA is a method used to reduce the dimensions of large data sets and is therefore a useful way to explore the naturally arising sample classes based on the expression profile. By including the top 50 miRNAs that have the largest variation across all samples, an overview of how the samples cluster based on this variance was obtained. If the biological differences between the samples are pronounced, this will be a primary component of the variation. This leads to separation of samples in different regions of a PCA plot corresponding to their biology. If other factors, e.g. sample quality, inflict more variation on the samples, the samples will not cluster according to the biology. The complete linkage method together with Euclidean

distance measurement was used to perform two way hierarchical clustering of normalised values of all 2089 targets for both colorectal cancer cell lines.

Based on expression levels, the number of miRNAs above a threshold value were identified for both colorectal cancer cell lines. miRNAs of interest were identified using public computative predictive models of gene pathway analysis (DIANA tools) [153]. Specific microRNAs were then validated in triplicate (three separate exosome isolates) by RQ-PCR as previously described.

2.10 Exosome Transfer

To visualise transfer of exosomes from one cell line to another confocal microscopy was used. Donor cells used were RFP labelled HT29 miR-379 over-expressing cells. Recipient cells used were HCT-116-WT cells. 4×10^5 recipient cells were seeded onto a glass coverslip which had been sterilised under UV light. The glass slips were placed in a 6 well plate and allowed to adhere overnight in an incubator at 37 °C. Exosomes were harvested from donor cells as previously described and were re-suspended in 30 µL of PBS. The PBS containing donor cell exosomes was then added to the recipient cells on a cover slip. 10 µL of exosome suspension was placed onto a separate cover slip without any recipient cells present to act as a control. A small peice of Parafilm was placed directly onto each coverslip to ensure the liquid covered all of the cells. Coverslips were placed into a humidification box. The humidification box was then incubated at 37 °C for 4 hours. Following incubation the coverslips were washed with PBS in a 6 well plate and then immersed in 4% Paraformaldehyde for 10 minutes to fix them. This was followed by another wash with PBS. The fixed cells are then immersed in 1 µg/mL DAPI solution for 5 minutes at room temperature and protected from the light using tinfoil. The slides were then washed again 3 times in PBS. 20 µL of Prolong Antifade (Lifetechnologies) was added to a glass microscope slide. 10 µL of mounting medium was added to the coverslip which was placed cell side down on the glass slide. The corners of the coverslip were sealed in place with nail polish. Slides were appropriately labelled and were protected from light until required. Immunofluorescent Z-stack images (0.1 µm steps) were captured using an Olympus IX81 Microscope fitted with an Andor Revolution Confocal system (Andor, Belfast, Northern Ireland), 60× oil immersion objective lens and an EMCCD Andor iXonEM + camera.

2.11 Assessing the functional effect of exosome transfer on recipient cell proliferation

To assess the functional effect of exosomes from different cell populations on recipient cells, proliferation assays were performed with HCT116 wild-type cells receiving exosomes isolated from various cell types. HCT116 wild-type cells were seeded at a density of 1 x 10^3 per well in a 96 well plate in vertical replicates of 8. To act as controls HCT116 cells were also grown in regular media and exosome free media on a 96 well plate. Exosomes were isolated from HCT116 wild type cells, HCT116 miR-379 over-expressing cells, and HCT116 NTC cells as previously described. Exosomes isolated from each cell type were added to the wells of HCT116 wild type cells and allowed to incubate for 48 hours. Following 48 hours incubation, 20 μL of MTS solution was added to each well. The plates were returned to the incubator for 3 hours and then read on a Bio-Rad microplate reader at 490 nm. Readings from replicates of 8 were averaged and assays were repeated in triplicate. Data were expressed as Mean +/- SEM of 3 experiments and were plotted based on absorbance value at 490nm following 48 hours. Statistical significance was assessed with a Students T test (One Way) and a p value < 0.05 was considered significant.

Chapter 3

Analysis of exosome encapsulated microRNAs secreted by colorectal cancer cells

3.1 Introduction

Intercellular communication is a complex process responsible for maintaining normal tissue homeostasis and can be altered in disease processes such as cancer. Communication in the tumour micro-environment occurs through cellular junctions, adhesions and paracrine release of soluble proteins [31]. Most cell types including colorectal cancer cells have been shown to release a variety of extracellular membrane vesicles including microvesicles known as exosomes. Exosomes are lipid vesicles with a diameter of 40-100nm which are highly stable, facilitating collection from various body fluids as well as cell culture media [32]. Exosomes were originally thought to function as a method of waste disposal to discard unwanted transferrin receptors but are now known to contain lipids, DNA and RNA [19]. They have recently been recognised as vehicles capable of facilitating intercellular communication [28, 29]. Emerging evidence indicates that exosomes may play a role in a range of biological processes including cell proliferation and migration through horizontal transfer of their contents from donor cells to recipient cells [28, 29]. Interestingly, exosomes have recently been found to contain microRNAs (miRNAs) [35].

miRNAs are a class of small non-coding RNA molecules 19-25 nucleotides in length. miRNAs regulate gene expression at a post transcriptional level by binding to the 3' untranslated regions (UTR), coding sequences or 5' UTR of target messenger RNAs. This leads to the inhibition of translation or mRNA degradation [80, 82]. Functional studies have shown miRNAs to participate in almost every cellular process including apoptosis, proliferation and differentiation [80 82]. Among exosomal contents, miRNAs appear to be a component that has one of the most significant functions in intercellular signalling and disease progression. Analysis of exosome miRNA signatures will further our knowledge and understanding of intercellular communication in the tumour micro-environment. To date, there is no existing data regarding the full microRNA content of colorectal cancer cell secreted exosomes.

To achieve advanced adenoma or early tumour detection, discovery and validation of minimally invasive tests as adjuncts to screening which accurately diagnose colorectal

cancer are required. In addition to being highly abundant in circulation, miRNAs show remarkable stability in both plasma and serum [104]. These properties make miRNAs ideal tumour markers for early detection in colorectal cancer. Recent studies, however, have reported a number of miRNAs believed to be released by colorectal tumours as circulating markers of disease are expressed by red blood cells and may be present in the plasma and serum secondary to haemolysis due to collecting procedures or storage methods [114, 115]. Tumour derived exosomal miRNAs may provide a more specific miRNA signature. A small number of studies have identified individual miRNAs present in colorectal cancer cell secreted exosomes (let-7a, miR-21, miR-34a, miR-143, miR-192, miR-1246, miR-215, miR-221) however the full range remains unknown and may include multiple novel targets suitable for further investigation [140, 141].

3.2 Aims

The aims of this study were

- To isolate exosomes secreted by two distinct colorectal cancer cell lines
- To characterise the exosomes isolated with Transmission Electron Microscopy (TEM) and Western Blotting for exosome-associated proteins
- To identify the full profile of exosome-encapsulated miRNAs secreted by two distinct colorectal cancer cell lines and validate a selection of miRNAs using RQ-PCR.

3.3 Materials and Methods

Two distinct colorectal cancer cell lines were used in this study: HT29 cells which grow in a columnar fashion and which have not undergone Epithelial-Mesenchymal Transition (EMT) and HCT116 cells which are more invasive and have partially undergone EMT. For the purpose of isolation of cell-secreted exosomes, the cells were cultured in exosome-depleted media to remove any potential FBS-derived exosomes, as described in section 2.6.

Exosomes secreted by both colorectal cancer cells lines were isolated separately from prepared exosome-free conditioned media using differential centrifugation, micro-

filtration and ultra-centrifugation as described previously (Section 2.6). To assess exosome morphology TEM was employed. Characterisation of exosome protein content was assessed firstly by using a Micro BCA™ Protein Assay kit to determine the protein concentration in isolated exosomes. Following this, Western Blotting for the exosome associated-protein CD63 was performed.

miRNA was extracted from exosomes using the commercially available miRVana™ miRNA isolation kit. The presence, concentration and quality of the miRNA fraction in exosomes was assessed using a NanoDrop ND-1000 Spectrophotometer® (NanoDrop technologies). MicroRNA profiling of exosomes secreted by both colorectal cancer cell lines was performed by Exiqon, Denmark using the miRCURY LNA™ microRNA Array. Hierarchical analysis was performed and miRNAs of interest were selected for validation in triplicate in subsequent exosome isolates using RQ-PCR.

3.4 Results

3.4.1 Exosome Morphology

In order to maintain the morphology and architecture of exosomes for visualisation so that their size could be accurately determined, a primary fixative was added to cell-conditioned media before the final ultra-centrifugation step of exosome isolation. Once isolated they were dehydrated in grades and embedded in resin. Debris from the primary fixative and the presence of resin accounts for the grainy appearance of the images.

Vesicles with a spherical morphology ranging in size between 40 and 100 nm consistent with exosomes were visualised in isolates from HT29 cells and HCT116 cells indicating successful isolation of exosomes secreted by both colorectal cancer cell lines (Figure 3.1)
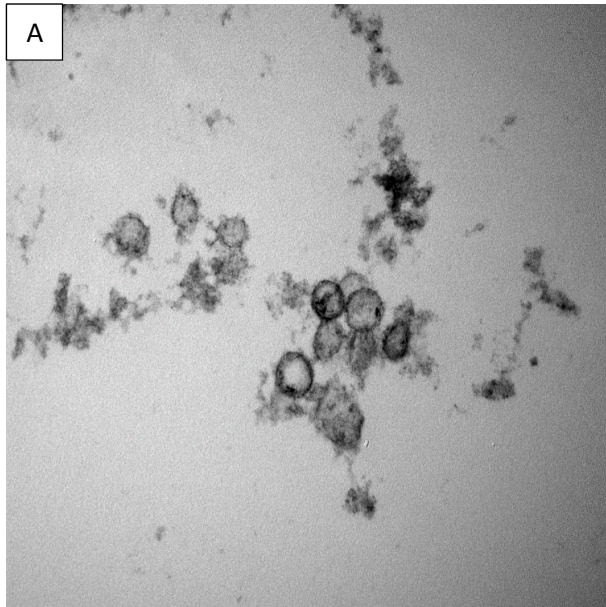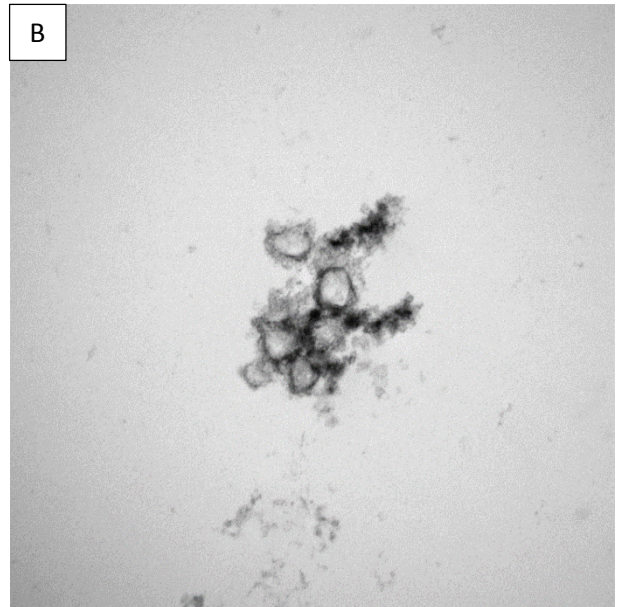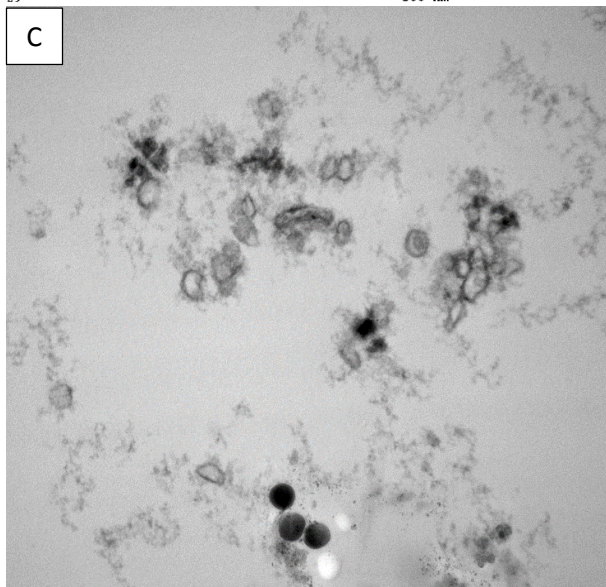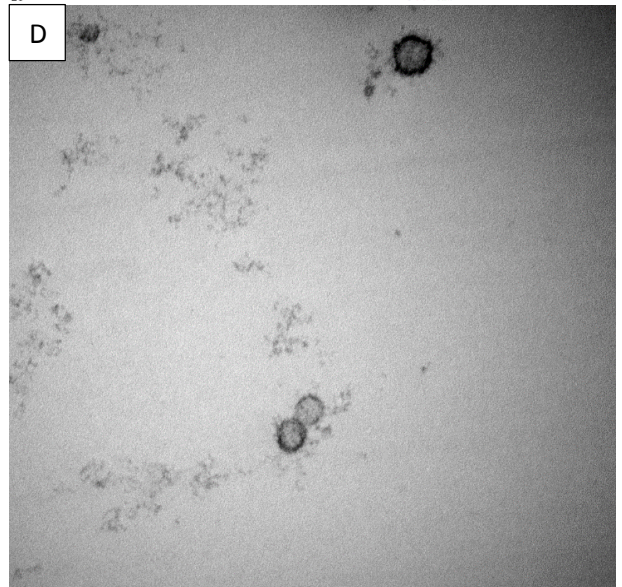
Figure 3.1: Transmission Electron Microscopy Images of Exosomes; A) HT29 exosomes - bar 500nm B) HT29 exosomes bar – 500nm, C) HCT116 exosomes – bar 500nm, D) HCT116 exosomes – bar 500nm

3.4.2 Western Blotting

Protein was extracted from exosome isolates prior to Western Blotting and the concentrations confirmed using the Micro BCA™ Protein Assay kit. Exosomes isolated from both colorectal cancer cell lines contained adequate protein concentrations to assess for exosomal protein markers with Western Blot. Western Blot for the characteristic exosome-associated tetraspanin protein CD63 revealed a band between 50-60 kilodaltons (kDa) in size as expected from protein extracted from HT29 and HCT116 exosomes (Figure 3.2). These results taken together with visualisation of exosome morphology using TEM confirmed the presence of exosomes in samples isolated from media of both HCT116 and HT29 colorectal cancer cell lines.
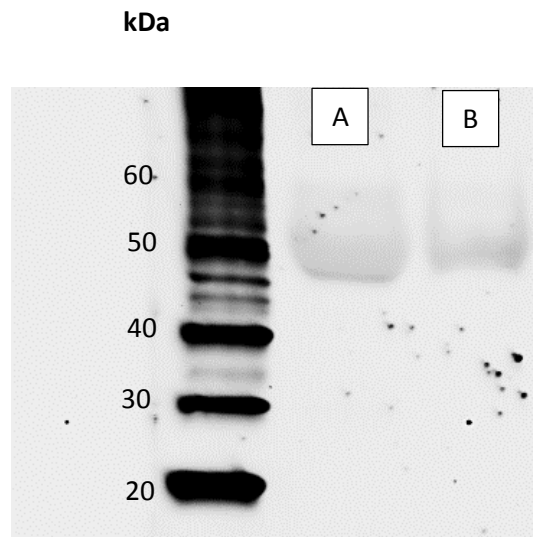
Figure 3.2: **Western Blot targeting exosome-associated protein CD63;** Protein was extracted from HT29 and HC116 colorectal cancer cell secreted exosomes; A) HT29 cell secreted exosomes and B) HCT116 cell secreted exosomes (kDa – KiloDaltons)

3.4.3 Analysis of exosomal miRNA content

miRNA array of exosomal miRNA content was performed by Exiqon, Denmark. Expression analysis using a background threshold figure to determine expression levels revealed the number of miRNAs present in the exosomes of both colorectal cancer cell lines to be within the expected range for human samples. The number present was consistent with the number of exosomal miRNAs present in several different malignant and non-malignant human cell line exosomes (Figure 3.3 A). The signal distribution of miRNAs from exosomes from a number of different malignant and non-malignant human cell lines correlated with that of the colorectal cancer cell line exosomes (Figure 3.3 B).
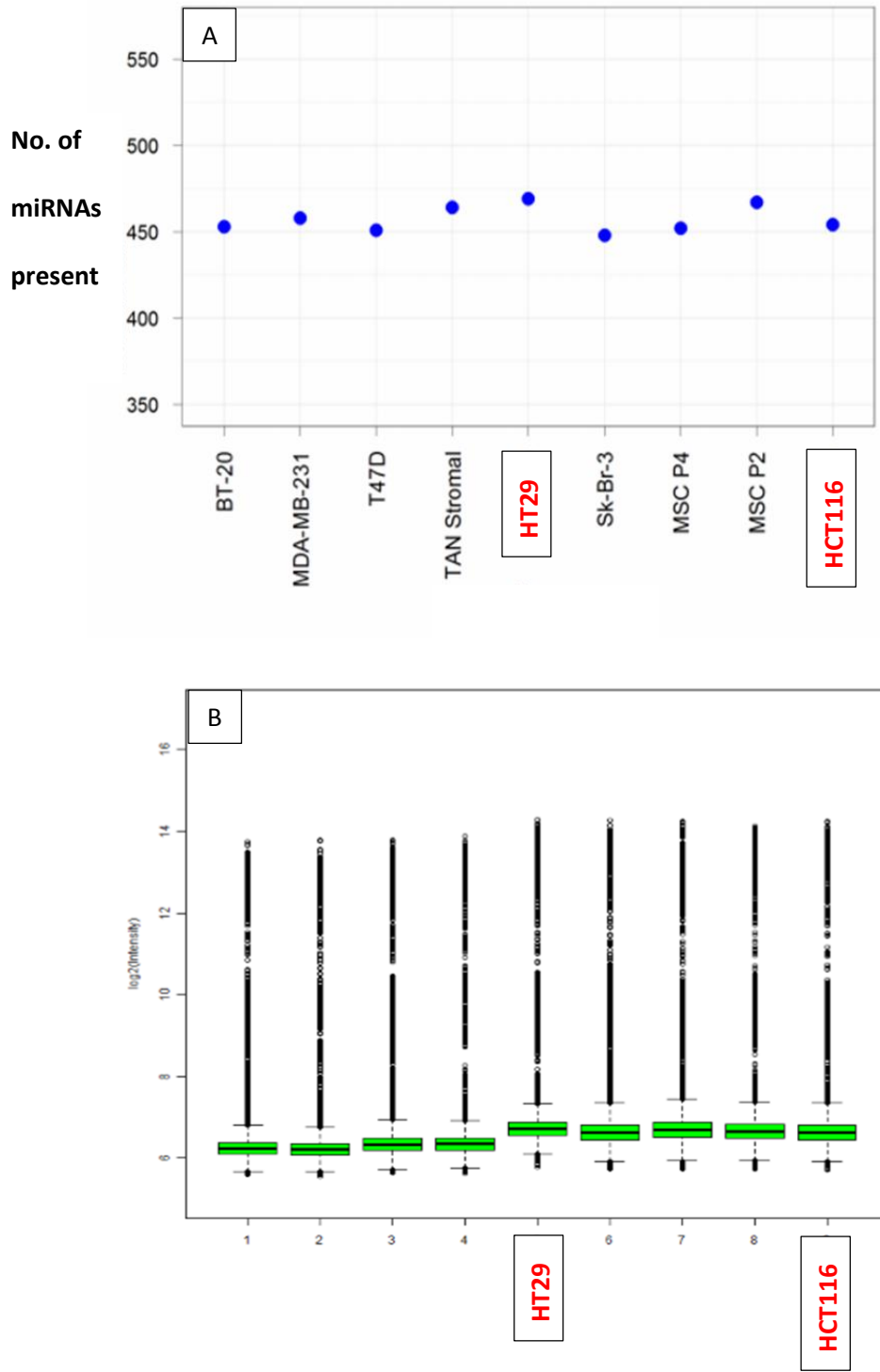
Figure 3.3: Detectable miRNAs in exosomes A) Plot showing number of microRNAs detectable above background threshold for each sample (out of a total of 2089 possible microRNAs) B) Signal distribution plot showing distribution of samples are similar across a number of malignant and non-malignant human cell line-secreted exosomes.

When non-human spike-in miRNAs were excluded array data revealed 409 miRNAs detected above threshold in HT29 derived exosomes. 393 miRNAs were detected in HCT116 exosomes, with 338 miRNAs common to exosomes from both HCT116 and HT29 cells (full list included) (Appendix 3).

A heat map was generated based on expression levels of miRNAs contained within colorectal cancer cell secreted exosomes. Significant differences were observed in certain areas of the heat map representing differential expression of certain miRNAs between cell secreted exosomes from different cell lines (Figure 3.4). Clusters of miRNAs were generated based on a dendrogram assessment of the heat map to identify any areas of interest to further explore (Figure 3.4).
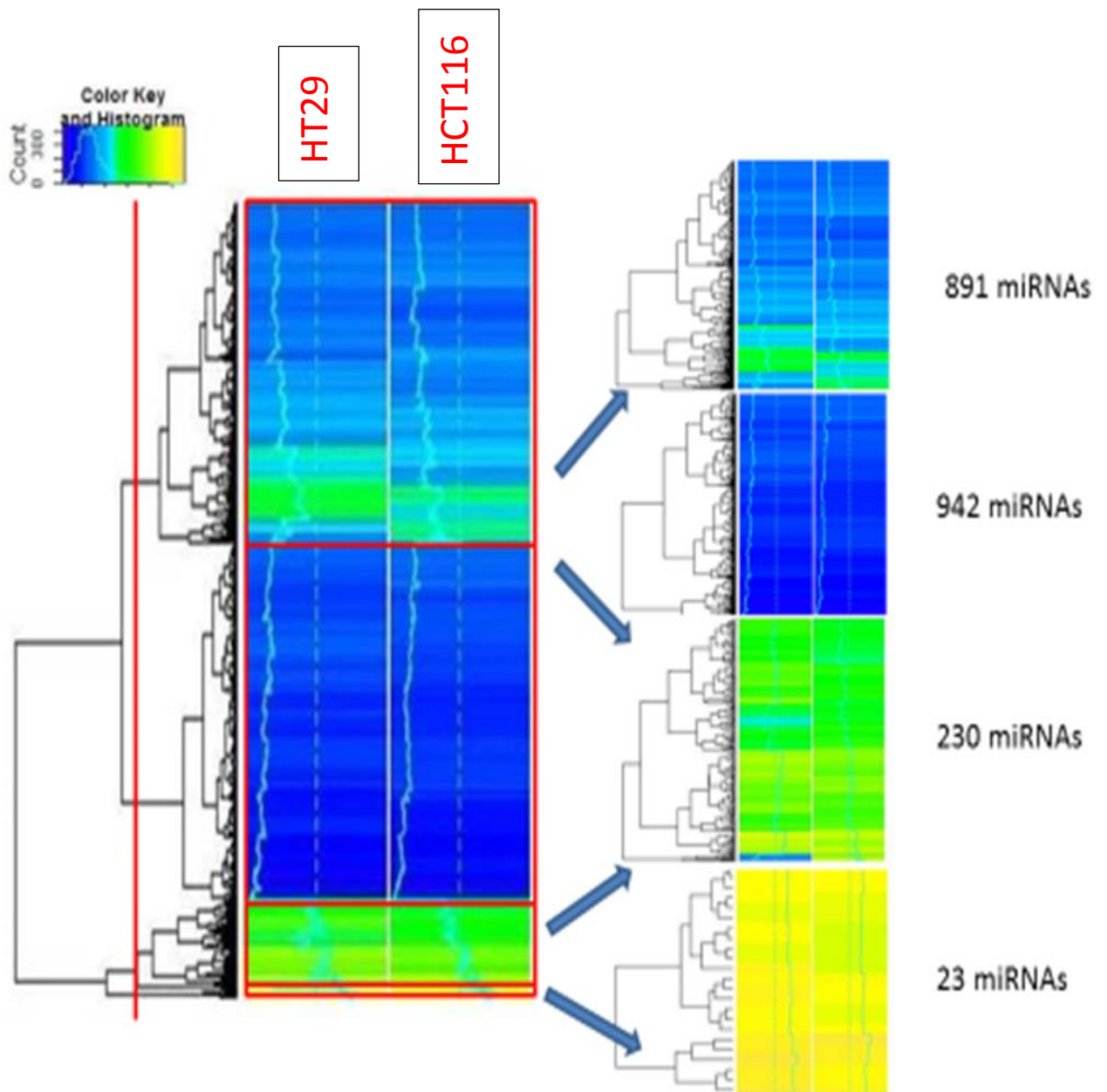
Figure 3.4: Heat map analysis of miRNA expression levels in exosomes. 2089 miRNAs targets were assessed in HT29 and HCT116 colorectal cancer cell line exosomes with subdivision of miRNAs into groups based on expression levels (Yellow represents the most highly expressed and blue the least highly expressed)

The 20 most highly expressed miRNAs common to both cell lines were identified through analysis of expression level data and a table compiled. (Table 3.1). The miRNAs which were found to have the highest difference in expression levels between the exosomes secreted by either HCT116 or HT29 cell lines were also identified (Table 3.2).

Most highly expressed microRNAs in both colorectal cancer cell secreted exosomes
miR-642b-5p
miR-10b-3p
miR-3679-3p
miR-4279
miR-943
miR-624-5p
miR-1248
miR-1249
miR-4723-3p
let7b-5p
miR-378c
miR-365
miR-30d-3p
miR-4274
miR-718
miR-361-5p
miR-1207-3p
miR-548k
miR-145-5p
miR-4312

Table 3.1: The 20 miRNAs found to be most highly expressed which were common to both colorectal cancer cell line exosomes.

HCT-116 cell secreted exosomes	HT29 cell secreted exosomes
microRNA	microRNA
miR-4497	miR-1250-5p
miR-377-5p	miR-511-5p
miR-3960	miR-1224-3p
miR-670-5p	miR-29c-5p
miR-4787-5p	miR-3144-3p
miR-1246	miR-513c-5p
miR-5100	miR-25-3p
miR-4454	miR-192-3p
miR-1260b	miR-1225-5p
miR-4708-3p	miR-1304-5p

Table 3.2: The 10 most differentially expressed microRNAs secreted in exosomes from HCT-116 or HT29 cells. (miRNAs which were highly expressed in one cell line exosome and had low expression levels in the other cell line exosomes)

3.4.4 Validation of miRNAs of interest from array data

Selected miRNAs from the highly expressed areas of the heat map (miR-10b, miR-143 and miR-149-5p) were validated in three separate exosome isolates from both HCT116 and HT29 cell secreted exosomes. miR-10b, miR143 and miR-149-5p, which all have well described roles in cancer and have target genes validated, were consistently detectable in exosome extracts from both cell lines using separate exosome samples isolated from separate experimental set-ups (Table 3.3).

MicroRNA	Role in cancer	Target Gene	Validated in ≥ 3 exosome samples
miR-10b	Initiation	APC [154]	✓
miR-143	Apoptosis	KRAS/BRAF [155]	✓
miR-149-5p	Apoptosis	GSK3a [156]	✓

Table 3.3: miRNAs of interest validated in triplicate with RQ-PCR and their associated roles in cancer.

Discussion

Dysregulated circulating miRNAs such as miR-18a, miR-21, miR-29a and miR-143 have been identified as promising biomarkers of colorectal cancer in several studies [106]. These results were not found to be reproducible in several other studies however [117, 118]. Issues such as pre-existing patient factors including disease states, smoking and ethnicity combined with differing views on the optimum medium from which miRNA should be extracted (whole blood/serum/plasma), and problems with haemolysis of red cells secondary to collection and storage have led to heterogeneous data. Identifying exosomal miRNA signatures from colorectal cancer cells may provide a tumour specific biomarker or panel which will negate some of these concerns.

A key factor in determining exosomal miRNA content and signatures is firstly proving the miRNAs are exosome-encapsulated and not derived from apoptotic bodies or larger microvesicles. While microvesicles and apoptotic bodies range between 50-1000 nm and 50-5000 nm respectively, exosomes tend to range in size between 40-100 nm. In addition, microvesicles and apoptotic bodies do not possess characteristic tetraspanin protein markers in their membranes such as CD 9, CD 63 or CD 81. The results shown in this study demonstrate vesicles between 40-100nm in size and Western Blot confirm the presence of CD 63 in samples. These results taken together are satisfactory evidence that the fraction isolated from conditioned media using micro-filtration and ultra-centrifugation consists of exosomes.

Previous studies have identified selected miRNAs present in exosomes secreted by colorectal cancer cells in vitro including miR-21, miR-192 and miR-221 [140]. A number of miRNAs have also been identified in exosomes isolated from the circulation of colorectal cancer patients including let-7a, miR-1224-5p, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a [141]. This is the first study to describe the full range of miRNAs present in both HCT116 and HT29 colorectal cancer cell-secreted exosomes, demonstrating a large range of miRNAs some of which have well described roles in cancer pathogenesis, and others which have only recently been discovered. Among the most highly expressed miRNAs in both colorectal cancer

cell line exosomes are several which have previously described roles in cancer (miR-642-5p, miR-10b-3p, miR-943, miR-1248, miR-1249, let7b-5p, miR-378c, miR-30d-3p, miR-718, miR-361-5p, miR-548k, miR-145-5p) [157 - 169]. In addition, included in the most highly expressed are some more recently discovered miRNAs with little or no available data regarding their association with disease states, associated gene targets, or functional roles (miR-3679-3p, miR-4279, miR-4723-3p, miR-4274, miR-1207-3p, miR-4312). These miRNAs may represent new targets as biomarkers worthy of further investigation. Among the miRNAs with previously described roles in cancer are some which have been implicated in the presence or progression of colorectal cancer. miR-378, identified as highly expressed in both colorectal cancer cell line exosomes in this study has previously been found to be a haemolysis-independent biomarker for disease presence in colorectal cancer [115]. miR-378 targets the tumour suppressor genes Sufu and Fus-1 and can enhance cell survival, tumour growth, and angiogenesis [115]. Levels of miR-378 are decreased following removal of the primary tumour suggesting its source may be tumour-derived. One potential reason for miR-378 being unaffected by haemolysis and decreased on removal of the primary tumour is that it is contained within tumour-derived exosomes in the circulation.

High levels of miR-10b have been associated with a higher incidence of lymphatic invasion and poor prognosis in colorectal cancer tissues [170]. Interestingly, exosome-mediated transfer of miR-10b to recipient non-invasive breast cancer cell lines has been shown to promote cell invasion in breast cancer [171]. In the current study, miR-10b was found to be among the most highly expressed miRNAs in both colorectal cancer cell line exosomes and was validated in multiple exosome samples from both colorectal cancer cell lines. The association of certain circulating miRNAs with advanced disease stage suggests a functional role of circulating miRNAs in metastasis or formation of a metastatic niche. Previous studies have shown tumour-secreted exosomes can modulate recipient cells towards a pre-metastatic phenotype mediated in part by exosome–encapsulated miRNAs [138]. Further investigation of some of the previously un-explored miRNA targets found in this study may reveal miRNAs with potential gene targets capable of preparing tissues for metastatic invasion.

The data presented identifies a wide range of miRNAs present in colorectal cancer cell secreted exosomes some of which have established roles in cancer pathogenesis and some which have only recently been discovered. There are, however, some limitations. miRNA array provides valuable information on the exosomal miRNA content of HCT116 and HT29 colorectal cancer cell lines and has identified previously unexplored targets. It is, however, only one snapshot of the miRNA content of exosomes and any target used for further investigation would require validation. Despite limitations, this study provides valuable information regarding miRNA profiling of colorectal cancer cell exosomes and will serve as a useful starting point for further investigation of miRNA targets involved in cancer pathogenesis and for use as biomarkers.

Chapter 4

The Effect of miR-379 in Colorectal Cancer

4.1 Introduction

Identification of potent tumour suppressor miRNAs in colorectal cancer is useful for furthering the understanding of cancer pathogenesis. In the context of exosomes, discovery of tumour suppressor miRNAs will expedite the development of new cancer therapies based on therapeutic gene delivery. miR-379 has been identified as a tumour suppressor in breast cancer through previous work in our laboratory [128]. miR-379 is located on chromosome 14q32.31 and is involved in regulation of interleukin-11 production in breast cancer cell lines [129]. Moreover, miR-379 is part of the delta-like 1 homolog-deiodinase, iodothyronine 3 (DLK1-DIO3) cluster which has been shown to be critical for embryonic development and epithelial to mesenchymal transition (EMT), a key process for tumour metastasis [172]. Interestingly, conflicting reports regarding the role of miR-379 have been shown in cancer. Elevated expression of miR-379 has been observed in bone metastatic prostate cancer cell lines and tissues, and miR-379 expression correlated with progression-free survival of patients with prostate cancer suggesting a tumour promoting role however the majority of evidence points towards a tumour suppressor role [172]. Work in our laboratory found in an examination of over 100 breast cancer tissue specimens compared to a control group of 30 normal tissue specimens that miR-379 levels were decreased in breast tumours. In addition, with increasing tumour stage, the level of miR-379 expression further decreased. Over-expression of miR-379 resulted in reduced breast cancer cell proliferation likely due to negative regulation of Cyclin-B1, a key initiator of mitosis in breast cancer [128]. Interestingly, a genome wide analysis of miRNAs imprinted on the 14q32.31 locus including miR-379 reveals downregulation of these miRNAs in human cancers indicating this entire miRNA cluster is a locus of tumour-suppressors [173]. Nothing is known of the effect of miR-379 in colorectal cancer. Given the effect of miR-379 on Cyclin-B1 and evidence that Cyclin-B1 regulates genes such as E-Cadherin which control colorectal cancer invasion, further investigation of the effect of miR-379 in colorectal cancer was undertaken [130]. Further, the apparent global tumour-suppressor effect of miRNAs imprinted on the 14q32.31 locus make miR-379 an ideal target to further investigate in colorectal cancer.

4.2 Aims

The aims of this study were:

- To successfully transduce colorectal cancer cells with a miR-379 over-expressing lentiviral vector and an identical non-targeting control lentiviral vector.
- To determine the effect of miR-379 over-expression in colorectal cancer cells in vitro
- To determine the levels of miR-379 in colorectal cancer patient tumour vs. normal tissue samples

4.3 Materials and Methods

To determine the effect of miR-379 on colorectal cancer cells HT29 and HCT116 colorectal cancer cells were transduced with a lentivirus vector forcing strong and stable over-expression of miR-379 as previously described (Section 2.3). For comparison, another identical lentivirus that does not over-express miR-379 but contains a non-targeting control (NTC) was used to transduce additional HCT116 and HT29 cells. The lentivirus used for this purpose was ThermoScientific SMARTchoice shMIMIC lentiviral microRNAs®. The lentiviral vectors used contained the human Cytomegalovirus (hCMV) which forces strong expression of both miR-379 and the NTC components. The vectors used also contained a red fluorescence protein which assists in tracking transduction and a Puromycin resistant gene which confers resistance of the transduced cell to the antibiotic Puromycin. Confirmation of transduction with fluorescence microscopy and counter-staining with (4',6-diamidino-2-phenylindole) DAPI was performed. Further confirmation of transduction with RQ-PCR was required to ensure adequate and stable transduction.

The functional effects of miR-379 over-expression in colorectal cancer cells were determined with proliferation and migration assays comparing miR-379 over-expressing, to non-targeting control cells. Proliferation assays were performed using a CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay (MTS, Promega) at 24, 48 and 72 hours as previously described (Section 2.4). Migration assays were performed using Corning® (New York, USA) Transwell ® porous membranes with a

6.5 mm diameter and a 0.8µm pore size. At the end of the experiment, membranes were harvested, stained, mounted onto slides, and migrated cells quantified via light microscopy in five fields of view (Section 2.5).

To determine miR-379 levels in patient tumour compared to normal samples, RNA which had been previously extracted from tumour and normal tissues was reverse transcribed for miR-16 (endogenous control) and miR-379. RQ-PCR was then performed to determine relative expression levels. All samples previously obtained and stored prospectively in the biobank were obtained with ethical approval and written informed consent (Appendix 2).

4.4 Results

4.4.1 Confirmation of Cell Line Transduction

HCT116 and HT29 cells were transduced with a miR-379 and NTC-mimic as described, and the successfully transduced cells were visualized using fluorescence microscopy following counter-staining with DAPI (Figure 4.1). Comparison of the cells demonstrating red fluorescence indicating transduction with the number of blue nuclei indicating all cells present confirmed successful transduction of cells.
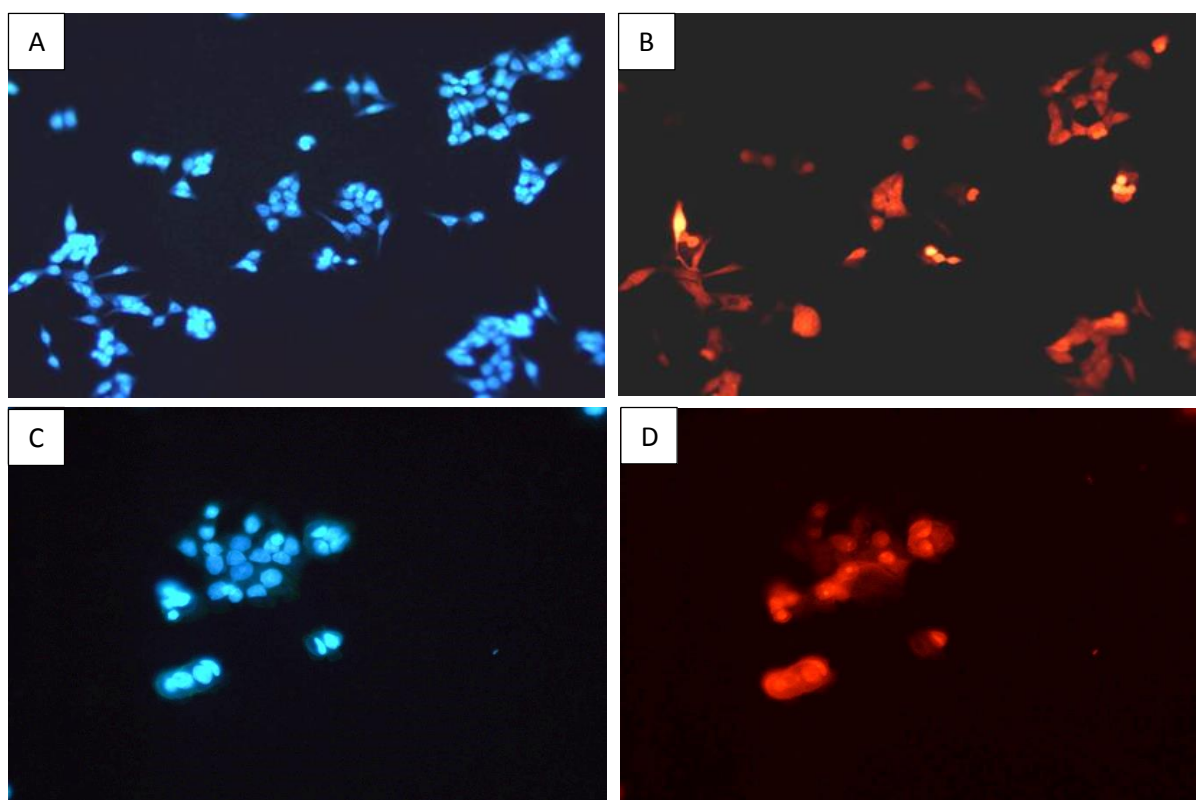
Figure 4.1: Fluorescence microscopy images of transduced cells; A) HCT-116-379 cell nuclei counterstained with DAPI, B) HCT-116-379 cells showing red-fluorescence indicating transduction C) HT-29-379 cell nuclei stained blue with DAPI, D) HT-29-379 cells showing red-fluorescence indicating transduction

Further confirmation of strong and stable over-expression of miR-379 was established using RQ-PCR. A significant elevation in miR-379 expression in HCT-116-379 cells (2.20 $_{log10}$ RQ increase relative to HCT-116-NTC) was confirmed at 1 and 2 weeks following transduction to establish stable over-expression of miR-379 (Figure 4.2).
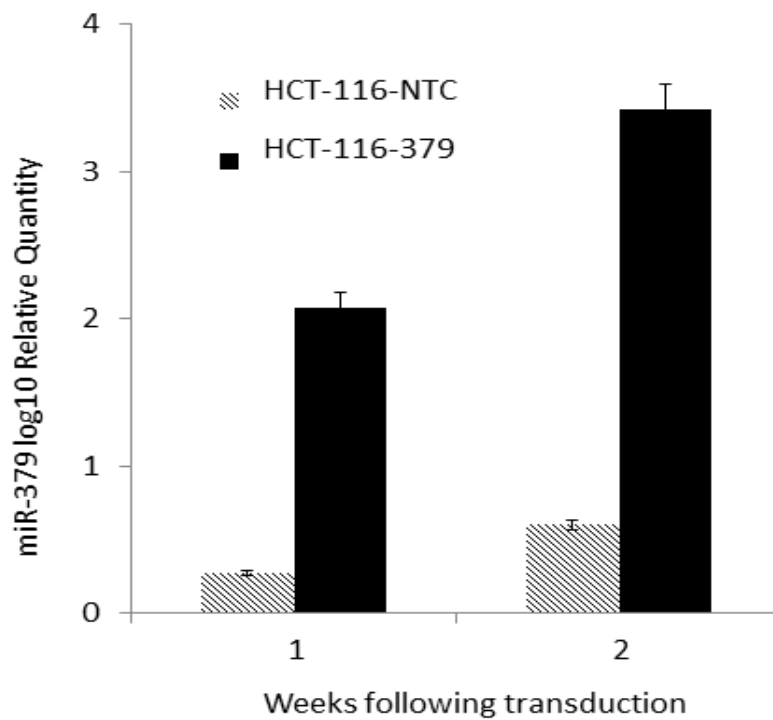
Figure 4.2: RQ-PCR analysis of miR-379 expression in transduced cells. Confirmation of elevated miR-379 expression in HCT-116-379 cells compared to HCT-116-NTC cells at 1 week and 2 weeks following transduction with a lentiviral vector over-expressing miR-379 or a non-targeting control.

4.4.2 Assessing the Functional Effects of miR-379 Over-Expression in Vitro

4.4.2.1 Cell Proliferation in response to miR-379

Following confirmation of successful transduction, cell proliferation at 24, 48 and 72 hours was determined in HCT-116--379 cells compared to HCT-116-NTC cells. Cells were seeded in parallel at the same density in a 96-well format and proliferation monitored over time. A statistically significant decrease in proliferation was observed in HCT-116-379 cells compared to HCT-116-NTC cells (12-15% decrease, ANOVA $p < 0.001$, Figure 4.3).
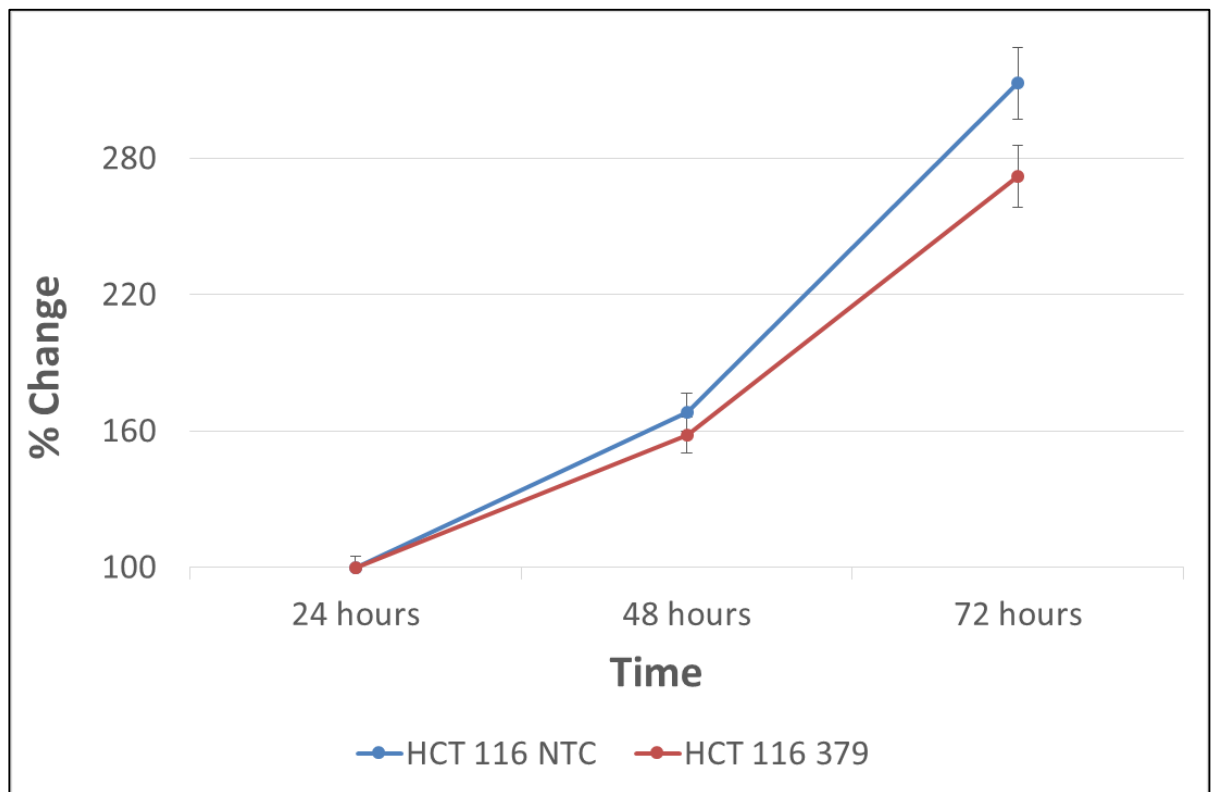
Figure 4.3: Assessment of cell proliferation in response to over-expression of miR-379. Overexpression of miR-379 was found to decrease the proliferation of HCT116 cells (HCT-116-379 – red line) compared to control cells (HCT-116-NTC blue line) (range 12-15%) (ANOVA p<0.001).

4.4.2.2 Cell Migration in response to miR-379

The effect of miR-379 over-expression on migration of cells toward a chemo-attractant was assessed. A statistically significant decrease of up to 86% was observed in HCT-116-379 cell migration towards a chemo-attractant (10% FBS) when compared to HCT-116-NTC cell migration (32-86% decrease, $p < 0.001$) (Figure 4.4).
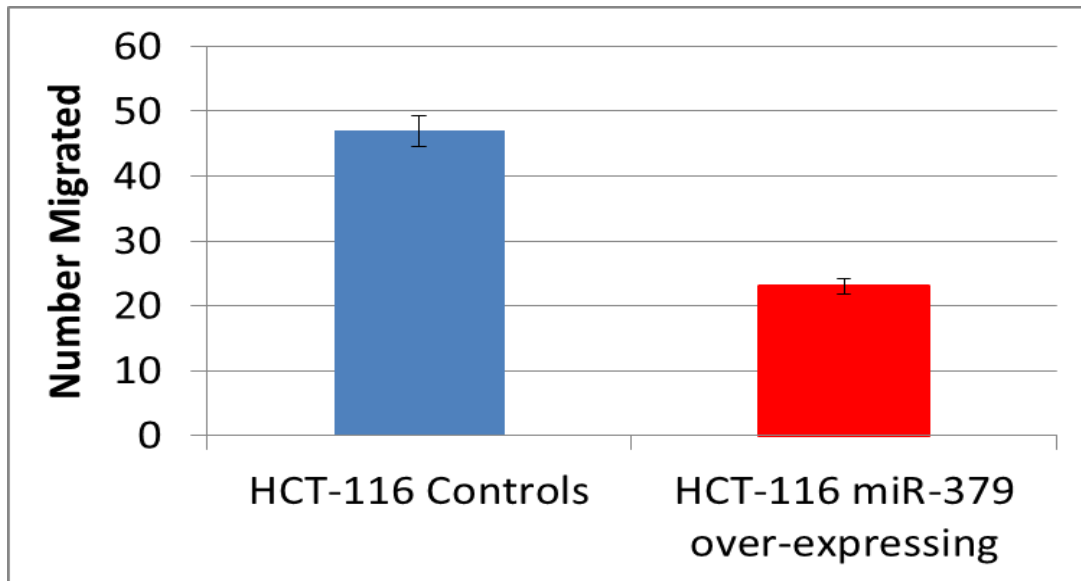
Figure 4.4: Cell migration in response to over-expression of miR-379. Results demonstrate a decrease in migration of HCT-116-379 cells (range 32-86% decrease) compared to HCT-116-NTC cells towards a chemoattractant (p<0.001 Student T Test)

4.4.3 miR-379 Expression in Patient Tumour Vs Normal Tissue Samples

RNA extracted from patient matched colon tumour and normal tissues harvested during surgical resection was stored in the biobank. miR-379 expression in 32 patient matched tumours and normal tissues was examined. miR-16 was used an endogenous control. MiR-379 and miR-16 were detectable in all 32 tumour samples and all 32 tissue samples. There was a trend towards lower expression of miR-379 in tumour samples compared to normal tissue although this was not statistically significant (Figure 4.5).
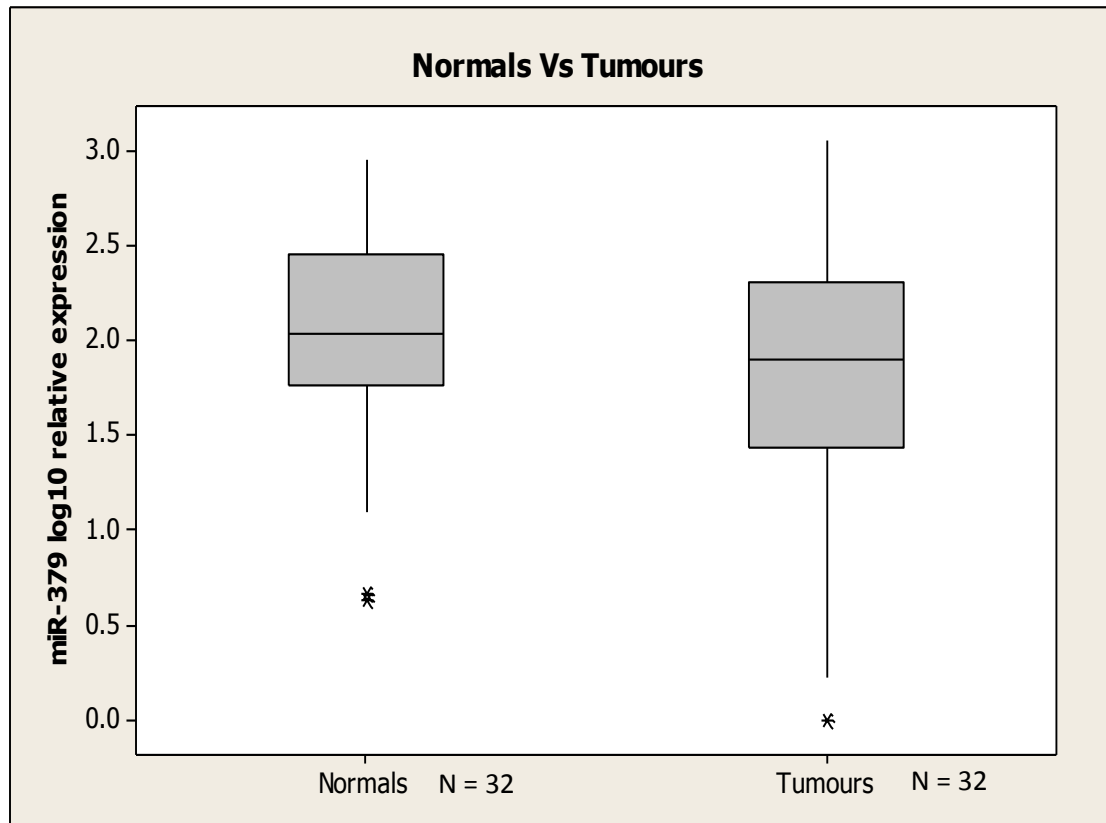
Figure 4.5: miR-379 expression in normal vs. tumour tissues (samples from 32 patient matched normal colon and colon tumour tissues).

Discussion

miR-379 represents an ideal target to further investigate as a tumour suppressor in a number of cancers based on pre-existing evidence. Firstly, the 14q32.31 cluster has proven to be a locus of tumour suppressor miRNAs in genome wide analysis of human cancer tissues [173]. On a genome wide scale between 12-30% of the total down-regulated miRNAs in a number of different cancers including glioblastoma, renal clear cell carcinoma, breast carcinoma and ovarian carcinoma were found to originate from the 14q32.31 cluster [173]. These data, taken in conjunction with work from our own laboratory showing compelling evidence that miR-379 is a tumour suppressor in breast cancer, indicate miR-379 is an ideal candidate for further investigation as a tumour suppressor in colorectal cancer. Furthermore, significant evidence exists demonstrating miR-379 regulates a number of genes with proven effects in colorectal cancer pathogenesis and metastasis. miR-379 down-regulates several genes involved in the TGF-β signalling pathway [174]. A large proportion of colorectal cancers are known to display abnormalities in TGF-β pathway factors [175]. Cyclin B1, which was found to be regulated by miR-379 in our laboratory, controls colorectal cancer invasion and metastases by regulating E-Cadherin [130]. Thus, investigation of any functional effects of miR-379 in colorectal cancer cells was undertaken.

A significant reduction in colorectal cancer cell proliferation in response to over-expression of miR-379 was observed in this study. Cells over expressing miR-379 were found to proliferate between 12-15% slower compared to controls after 72 hours incubation. Interestingly, a number of the predicted target genes such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) have been found to be up-regulated in colorectal cancer, in keeping with its evident anti-proliferative effect in this study [176, 177]. Transient knockdown of PDK1 suppresses cellular growth, induces cellular apoptosis and causes abnormal cell cycle distribution [177]. It is likely that over-expression of miR-379 within colorectal cancer cells produces a myriad of effects leading to an over-all decrease in proliferation which was observed in this study.

A marked reduction in migration of colorectal cancer cells in response to over-expression of miR-379 was observed ranging as high as 86%. This also accounts for chemokinesis or random movement of cells. This very significant effect is interesting as miR-379 has a documented effect on Cyclin B1 as previously discussed [130]. Cyclin B1 in turn has a documented inhibitory effect on E-Cadherin leading to induction of colorectal cancer cell migration and invasion [130]. While over-expression of Cyclin B1 has been found in colorectal cancer tissues, elevated expression of Cyclin B1 is negatively associated with lymph node metastases, distant organ metastases and advanced disease stage [130]. Inhibition of Cyclin B1 in colorectal cancer cells has in fact proved to reduce cell migration and invasion of three distinct colorectal cancer cell lines. While the marked effect of miR-379 on colorectal cancer cell migration in this study are again likely multifactorial, further examination of Cyclin B1 expression in colorectal cancer cells over-expressing miR-379 would be worthwhile.

miR-379 expression levels in tumour tissues compared to patient matched normal samples were not significantly different in this study. There was however a trend towards lower expression of miR-379 in tumours which may have become more significant with increasing patient samples. Unfortunately, data regarding the disease stage and presence of metastases was not available for all patient matched samples but given the marked effect of miR-379 on migration, it may be worthwhile investigating a link between miR-379 and factors such as advanced disease stage, lymph node metastases and distant organ metastases.

The results demonstrated in this study show miR-379 has a significant effect on colorectal cancer cell proliferation and a marked effect on colorectal cancer cell migration. There was no significant difference in expression levels of miR-379 in tumours compared to normal tissue although patient numbers were small. Given the functional effects exerted by miR-379 on colorectal cancer cells, further investigation of miR-379 target genes involved in EMT including E-Cadherin is warranted as this plays such a central role in cancer metastases. Further evaluation of miR-379 levels in

metastatic colorectal cancer compared to non-invasive or early stage malignancy may reveal additional evidence of its effect in promoting EMT.

Chapter 5

Engineering and Transfer of Exosomes Over-Expressing miR-379

5.1 Introduction

The use of exosomes as a vehicle for the administration of anti-tumour compounds including genetic material or drug therapies has gained significant interest recently. Exosomes are immunologically inert when purified from a compatible cell source and possess an innate ability to pass biological barriers [178]. These properties make them ideal vehicles for delivery of therapeutics. Animal models have already shown exosomes can be used to deliver chemotherapeutics such as Doxorubicin to specifically target breast tumour cells which results in decreased tumour cell proliferation [179]. One of the most exciting applications of exosome therapy is the use of therapeutic miRNAs. Delivery of tumour suppressor miRNAs ''miRNA replacement therapy'', has been somewhat hindered by the requirement for a delivery vehicle to transfer miRNAs to diseased or cancerous tissue. A growing body of evidence supports the use of exosomes to efficiently deliver anti-tumour miRNAs to cancer tissues in vivo. Ohno et al [143] have shown intravenously injected exosomes can deliver let-7a miRNA to EGFR-expressing breast cancer xenografts in mice. This subsequently resulted in inhibition of tumour development in vivo [143]. Further, Iguchi et al. [144] found exosomal miR-16 was transferred into prostate cancer PC-3M cells subcutaneously xenografted in nude mice following intra-tumoral injection, resulting in the suppression of its target gene. To date, there are no studies investigating the potential for exosomal miRNA based therapies in colorectal cancer.

The first step in the development of gene therapies based on tumour-suppressor miRNAs in colorectal cancer is to determine if exosomes can be engineered to over-express specific miRNAs and if these exosomes can effectively be delivered to target cells. Once delivered to target cells, it is then necessary to determine any functional effect the enriched exosomes may exert.

5.2 Aims

The aims of this study were:

- To engineer colorectal cancer cells to secrete exosomes enriched with miR-379

- To visualise the transfer of exosomes from one cell population to another
- To investigate any functional effect engineered exosomes may exert on recipient cells

5.3 Materials and Methods

As discussed in section 2.6, HCT-116-379 cells were cultured in specially prepared exosome free media to ensure only exosomes secreted by engineered cells were present. Exosomes secreted by HCT-116-379 cells were then isolated as previously described using differential centrifugation, microfiltration and ultracentrifugation. Following reverse transcription for miR-379 and miR-16, RQ-PCR was then performed on HCT-116-379 cells and exosomes, HCT-116-NTC cells and exosomes and HCT-116 WT cells and exosomes to determine expression levels of miR-379.

To visualise exosome transfer from one cell population to another, exosomes were isolated as previously described from transduced cells, as these exosomes were labelled with a red fluorescent protein. These exosomes were incubated for 4 hours or 24 hours with WT cells in separate experiments in a humidification box. Following fixation, confocal microscopy using an Olympus IX81 Microscope fitted with an Andor Revolution Confocal system (Andor, Belfast, Northern Ireland), 60× oil immersion objective lens and an EMCCD Andor iXonEM + camera was then used to image exosome transfer to recipient cells.

To determine any functional effect an excess or depletion of exosomes may have on recipient cells, proliferation assays were performed with HCT-116 WT cells incubated in exosome depleted media, standard media and media with an exosome spike-in. Proliferation assays were performed as previously described using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega) at 48 hours. Experiments were performed in triplicate.

To determine any functional effect miRNA enriched exosomes have on recipient cells proliferation assays were performed on HCT116 WT cells receiving miR-379 enriched exosomes and WT exosomes for comparison. Cell proliferation was measured at 48 hours. Experiments were performed in triplicate.

5.4 Results

5.4.1 Confirmation of miRNA Enrichment of Exosomes using RQ-PCR

Exosomes were isolated from HCT-116-379 cells, HCT-116-NTC cells and HCT-116 WT cells. miRNA was extracted from the exosomes and reverse transcription and RQ-PCR were performed. miR-379 was detectable in exosomes isolated from HCT-116-379 cells but was undetectable in HCT-116-NTC and HCT-116 WT cells confirming successful engineering of exosomes to over-express miR-379 (Table 5.1).

Cell line	miR-379 detected in cells	miR-379 detected in conditioned medium	miR-379 detected in exosomes
HCT-116-WT	✓	✗	✗
HCT-116_NTC	✓	✗	✗
HCT-116-379	✓	✗	✓

Table 5.1: miR-379 expression levels in cells, conditioned medium and exosomes following transduction

5.4.2 Transfer of Exosomes Between Cell Populations

Exosomes isolated from transduced cells were placed onto cover slides and fixed without any donor cells present to demonstrate that the exosomes were isolated from the donor cells. Images taken of the fraction isolated from donor cells demonstrate fluorescent red vesicles in keeping with exosomes (Figure 5.1)
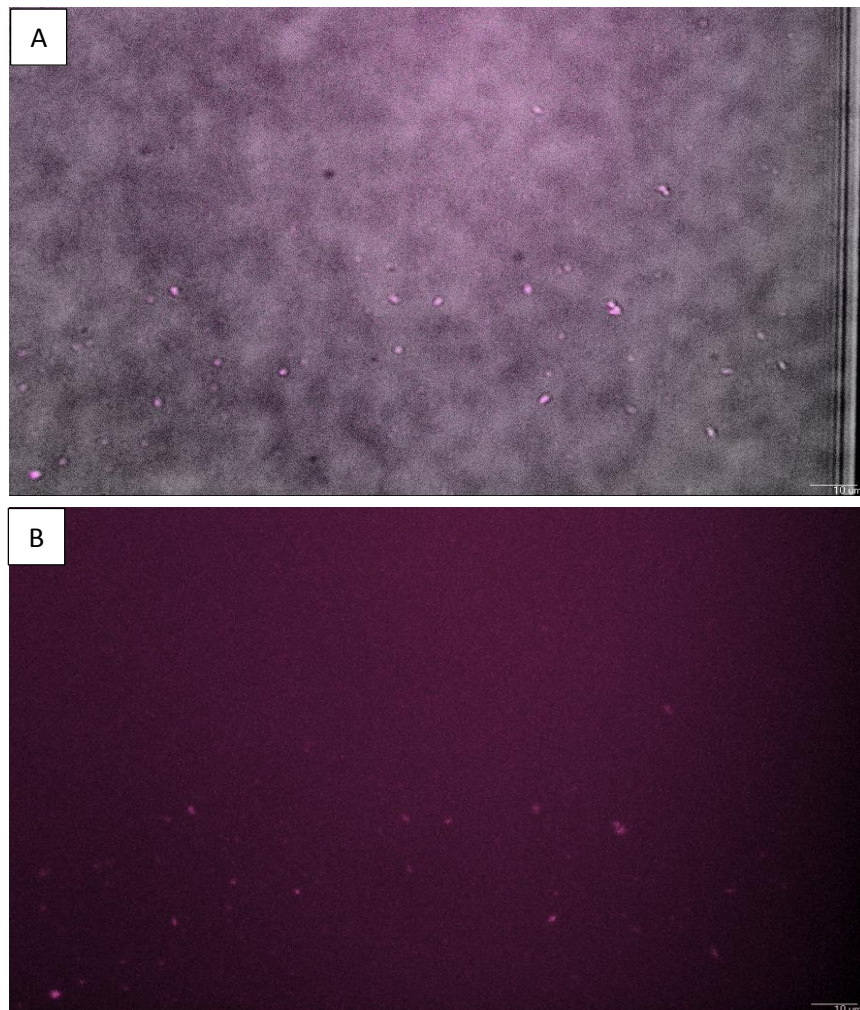
Figure 5.1: Fluorescence microscopy images of exosomes; A) Brightfield images of exosomes labelled with a red fluorescent protein, B) Fluorescence microscopy images of exosomes labelled with a red fluorescent protein (magnification 60,000X).

Exosomes isolated from HCT-116-379 cells were transferred to HT29 WT colorectal cancer cells. Two separate set-ups were used in which recipient cells were incubated in humidifiers for 4 and 24 hours respectively using a similar amount of exosomes. Following 4 hours incubation, RFP-labelled donor cell exosomes were visualised clustering around recipient cells indicative of transfer to the recipient cell and fusion with the membrane (Figure 5.2 A). Similar transfer of exosomes occurred following 24 hours incubation in a separate experiment. Again, RFP-labelled donor cell exosomes were visualised clustering around recipient cells indicative of transfer to the recipient cell. Following 24 hours incubation there appears to be a more extensive interaction between exosomes and cell membranes indicative of further fusion with the membrane (Figure 5.2 B).
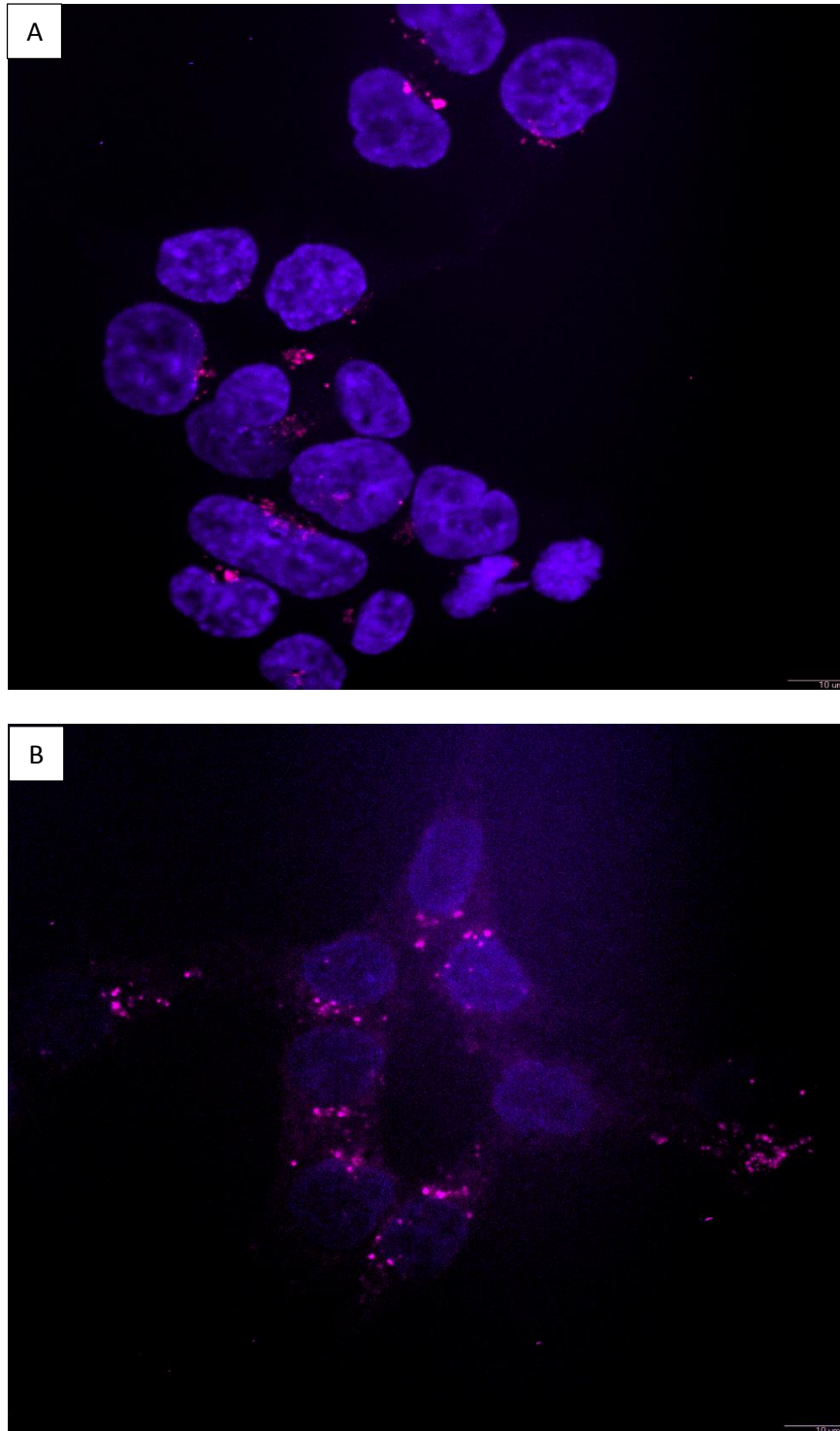
Figure 5.2: Exosome transfer to recipient cells. Confocal microscopy image of exosomes isolated from miR-379 over expressing cells (red due to RFP labelling) clustering around WT cells (blue DAPI stained nuclei) following A) 4 hours incubation and B) 24 hours incubation. All Z stack images (0.1 μm steps) were processed using Andor IQ 2.3 software and all images are presented as maximum intensity projections (magnification – 60,000X)

5.4.3 The Effect of Exosomes on Recipient Cell Proliferation

To determine the functional effect of exosomes on recipient cells HCT-116 wild type cells were incubated in exosome free media, standard media and media with an exosome spike in from the identical cell line. While there are apparent differences observed between proliferation levels, particularly between cells grown with additional exosomes and cells grown in exosome depleted media, this was not statistically significant (P = 0.112, Figure 5.3).
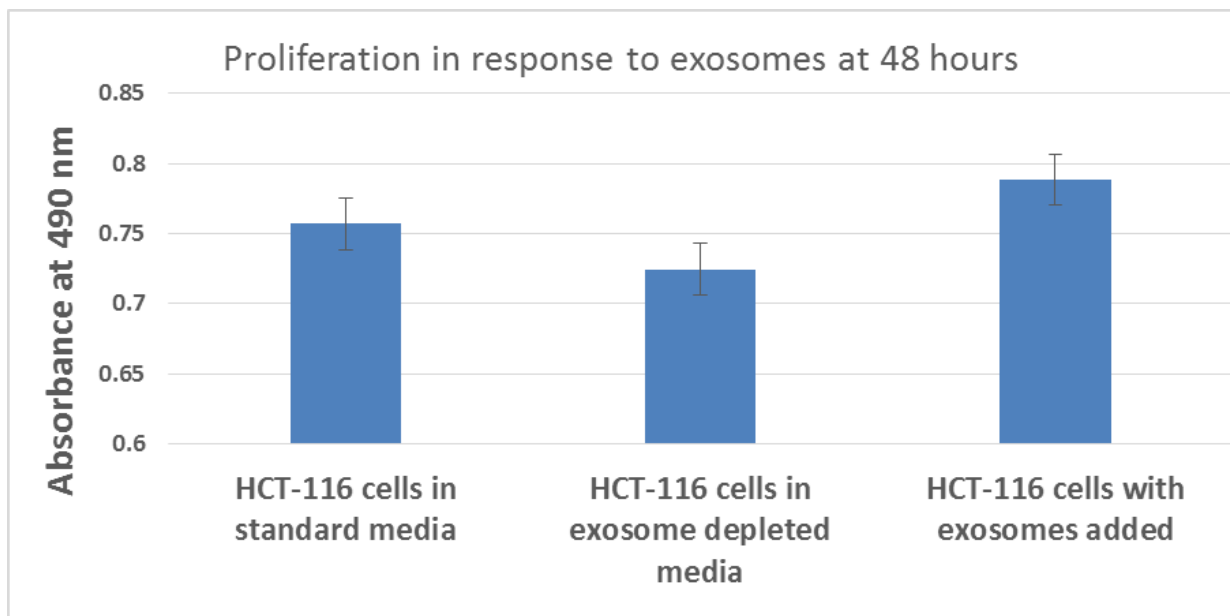
Figure 5.3: Proliferation of HCT-116-WT cells in standard media, exosome depleted media and media with exosomes spiked in

5.4.4 The Effect of miR-379 Enriched Exosomes on Recipient Cell Proliferation

To determine the functional effect of miRNA enriched exosomes on recipient cells HCT-116 wild type cells were incubated in media containing exosomes enriched in miR-379, HCT-116-NTC exosomes and WT exosomes. An observed difference in proliferation of WT cells in response to exosomes containing miR-379 compared to WT cells receiving NTC cell secreted exosomes was identified although it was not statistically significant (P = 0.538, Figure 5.4).
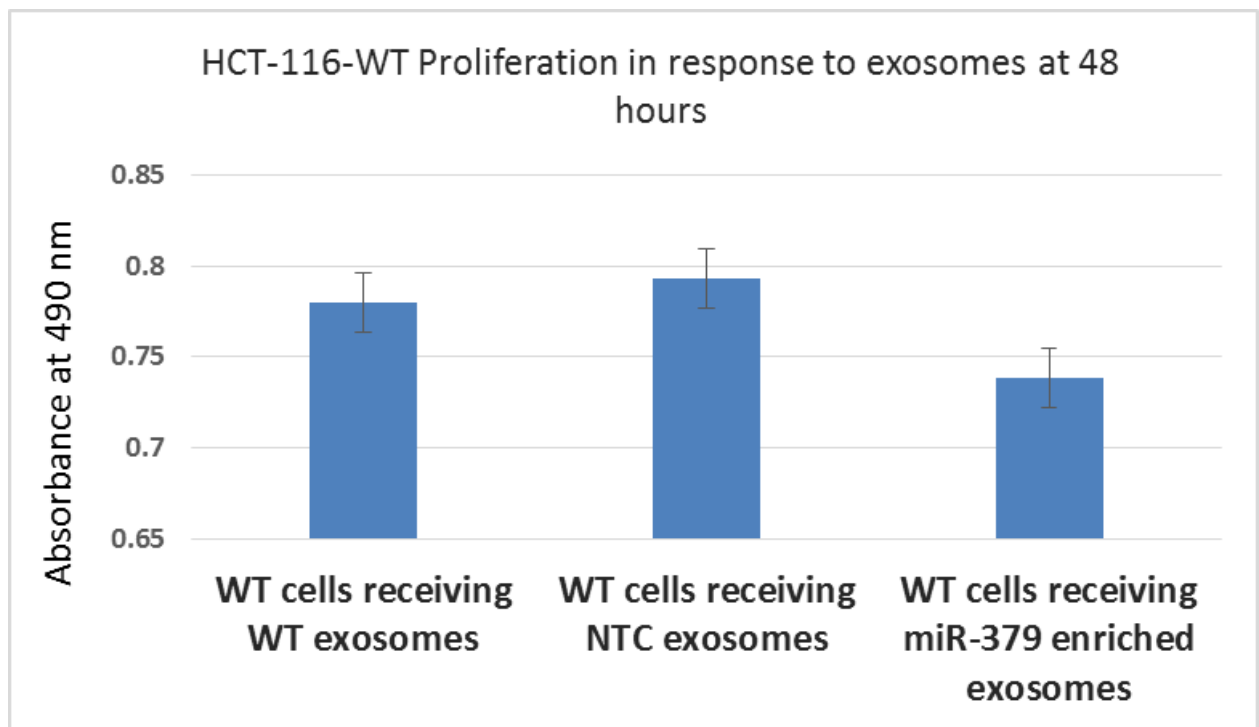
Figure 5.4: Proliferation of HCT-116 WT cells in response to WT cell secreted exosomes, NTC cell secreted exosomes and miR-379 enriched exosomes

Discussion

Based on the proven impact of exosomes on cell phenotype, there is growing interest in the potential of exosomes in the therapeutic setting. Following confirmation that transfection with miR-146b reduces glioma cell invasion, migration and viability Katakowski et al. [180] have demonstrated that exosomes from stromal cells engineered to express miR-146b, inhibited glioma growth in rats [180]. Functionally competent miRNAs contained within exosomes represents an exciting potential therapeutic delivery vehicle for tumour suppressor miRNAs in other cancers including colorectal. This study aimed to evaluate if a similar effect could be seen with colorectal cancer cells.

miR-379 is a reported tumour suppressor in the breast cancer setting and has demonstrated similar properties in colorectal cancer cells as previously described in this work. A statistically significant decrease in proliferation and migration of colorectal cancer cells was observed in the presence of miR-379 which has not previously been shown in colorectal cancer cells. Thus, this aspect of the study sought to determine if exosomes could be enriched with miR-379 and delivered to recipient colorectal cancer cells. Exosomes which were secreted from miR-379 over-expressing cells were found to contain detectable levels of miR-379 compared to exosomes secreted by HCT-116-NTC and HCT-116-WT cells. In addition, miR-379 was not detected through miRNA array performed on HCT-116 WT exosomes as previously described. These results demonstrate that exosomes can be engineered to over-express miRNAs which are not naturally occurring within the donor cell and raises exciting possibilities for engineering of exosomes which contain potent tumour-suppressors applicable to a wide range of cancers.

Once the successful engineering of exosomes to over express miR-379 was established, confirmation that exosomes are transferred to recipient cell lines was required. The images taken of red fluorescent exosomes and recipient cell nuclei at 4 hours showed exosomes clustering around recipient cells. Following 24 hours there was a larger amount of exosomes which appeared to be clustering around recipient cells indicating further uptake. These results strongly indicate that exosomes are

transferred to recipient cells and the fluorescent red staining around recipient cell nuclei represents exosomes which have merged with recipient cell membranes.

Following confirmatory imaging of transfer the functional effects of miR-379 enriched exosomes on recipient cells were investigated. As shown previously, over-expression of miR-379 produced a 12-15% decrease ($p < 0.001$) in cell proliferation following 72 hours. Incubation of HCT-116 WT cells in media containing exosomes enriched in miR-379 produced an observable decrease in proliferation however it was not significant. As the effect of miR-379 over-expression on migration was very significant, ranging from 32-86%, further investigation of the effect of miR-379 enriched exosomes on cell migration may yield a more pronounced effect. Determining a target gene for miR-379 in colorectal cancer cells and identifying knockdown of that gene in recipient cells could provide further confirmatory evidence of functional effects of miRNA transfer. A similar experiment by Katakowski et al. [180] showed transfer of exosomal miR-146 to glioma cells reduced expression of EGFR and NF-κB protein in recipient cells. There are also a number of caveats however when considering the effect of exosomal miRNAs on recipient cells. Effects exerted on recipient cells by exosomes cannot be entirely attributed to miRNAs and is likely multi-factorial and extremely complex. In this study, forced miR-379 over-expression in cells may significantly alter the contents of the exosomes they secrete. Exosomal content may reflect cell phenotype and alteration of cell phenotype with transduction may have a vast array of effects altering protein and mRNA content in exosomes. Further, the effect exerted on recipient cells by exosomes may be significantly dependant on the number of exosomes used. Studies vary significantly in terms of the amount of exosomes applied for experimental purposes [179, 180]. Some studies have shown exosomes exert effects in a dose dependant manner thus experimental data available currently may not be comparable [181]. A more pronounced effect on recipient cell proliferation may have been observed in this study with increasing number of exosomes. This work does however add to the growing body of evidence that it is possible to engineer exosomes to over-express miRNAs and these can be transferred to recipient cells to exert effects.

Chapter 6

Discussion

Discussion

The interaction between colorectal cancer epithelial cells and surrounding tumour stroma is complex. Understanding these complex interactions between cell types in the tumour micro-environment will ultimately lead to a better understanding of cancer pathogenesis, development of biomarkers and efficacious therapeutics. miRNAs have been the focus of very significant interest as they appear to regulate large numbers of target genes, show tumour suppressor and tumour promoting capabilities in tissues and are stable in the circulation. Their functional role in circulation has until recently remained unknown. The discovery that exosomes can transfer functionally competent miRNAs to recipient cells both locally and systemically has opened up a vast area for further investigation. Exosome encapsulated miRNAs may be responsible for promoting tumour growth in the tumour micro-environment and creating a metastatic niche systemically. Determining what miRNAs are secreted within colorectal cancer cell exosomes is essential in advancing our understanding of these processes and can be achieved with exosomal miRNA profiling as performed in this study. While investigation has been carried out on the functional roles of exosome encapsulated miRNAs in cancers such as breast and prostate, there is no existing data to date investigating the range of miRNAs contained in colorectal cancer cell secreted exosomes [143, 144]. This study is the first to describe the full range of miRNAs secreted by both HCT116 and HT29 colorectal cancer cells.

An essential aspect in determining exosomal miRNA content and signatures is to prove the miRNAs are exosome-encapsulated and not derived from apoptotic bodies or larger microvesicles. As previously described, microvesicles and apoptotic bodies range between 50-1000 nm and 50-5000 nm respectively whereas exosomes are sized between 40-100 nm. Exosomes also possess characteristic tetraspanin protein markers in their membranes such as CD 9, CD 63 or CD 81. The results shown in this study demonstrate vesicles between 40-100nm in size visualised with Transmission Electron Microscopy (TEM). Western Blot targeting the exosome associated protein CD 63 confirms the presence of CD 63 in samples. These results taken together are satisfactory evidence that the fraction isolated from conditioned media of HCT116 and HT29 colorectal cancer cells using micro-filtration and ultra-centrifugation consists of exosomes secreted by the cell lines.

The profiling of miRNAs secreted within exosomes by both HCT116 and HT29 colorectal cancer cells has identified a large number of miRNAs to be detectable above a threshold level indicating their presence. Overall, when non-human spike-in miRNAs were excluded, array data revealed 409 miRNAs detected above threshold in HT29 derived exosomes. 393 miRNAs were detected in HCT116 exosomes, with 338 miRNAs common to exosomes from both HCT116 and HT29 cells. Analysis of miRNA expression levels in both colorectal cancer cell secreted exosomes interestingly show some areas with distinct differences. Exosomes have been shown to confer phenotypic traits representing their cells of origin to recipient cells. Potentially, the differences between the miRNA profiles of exosomes secreted by these two distinct colorectal cancer cell lines may represent differences in growth pattern and phenotype [182]. Some miRNAs which have well described roles in cancer pathogenesis, and others which have only recently been discovered were included among those identified in exosomes. Among the most highly expressed miRNAs in both colorectal cancer cell line exosomes are several which have previously described roles in cancer (miR-642-5p, miR-10b-3p, miR-943, miR-1248, miR-1249, let7b-5p, miR-378c, miR-30d-3p, miR-718, miR-361-5p, miR-548k, miR-145-5p) [151 - 163]. In addition, included in the most highly expressed are some more recently discovered miRNAs with little or no available data regarding their association with disease states, associated gene targets, or functional roles (miR-3679-3p, miR-4279, miR-4723-3p, miR-4274, miR-1207-3p, miR-4312). The wealth of information provided in the miRNA profiling of these exosomes provides the basis to investigate both well-known and previously unexplored miRNAs and their role in colorectal cancer initiation and progression. Furthermore, identifying miRNA signatures secreted by colorectal cancer cells within exosomes can expedite the search for circulating biomarkers which are tumour derived and specific.

miRNAs have been extensively investigated as circulating biomarkers and have shown significant promise. Issues such as pre-existing patient factors including disease states, smoking and ethnicity combined with differing views on the optimum medium from which miRNA should be extracted (whole blood/serum/plasma), and problems with haemolysis of red cells secondary to collection and storage have led to heterogeneous data. Identifying exosomal miRNA signatures from colorectal cancer cells may

provide a tumour specific biomarker or panel which will negate some of these concerns. Interestingly, some of the miRNAs which were found to be highly expressed in both colorectal cancer cell secreted exosomes have been described as haemolysis independent miRNAs capable of identifying colorectal cancer in patients. miR-378, identified as highly expressed in both colorectal cancer cell line exosomes in this study has previously been found to be a haemolysis-independent biomarker for disease presence in colorectal cancer [115]. Levels of miR-378 are decreased following removal of the primary tumour suggesting a tumour derived source which is backed up by the findings of the exosomal miRNA content in this current study. The miRNA profiling of exosomes from colorectal cancer cells serves as an ideal platform to further investigate miRNAs including miR-378 as tumour-derived biomarkers. Although array data only represents one snapshot of the miRNA content of exosomes and this may be subject to alterations based on cell growth patterns and other factors, validation of several miRNAs with different threshold levels was performed in this study indicating reliable and reproducible data. MiR-10b, miR-143 and miR-149-5p were validated in 3 separate exosome isolates from both colorectal cancer cell lines and found to be consistently present. Importantly, miR-143 and miR-149-5p were not among the most highly expressed miRNAs but were still consistently detectable with RQ-PCR confirming reproducibility of data. Thus far, exosomal miRNAs have shown significant promise as biomarkers. Exosomal miR-34a has been found to be a biomarkers predictive of response to chemotherapeutics in prostate cancer [142]. An exosomal biomarker panel has been found to differentiate between oesophageal adenocarcinoma, Barrett's oesophagus and healthy controls [183]. Investigating some of the highly expressed miRNAs identified in this study may allow similar applications in colorectal cancer for development of sensitive and specific biomarkers.

Not only do exosome encapsulated miRNAs represent ideal biomarkers but also have vast potential in development of gene based therapies. As previously discussed, work in our laboratory has identified miR-379 as a tumour suppressor in breast cancer [128]. Furthermore, miR-379 originates from the 14q32.31 cluster which has proven to be a locus of tumour suppressor miRNAs in genome wide analysis of human cancer tissues [167]. To this end, it represents an excellent miRNA for investigation of functional effects in colorectal cancer. To examine the functional effects of miR-379, cells were

engineered to over-express miR-379 or a non-targeting control miRNA. A significant reduction in colorectal cancer cell proliferation in response to over-expression of miR-379 was observed in this study. Cells over expressing miR-379 were found to proliferate between 12-15% slower than compared to controls after 72 hours incubation. Predicted target genes, such as 3-phosphoinositide-dependent protein kinase 1 (PDK1) have been found to be up-regulated in colorectal cancer, in keeping with its evident anti-proliferative effect in this study [171]. A marked reduction in migration of colorectal cancer cells in response to over-expression of miR-379 was observed ranging as high as an 86% decrease in migration compared to the control cell line accounting for chemokinesis or random movement of cells. This very significant effect is interesting as miR-379 has a documented effect on Cyclin B1 as previously discussed [130]. While over-expression of Cyclin B1 has been found in colorectal cancer tissues, elevated expression of Cyclin B1 is negatively associated with lymph node metastases, distant organ metastases and advanced disease stage [130]. Once the functional effects of miR-379 over-expression were confirmed in colorectal cancer cells, expression levels of miR-379 in tissues were examined. miR-379 expression levels in tumour tissues compared to patient matched normal samples were not significantly different in this study. There was however a trend towards lower expression of miR-379 in tumours which may have become more significant with increasing patient samples. Having established the anti-proliferative and anti-migratory effect of miR-379 in vitro, the potential to force over-expression of miR-379 in exosomes and transfer them to recipient cells was examined. miR-379 was found to be present in colorectal cancer cell secreted exosomes when RQ-PCR was performed in triplicate. It was not detectable in wild type or non-targeting control cells indicating successful engineering of exosomes to over-express miR-379. These exosomes were then imaged being transferred to recipient cells using fluorescence microscopy suggesting they are taken up by recipient cells. Following confirmatory imaging of transfer the functional effects of miR-379 enriched exosomes on recipient cells were investigated. Incubation of HCT-116 WT cells in media containing exosomes enriched in miR-379 produced an observable decrease in proliferation however it was not significant. As the effect of miR-379 over-expression on migration was very significant, ranging from 32-86%, further investigation of the effect of miR-379 enriched exosomes on cell migration may yield a more pronounced effect. In addition, increasing the number of exosomes used for transfer may produce a more

pronounced effect as previous studies have suggested exosomes produce effects in a dose dependant manner [181].

Results confirming the potential to force over-expression of miRNAs within exosomes and subsequently transfer them to recipient cells further promote the exciting potential of exosome encapsulated miRNAs as novel gene therapies. There are also a number of caveats however when considering the effect of exosomal miRNAs on recipient cells. miRNAs regulate a large number of target genes and where desirable effects are produced through regulation of one gene, undesirable effects could be produced in other cells through regulation of other genes. This is of particular relevance to delivery of systemic miRNAs. Effects exerted on recipient cells by exosomes cannot be entirely attributed to miRNAs and are likely multi-factorial and extremely complex. Until the full range of effects exerted by exosomal contents on recipient cells is understood there will be doubt regarding their safety in systemic delivery. This work does however add to the growing body of evidence that it is possible to engineer exosomes to over-express miRNAs and these can be transferred to recipient cells to exert effects which has exciting potential for development of novel therapies.

In conclusion, this study is the first of its kind to provide a full miRNA profile for exosomes secreted by HT29 and HCT116 colorectal cancer cells. This data provides an invaluable platform for further investigating well known and previously undescribed miRNAs and their role in intercellular communication. In addition, further investigation of miRNAs secreted by colorectal cancer cells identified in this study may provide robust, tumour-specific biomarkers. Functional effects of miR-379 have been established and the potential to enrich exosomes with miR-379 and transfer them to recipient cells has been shown further supporting the development of exosome encapsulated miRNA based therapies. Further experiments looking at dose dependant functional effects of exosomal miR-379 on recipient colorectal cancer cells may yield interesting results. Establishing the effect of exosomal miRNAs on target genes in recipient colorectal cancer cells could also provide valuable information on cancer pathogenesis and expedite the development of new therapies.

Chapter 7

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Chapter 8

Appendices

Appendix 1

Merlin Park University Hospital
Ospidéal na h-Ollscoile, Páirc Mheirlinne
GALWAY UNIVERSITY HOSPITALS

Clinical Research Ethics Committee
Main Administration Building
Merlin Park Hospital
Galway.

16ᵗʰ May, 2014.

Professor Michael J. Kerin
Head of Discipline of Surgery
School of Medicine
Clinical Science Institute
National University of Ireland
Galway.

Amendment to Protocol Number 45/05 and C.A. 151: - Approved 16ᵗʰ May, 2014
"The Provision of a breast cancer biobank research resource for use in molecular and cellular studies and clinical trials"

Dear Professor Kerin,

I have considered the above amendment, and I wish to confirm Chairman's approval to proceed. The following documentation was reviewed:

- Amendment 1: - The introduction of an edited Patient Information Leaflet and Consent Form
- Amendment 2: - The introduction of a Questionnaire

Yours sincerely,

PP

Dr. Shaun T. O'Keeffe
Chairman Clinical Research Ethics Committee.

c.c. Ms. Eimear Ramphul, Research Assistant, Discipline of Surgery, School of Medicine, Clinical Science Institute, NUI, Galway.

Merlin Park University Hospital, OSPIDÉAL NA H-OLLSCOILE, PÁIRC MHEIRLINNE, Galway, Ireland. Tel: 00 353 (0)91 757631

Appendix 2

GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT
Patient Information

Introduction
We would like to invite you to participate in a clinical research initiative at Galway University Hospitals to establish a BioBank. The purpose of the BioBank is to set up a resource that can support a diverse range of research programmes intended to improve the prevention, diagnosis and treatment of cancer. You are under no obligation to take part and if, having read the information below, you would prefer not to participate, we will accept your decision without question.

Although major advances have been made in the management of cancer, many aspects of the disease are not fully understood. It is hoped that our understanding of the disease will be improved through research. Galway University Hospitals are actively involved in research that aims to identify markers that will predict how a cancer develops, progresses and responds to a variety of treatments. This type of work requires the use of tissue and blood samples. It is hoped that it will eventually lead to improvements in the diagnosis, treatment and outcome for those who have cancer. Although this study may have no direct benefit to you, it is hoped that the results may benefit patients like you in the future.

Your Involvement
If you volunteer to participate in our BioBank, there will be no additional risks to you outside those of your standard investigation and treatment. Your identity will remain confidential. Your name will not be published or disclosed to anyone outside the study group. All research is covered by standard institutional indemnity insurance and is approved by a Research Ethics Committee that ensures the ethical nature of the research. Nothing in this document restricts or curtails your rights. You may withdraw your consent at any time. If you decide not to participate, or if you withdraw your consent, your standard of treatment will not be affected in any way.

Procedure
We invite all patients who are undergoing treatment and/or investigation to participate. All samples for research will be taken at the time you are attending the hospital for routine diagnostic tests.

(i) Tissue Samples
By participating, you give us consent to retain small pieces of your tissue obtained at the time of surgery. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland. This will not affect your diagnosis in any way.

(ii) Blood Samples
By participating, you give us consent to take an extra blood sample (equivalent of 4 teaspoonfuls) at the same time that your blood is being taken for routine tests. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland.

(iii) Clinical Information
By participating, you give us consent to store information relating to your diagnosis and treatment on a database. This information is only accessed by personnel directly involved in research within the Surgical Research Unit.

Further Information
If you would like further information about our BioBank, your participation and your rights, please contact the Surgical Research Unit (Tel: 091 524390).

If you would like further information about research projects that may be conducted, please contact your Consultant.

Thank you in anticipation of your assistance. Please read and sign the Consent section.

I have read the attached information sheet on the above project, dated _____

Please Initial Box

GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

PARTICIPANT DECLARATION

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor. I understand that I may withdraw from the study at any time.

(Name of sponsor): ....

PARTICIPANT'S NAME: ....

CONTACT DETAILS: ....

PARTICIPANT'S SIGNATURE: ....

DATE: ....

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the participant is a minor (under 18 years old) the signature of parent or guardian must be obtained:

NAME OF CONSENTER, PARENT, OR
GUARDIAN: ....

SIGNATURE: ....

RELATION TO PARTICIPANT: ....

DECLARATION OF INVESTIGATOR'S RESPONSIBILITY
I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

NAME OF RESEARCH NURSE OR ....
INVESTIGATOR:

SIGNATURE: ....

DATE: ....

CONSULTANT: ....

Keep the original of this form in the investigators file, give one copy to the participant, and send one copy to the sponsor (if there is a sponsor).

Appendix 3

ProbeID	Annotation	HT29	HCT116
168870	hsa-miR-1246	#####	8.671
168878	hsa-miR-5100	#####	8.263
169028	hsa-miR-4708-3p	#####	8.206
169050	hsa-miR-4787-5p	#####	7.898
169024	hsa-miR-3960	#####	7.781
146006	hsa-miR-670-5p	#####	7.663
169110	hsa-miR-4497	#####	7.643
42899	hsa-miR-377-5p	#####	7.536
168619	hsa-miR-1260b	#####	7.395
146089	hsv1-miR-H8-5p	#####	7.214
147606	hsa-miR-4259	#####	7.066
19013	SNORD14B	#####	6.973
147667	hsa-miR-3182	#####	6.924
147654	hsa-miR-3138	#####	6.806
148504	hsa-miR-874-5p	#####	6.779
168978	hsa-miR-371b-5p	#####	6.646
42615	hsa-miR-135b-3p	#####	6.621
17492	sv40-miR-S1-5p	#####	6.592
46501	hsa-miR-1305	#####	6.549
147562	hsa-miR-4253	#####	6.517
148474	hsa-miR-3622a-5p	#####	6.510
46404	hsa-miR-1244	#####	6.485
145946	hsa-miR-449a	#####	6.455
169244	hsa-miR-5572	#####	6.445
147671	hsa-miR-4323	#####	6.439
169285	hsa-miR-4467	#####	6.438
148156	hsa-miR-3686	#####	6.412
146085	hsa-miR-3170	#####	6.406
168648	hsa-miR-4687-5p	#####	6.396
147906	hsa-miR-4322	#####	6.367
148628	hsa-miR-3199	#####	6.348
46335	hsa-miR-548m	#####	6.342
147881	hsa-miR-3115	#####	6.336
148351	hsa-miR-3945	#####	6.325
42769	hsa-let-7b-3p	#####	6.313
42824	ebv-miR-BART9-5p	#####	6.304
147683	hsa-miR-3188	#####	6.280
13147	hsa-miR-96-5p	#####	6.275
147823	hsa-miR-3146	#####	6.264
147915	hsa-miR-3174	#####	6.261
168689	hsa-miR-361-3p	#####	6.216

146137	hsa-miR-133a-3p	#####	6.203
148678	hsa-miR-301a-5p	#####	6.183
33902	hsa-miR-128-3p	#####	6.175
14272	hsa-miR-542-3p	#####	6.174
17298	hsa-miR-548b-3p	#####	6.150
169135	hsa-miR-3173-5p	#####	6.139
146010	hsa-miR-2116-5p	#####	6.139
146107	hsa-miR-762	#####	6.124
13140	hsa-miR-138-5p	#####	6.111
147712	hsv1-miR-H1-5p	#####	6.110
42956	hsa-miR-545-5p	#####	6.108
46751	hsa-miR-2278	#####	6.105
46766	hsa-miR-1208	#####	6.092
169082	hsa-miR-1275	#####	6.085
148669	hsa-miR-185-3p	#####	6.084
168672	hsa-miR-1587	#####	6.082
169120	hsa-miR-4787-3p	#####	6.082
32891	hsa-miR-769-5p	#####	6.071
42838	hsa-miRPlus-C1076	#####	6.069
17875	hsa-miR-500a-5p	#####	6.066
169034	hsa-miR-642b-5p	9.554	9.196
42969	hsa-miR-10b-3p	9.291	9.016
148038	hsa-miR-3679-3p	9.259	9.447
147767	hsa-miR-4279	9.232	9.273
42696	hsa-miR-943	9.204	8.908
42557	hsa-miR-624-5p	9.161	8.937
46427	hsa-miR-1248	9.105	8.749
42641	hsa-miR-145-5p	9.002	8.523
46210	hsa-miR-1249	9.001	8.794
169323	hsa-miR-4723-3p	8.998	8.873
147165	hsa-let-7b-5p	8.983	8.690
42522	ebv-miR-BART19-3p	8.930	8.668
147755	hsa-miR-378c	8.899	8.771
11078	hsa-miR-365a-3p/hsa-miR-365b-3p	8.855	8.557
17853	hsa-miR-30d-3p	8.808	8.555
147751	hsa-miR-4274	8.759	8.648
42624	kshv-miR-K12-10b	8.739	8.800
146064	hsa-miR-718	8.736	8.723
17858	hsa-miRPlus-A1073	8.725	8.748
46705	hsa-miR-548k	8.709	8.484
14301	hsa-miR-361-5p	8.707	8.540
46345	hsa-miR-1207-3p	8.706	8.631
42656	kshv-miR-K12-10a-3p	8.624	8.641
147664	hsa-miR-4311	8.595	8.118
147907	hsa-miR-4312	8.544	8.456

46368	hsa-miR-1282	8.542	8.107
146116	hsa-miR-2116-3p	8.530	8.414
148622	hsa-miR-877-3p	8.512	8.410
42892	hsa-miR-450b-3p	8.506	8.385
10954	hsa-miR-147a	8.502	8.298
42810	hsa-miR-149-5p	8.435	7.964
46634	hsa-miR-1281	8.433	8.360
27740	hsa-miR-574-5p	8.417	8.246
146103	hsa-miR-1913	8.403	8.349
42504	hsa-miR-593-3p	8.373	8.140
17306	ebv-miR-BART12	8.324	8.147
147701	hsa-miR-491-3p	8.283	7.668
42729	hsa-miR-34c-3p	8.281	8.023
32946	hsa-miR-486-5p	8.274	7.906
10947	hsa-miR-142-3p	8.273	7.900
46850	hsa-miR-1237-3p	8.228	8.444
46416	hsa-miR-1293	8.213	8.165
11140	hsa-miR-508-3p	8.213	7.863
42490	hsa-miR-505-5p	8.211	7.960
30033	hsa-miR-877-5p	8.185	7.872
46789	hsa-miR-513b-5p	8.179	8.021
147897	hsa-miR-3136-5p	8.171	7.673
148682	hsa-miR-483-3p	8.166	8.360
11182	hsa-miR-98-5p	8.166	7.996
169182	hsa-miR-4728-3p	8.156	8.618
46624	hsa-miR-1236-3p	8.135	7.930
46738	hsa-miR-1182	8.135	7.987
42898	hsa-miR-124-5p	8.132	7.215
146090	hsv1-miR-H7-3p	8.131	8.001
147981	hsa-miR-4325	8.117	7.814
145973	hsa-miR-664a-3p	8.115	8.315
42581	hsa-miR-513a-5p	8.102	8.151
27536	hsa-miR-190a-5p	8.093	7.439
145820	hsa-let-7c-5p	8.088	7.944
42897	ebv-miR-BART15	8.077	7.967
145705	hsa-miR-431-5p	8.068	7.689
46806	hsa-miR-1227-3p	8.064	7.801
17302	hsa-miR-578	8.057	7.812
14962	hsa-miR-581	8.045	7.257
169282	hsa-miR-4290	8.039	8.291
147935	hsa-miR-3125	8.024	7.819
11141	hsa-miR-509-3p	8.022	7.981
146140	hsa-miR-1976	8.020	7.852
146058	hsv1-miR-H3-3p	8.018	7.801
46917	hsa-miR-205-5p	8.015	7.614
46443	hsa-miR-193a-5p	8.012	8.041

46222	hsa-miR-1228-5p	8.012	7.948
146069	hsa-miR-1915-5p	8.011	7.609
11005	hsa-miR-204-5p	7.974	7.567
4040	hsa-miR-9-5p	7.965	7.853
46556	hsa-miR-623	7.961	7.934
10990	hsa-miR-196a-5p	7.946	8.033
14313	hsa-miR-499a-5p	7.928	6.993
27720	hsa-miR-15a-5p	7.927	7.367
17752	hsa-let-7f-5p	7.925	7.558
46221	hsa-miR-519d-3p	7.922	7.790
148065	hsa-miR-3689b-3p/hsa-miR-3689c	7.921	7.806
42672	hsa-miR-323b-5p	7.915	7.131
42839	hsa-miR-135a-5p	7.912	7.499
11245	hsa-miR-433-5p	7.908	7.389
146042	hsv1-miR-H8-3p	7.900	7.884
17810	hsa-miR-29b-1-5p	7.877	6.697
169211	hsa-miR-5704	7.873	8.029
145647	hsa-miR-584-5p	7.870	7.654
147919	kshv-miR-K12-12-3p	7.863	7.687
17349	hsa-miR-595	7.839	7.813
46266	hsa-miR-1825	7.836	7.563
11111	hsa-miR-432-5p	7.807	7.022
42523	hsa-miR-26b-3p	7.795	8.081
17898	hsa-miR-99b-3p	7.794	7.254
145666	SNORD110	7.787	7.590
46440	hsa-miR-1287-5p	7.786	7.535
11024	hsa-miR-223-3p	7.773	7.754
17336	hsa-miR-618	7.763	7.222
145690	hsa-miR-512-5p	7.741	7.036
17824	hsa-miRPlus-A1025	7.738	7.463
147821	hsa-miR-3169	7.734	7.980
46565	hsa-miR-1207-5p	7.732	7.899
11022	hsa-miR-221-3p	7.732	7.641
17854	hsa-miR-106b-3p	7.732	6.508
147334	hsa-miR-3613-5p	7.727	7.036
46259	hsa-miR-885-5p	7.723	7.701
9938	hsa-let-7i-5p	7.716	7.042
19603	SNORD13	7.697	7.619
146052	hsa-miR-1471	7.693	7.202
46462	hsa-miR-1224-5p	7.689	7.584
42848	hsa-miR-1180-3p	7.675	7.447
46689	hsa-miR-1229-3p	7.668	7.495
145956	hsa-miRPlus-A1083	7.667	7.273
147576	hsv1-miR-H1-3p	7.666	7.888
168950	hsa-miR-4646-3p	7.663	8.043
146077	hsv2-miR-H3	7.662	7.985

17961	hsa-miR-629-5p		7.658	7.370
147203	hsa-miR-302a-3p		7.657	7.278
46788	hsa-miR-1299		7.654	7.204
17619	hcmv-miR-US25-2-5p		7.645	7.110
148187	hsa-miR-410-5p		7.625	7.336
145753	hsa-miR-484		7.614	7.724
27838	hsa-miR-302d-3p		7.608	7.728
17623	ebv-miR-BART14-3p		7.607	7.017
10977	hsa-miR-183-5p		7.604	6.896
46866	hsa-miR-1321		7.604	6.936
46752	hsa-miR-1270		7.590	7.325
148228	hsa-miR-3656		7.583	7.680
17283	ebv-miR-BART11-5p		7.582	7.317
17848	hsa-miRPlus-A1087		7.573	7.565
146178	hsa-miR-502-3p		7.571	6.993
42485	ebv-miR-BART10-5p		7.554	7.382
42592	hsa-miR-338-3p		7.544	6.903
19585	hsa-miR-148b-3p		7.534	7.678
11139	hsa-miR-507		7.527	6.846
11007	hsa-miR-206		7.520	6.684
42749	hsa-miR-659-3p		7.517	7.013
145934	hsa-miRPlus-B1114		7.509	7.782
28884	hsa-miR-876-3p		7.507	6.836
145843	hsa-miR-330-5p		7.494	6.830
168869	hsa-miR-5010-3p		7.485	7.966
46822	hsa-miR-1178-3p		7.482	7.200
29490	hsa-miR-7-5p		7.482	7.411
148413	hsa-miR-3614-3p		7.478	7.638
145717	hsa-miR-516a-3p/hsa-miR-516b-3p		7.464	7.774
147591	hsa-miR-4283		7.442	7.460
46750	hsa-miR-1254		7.430	7.081
17614	sv40-miR-S1-3p		7.429	7.168
148282	hsa-miR-3714		7.428	7.325
148361	hsa-miR-3911		7.420	7.202
10964	hsa-miR-155-5p		7.419	7.539
46541	hsa-miR-1225-5p		7.411	6.497
147186	hsa-miR-200b-3p		7.410	6.813
46215	hsa-miR-1301-3p		7.407	6.962
17490	hsa-miR-571		7.402	7.285
147900	hsv2-miR-H6-3p		7.396	6.867
148663	hsa-miR-557		7.396	6.725
147631	hsa-miR-4258		7.369	7.456
146159	hsv1-miR-H4-3p		7.368	6.577
42610	hcmv-miR-UL36-3p		7.366	7.052
14328	hsa-miR-124-3p		7.364	6.911

42951	ebv-miR-BHRF1-2-3p	7.364	6.621
46479	hsa-miR-1304-5p	7.363	6.394
46744	hsa-miR-526b-5p	7.362	6.911
11058	hsa-miR-325	7.357	7.218
11104	hsa-miR-422a	7.352	6.807
11077	hsa-miR-363-3p	7.345	6.781
148273	hsa-miR-3150b-3p	7.340	7.184
145962	hsa-miR-639	7.332	6.933
46467	hsa-miR-143-5p	7.331	6.583
11011	hsa-miR-211-5p	7.321	7.655
148243	hsa-miR-3689a-3p	7.310	6.966
46675	hsa-miR-1181	7.307	7.133
42782	hcmv-miR-UL148D	7.299	7.536
42507	hsa-miR-202-5p	7.284	7.163
147864	hsv2-miR-H24	7.282	7.589
46860	hsa-miR-1205	7.261	7.300
46875	hsa-miR-2276-3p	7.250	#NUM!
148645	hsa-miR-129-5p	7.242	6.581
146115	hsa-miR-940	7.239	6.684
19606	SNORD12	7.226	6.923
147806	hsa-miR-3149	7.221	7.112
13485	hsa-miR-10a-5p	7.212	6.403
46695	hsa-miR-1228-3p	7.207	7.463
11074	hsa-miR-34c-5p	7.183	6.760
42502	hsa-miR-204-3p	7.180	8.354
42442	hsa-miR-498	7.178	7.138
46531	hsa-miR-1231	7.172	6.527
46810	hsa-miR-1827	7.170	7.169
11038	hsa-miR-299-5p	7.165	#NUM!
14271	hsa-miR-539-5p	7.163	7.157
42609	hsa-miR-135a-3p	7.151	6.832
146117	hsv1-miR-H6-3p	7.146	7.180
148482	hsa-miR-874-5p	7.132	7.119
19591	hsa-miR-199b-5p	7.130	6.517
46661	hsa-miR-1294	7.113	7.236
147803	hsv2-miR-H10	7.110	7.263
10306	hsa-miR-146b-5p	7.109	6.456
148577	hsa-miR-3943	7.096	7.322
29379	hsa-miR-452-5p	7.085	6.679
17506	hsa-miR-24-3p	7.085	6.680
145701	hsa-miR-668-3p	7.084	6.509
146180	hsa-miR-1909-3p	7.067	7.054
21498	hsa-miR-654-3p	7.050	6.649
11165	hsa-miR-520a-3p	7.046	6.916
42532	hsa-miR-22-5p	7.040	6.862
46791	hsa-miR-1204	7.038	6.811

148317	hsa-miR-3621	7.024	6.925
145889	hsa-miR-196b-5p	7.021	6.812
42674	hsa-miR-431-3p	6.994	6.911
17280	hsa-miR-15b-5p	6.993	6.771
29575	hsa-miR-32-3p	6.986	6.425
10943	hsa-miR-136-5p	6.984	6.717
17402	ebv-miR-BART2-5p	6.983	6.382
145708	hsa-miR-324-3p	6.980	6.550
146131	hsa-miR-2117	6.972	6.529
42773	ebv-miR-BART17-3p	6.969	7.474
42476	hsa-miR-374b-3p	6.969	6.283
145990	ebv-miR-BART21-3p	6.960	7.615
42682	hsa-miR-25-3p	6.958	6.095
28759	hsa-miR-758-3p	6.952	6.318
145675	hsa-miR-501-5p	6.948	6.334
46690	hsa-miR-1238-3p	6.941	6.639
17841	hsa-miRPlus-C1100	6.929	6.196
17444	hsa-miR-632	6.927	6.278
46872	hsa-miR-1262	6.921	6.432
148358	hsa-miR-3657	6.918	6.365
148052	hsa-miR-374c-3p	6.912	6.587
17946	hsa-miR-192-3p	6.911	6.080
46918	hsa-miR-375	6.895	6.299
148621	hsa-miR-892a	6.891	7.185
146185	bkv-miR-B1-5p	6.880	6.309
148641	hsa-miR-518b	6.879	6.725
147975	hsa-miR-487a-5p	6.879	6.860
27533	hsa-miR-320a	6.867	6.502
17563	hsa-miR-644a	6.867	6.851
42493	hsa-miR-892b	6.866	7.333
46944	hsa-miR-1297	6.857	6.736
46398	hsa-miR-513c-5p	6.856	#NUM!
146066	hsa-miR-3116	6.855	6.448
145977	hsa-miR-1247-5p	6.851	6.469
10946	hsa-miR-141-3p	6.849	6.639
147887	hsa-miR-3147	6.848	6.253
46406	hsa-miR-500a-3p	6.842	6.470
147930	hsa-miR-3144-3p	6.837	#NUM!
14300	hsa-miR-29c-5p	6.832	#NUM!
148624	hsa-miR-942-5p	6.829	6.208
27672	hsa-miR-615-3p	6.825	6.783
33596	hsa-miR-126-5p	6.824	6.204
17272	hsa-miR-551a	6.823	7.427
45775	hsa-miR-1279	6.821	6.275
10919	hsa-miR-103a-3p	6.815	6.277
46832	hsa-miR-1202	6.813	6.924

145838	hsa-miR-125b-1-3p	6.811	6.495
169171	hsa-miR-4436b-5p	6.788	6.692
148049	hsa-miR-3924	6.781	6.706
17353	hsa-miR-609	6.780	6.185
46558	hsa-miR-1268a/hsa-miR-1268b	6.774	6.956
46381	hsa-miR-1298-5p	6.763	6.980
17508	hcmv-miR-UL112-3p	6.761	6.189
42466	ebv-miR-BART18-3p	6.753	6.209
147682	hsv1-miR-H6-5p	6.752	6.540
46584	hsa-miR-1468-5p	6.751	6.146
148363	hsa-miR-3652	6.742	6.532
6880	hsa-miR-297	6.741	7.403
145678	hsa-miR-150-5p	6.739	6.680
146005	hsa-miR-3129-5p	6.734	6.916
42567	hsa-miR-590-3p	6.731	6.272
10936	hsa-miR-130b-3p	6.728	6.563
42492	hsa-miRPlus-A1031	6.727	7.685
42870	hsa-miR-616-3p	6.727	6.640
146160	hsa-miR-133b	6.726	6.825
46800	hsa-miR-1224-3p	6.722	#NUM!
46205	SNORD48	6.720	7.002
10997	hsa-miR-19a-3p	6.713	6.598
11168	hsa-miR-520d-3p	6.712	6.484
17851	hsa-miR-200c-5p	6.706	6.475
145821	hsa-miR-518c-5p	6.702	6.786
46803	hsa-miR-503-5p	6.694	6.476
148371	hsa-miR-3620-3p	6.693	7.297
148154	hsa-miR-3922-3p	6.690	6.275
42965	hsa-miR-424-5p	6.688	7.284
42570	hsa-miR-194-3p	6.681	6.395
17835	hsa-miR-450a-5p	6.676	6.941
11085	hsa-miR-373-3p	6.676	6.394
145640	hsa-miR-328-3p	6.675	6.132
169188	hsa-miR-4443	6.670	6.884
145999	hsa-miR-517a-3p/hsa-miR-517b-3p	6.667	6.609
4610	hsa-miR-126-3p	6.662	6.272
19607	SNORD15A	6.657	7.167
145846	hsa-let-7e-5p	6.654	6.413
169305	hsa-miR-4455	6.651	6.121
17630	hsa-miR-588	6.648	#NUM!
147589	hsa-miR-1193	6.646	6.474
17332	ebv-miR-BART9-3p	6.635	#NUM!
27565	hsa-miR-423-5p	6.624	6.529
46929	hsa-miR-548n	6.621	#NUM!
147694	hsa-miR-4281	6.617	7.072

146169	mcv-miR-M1-3p	6.606	6.649
17488	kshv-miR-K12-6-3p	6.600	6.128
17932	hsa-miR-381-5p	6.593	6.847
169325	hsa-miR-4446-5p	6.591	6.530
147698	hsv2-miR-H22	6.582	7.505
28966	hsa-miR-574-3p	6.573	6.418
19596	hsa-miR-30d-5p	6.566	6.421
148331	hsa-miR-3655	6.565	6.743
42859	hsa-miR-675-3p	6.565	6.468
42591	hsa-miR-634	6.563	6.815
148206	hsa-miR-3664-5p	6.555	6.255
146061	hsa-miR-1914-3p	6.554	6.164
148393	hsa-miR-676-3p	6.551	6.258
18739	hsa-miR-186-5p	6.551	6.403
147891	hsa-miR-3175	6.546	6.282
169223	hsa-miR-4680-3p	6.546	#NUM!
10916	hsa-miR-1	6.545	6.216
147276	hsa-miR-3616-3p	6.541	6.248
32731	hsa-miR-190b	6.540	6.557
168943	hsa-miR-4769-3p	6.536	7.836
11044	hsa-miR-302c-3p	6.531	6.204
145637	hsa-miR-187-3p	6.524	6.209
148380	hsa-miR-3913-5p	6.522	6.258
42453	hsa-miR-376b-5p/hsa-miR-376c-5p	6.517	#NUM!
46214	hsa-miR-1250-5p	6.517	#NUM!
42635	hsa-miR-541-3p	6.515	6.615
42613	ebv-miR-BART19-5p	6.514	7.303
168989	hsa-miR-4447	6.509	6.501
147772	hsa-miR-4303	6.508	6.158
147718	hsv1-miR-H3-5p	6.507	6.243
11154	hsa-miR-517c-3p	6.507	6.731
168809	hsa-miR-5698	6.505	#NUM!
147632	hsa-miR-4297	6.500	6.275
169083	hsa-miR-371b-3p	6.494	7.276
147790	hsv2-miR-H7-3p	6.491	6.114
147786	hsa-miR-3198	6.488	6.336
147796	hsa-miR-4327	6.487	6.603
42705	hsa-miR-191-3p	6.487	6.987
46274	hsa-miR-3187-3p	6.486	#NUM!
11260	hsa-miR-151a-5p	6.484	#NUM!
147600	hsa-miR-4292	6.477	6.450
145638	hsa-miR-29a-5p	6.474	#NUM!
148514	hsa-miR-365a-5p	6.471	#NUM!
42970	hsa-miR-744-3p	6.465	6.590
42666	hsa-miR-26a-2-3p	6.464	6.174

146179	hsa-miR-2113	6.462	6.391
30687	hsa-miR-93-5p	6.456	6.293
148620	hsa-miR-454-3p	6.456	6.320
46231	hsa-miR-519b-3p	6.450	#NUM!
11018	hsa-miR-218-5p	6.449	#NUM!
10925	hsa-miR-10b-5p	6.447	6.511
42509	hsa-miR-219a-5p	6.447	6.267
148247	hsa-miR-2355-3p	6.440	6.117
145643	hsa-miR-382-5p	6.438	#NUM!
42827	hsa-miR-652-3p	6.436	#NUM!
46326	hsa-miR-1233-3p	6.434	7.347
19605	SNORD6	6.426	#NUM!
17299	hcmv-miR-UL22A-3p	6.425	7.098
27229	hsa-miR-511-5p	6.422	#NUM!
42732	hsa-miR-532-3p	6.420	7.137
147889	hsv1-miR-H14-3p	6.417	#NUM!
46223	hsa-miR-1306-3p	6.416	7.200
169170	hsa-miR-4472	6.414	#NUM!
11134	hsa-miR-502-5p	6.403	#NUM!
147742	hsa-miR-4265	6.397	6.482
145789	hsa-miR-550a-3-5p/hsa-miR-550a-5p	6.391	6.750
27541	hcmv-miR-UL70-3p	6.388	6.823
46361	hsa-miR-1278	6.378	#NUM!
42811	hsa-miR-542-5p	6.377	#NUM!
27551	hsa-miR-612	6.374	6.411
42497	hcmv-miR-UL70-5p	6.374	#NUM!
17571	kshv-miR-K12-11-3p	6.363	#NUM!
17641	hsa-miR-573	6.362	#NUM!
10972	hsa-miR-181b-5p	6.362	#NUM!
46829	hsa-miR-664a-5p	6.352	6.556
168708	hsa-miR-296-5p	6.347	7.184
146152	hsa-miR-3179	6.343	6.154
42788	hsa-miR-924	6.340	#NUM!
145986	hsa-miR-2052	6.338	#NUM!
147760	hsa-miR-4316	6.326	#NUM!
28047	hsa-miR-890	6.322	6.392
17312	hsa-miR-592	6.319	6.549
147768	hsa-miR-4257	6.312	#NUM!
11083	hsa-miR-371a-3p	6.310	6.551
27546	hsa-miR-380-3p	6.309	#NUM!
17493	hsa-miR-622	6.308	6.432
148042	hsa-miR-3662	6.307	#NUM!
145859	hsa-miR-33a-5p	6.304	6.349
148687	hsa-miR-1908-5p	6.303	6.505
145722	hsa-miR-520e	6.300	#NUM!

29190	hsa-miR-708-5p	6.294	#NUM!
33043	hsa-miR-544a	6.292	#NUM!
42718	hsa-miR-130a-5p	6.291	6.504
148227	hsa-miR-1251-3p	6.286	6.065
169015	hsa-miR-4454	6.286	9.125
147885	kshv-miR-K12-5-5p	6.283	6.225
145634	hsa-miR-132-5p	6.275	6.194
42451	hsa-miR-139-3p	6.275	6.261
148627	hsa-miR-615-5p	6.270	6.263
148466	hsa-miR-3937	6.267	6.721
17613	hsa-miR-645	6.267	#NUM!
145751	hsa-miR-23b-5p	6.266	#NUM!
168636	hsa-miR-122-5p	6.259	#NUM!
148562	hsa-miR-128-1-5p	6.259	#NUM!
17327	hsa-miR-630	6.258	#NUM!
145741	hsa-miR-545-3p	6.254	6.124
10923	hsa-miR-107	6.249	6.481
31349	hsa-miR-524-3p	6.244	6.161
147809	hsa-miR-514b-3p	6.236	6.326
46614	hsa-miR-1323	6.236	#NUM!
42865	hsa-miR-181a-5p	6.235	6.474
46272	hsa-miR-1200	6.235	#NUM!
17463	hsa-miR-151a-3p	6.227	6.094
11138	hsa-miR-506-3p	6.223	6.773
147731	hsa-miR-3189-3p	6.221	6.482
145633	hsa-let-7d-3p	6.215	6.160
46880	hsa-miR-1183	6.214	6.347
169091	hsa-miR-5006-5p	6.209	#NUM!
31038	hiv1-miR-N367	6.200	7.295
17393	hsa-miR-603	6.199	#NUM!
147917	hsa-miR-3151-5p	6.195	#NUM!
46454	hsa-miR-520g-3p	6.194	#NUM!
147776	hsa-miR-4317	6.191	6.225
42681	hsa-miR-1307-3p	6.185	#NUM!
42549	hsa-miR-19a-5p	6.185	#NUM!
145827	hsa-miR-200a-5p	6.177	#NUM!
146105	hsa-miR-1539	6.177	#NUM!
146008	hsa-miR-26b-5p	6.175	6.266
46729	hsa-miR-302d-5p	6.170	#NUM!
147979	hsa-miR-3150a-3p	6.169	#NUM!
146020	hsa-miR-449c-3p	6.167	6.628
145845	hsa-miR-20a-5p	6.166	#NUM!
42874	hsa-miR-16-2-3p	6.166	6.193
27537	ebv-miR-BART13-3p	6.165	6.097
42902	hsa-miR-185-5p	6.163	#NUM!
42617	hsa-miR-541-5p	6.157	6.314

46408	hsa-miR-1322	6.152	6.830
145980	hsa-miR-939-5p	6.142	6.613
42869	hsa-miR-936	6.142	#NUM!
145696	hsa-miR-655-3p	6.140	#NUM!
146168	hsa-miR-1912	6.132	6.298
17289	hsa-miR-616-5p	6.132	6.596
145715	hsa-miR-648	6.127	#NUM!
42852	hsa-miR-760	6.127	#NUM!
145852	hsa-miR-210-3p	6.126	#NUM!
145693	hsa-miR-92a-3p	6.123	#NUM!
169243	hsa-miR-3682-5p	6.120	#NUM!
42460	hsa-miR-223-5p	6.118	6.310
147898	hsa-miR-2861	6.115	6.614
17460	hsa-miR-657	6.113	#NUM!
5250	hsa-miR-105-5p	6.110	6.093
147722	hsa-miR-4306	6.107	6.538
147942	hsa-miR-4268	6.102	6.326
147556	hsa-miR-4254	6.100	6.119
146114	hsa-miR-1538	6.095	#NUM!
147871	hsa-miR-3180-5p	6.093	6.443
17882	hsa-miR-20b-3p	6.092	6.116
145974	hsa-miR-200b-5p	6.090	#NUM!
46450	hsa-miR-548o-3p	6.087	6.579
145826	hsa-miR-18b-3p	6.085	6.122
19011	SNORD10	6.083	6.383
147376	hsa-miR-3679-5p	6.081	6.690
147894	hsa-miR-3137	6.079	6.253
15619	hsa-miR-649	6.078	#NUM!
42654	hsa-miR-483-5p	6.068	6.178
11053	hsa-miR-32-5p	6.067	#NUM!
28480	hsa-miR-504-5p	6.067	#NUM!
42584	hsa-miR-432-3p	6.066	#NUM!
147493	hsa-miR-3944-3p	6.062	#NUM!
42721	hsa-miR-503-3p	6.061	#NUM!

Literature Review

The use of circulating microRNAs as diagnostic biomarkers in colorectal cancer

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Abstract.
BACKGROUND: Abnormal levels of microRNAs (miRNAs) have been found in the blood or its components in a number of different cancers including colorectal cancer. In addition to being abundant in circulation, miRNAs show remarkable stability in both plasma and serum making miRNAs ideal markers for early detection in colorectal cancer. Several miRNAs have been identified as potential circulating biomarkers although none have been incorporated into clinical practice.
OBJECTIVE: To identify the most consistently dysregulated circulating miRNAs in colorectal cancer patients according to current literature and postulate reasons for heterogeneity in results.
METHODS: A literature review was performed using the electronic databases PubMed, Embase and the Cochrane Library.
RESULTS: The 6 circulating miRNAs most frequently found to be dysregulated in colorectal cancer are miR-18a-5p, miR-21-5p, miR-29a-5p, miR-92a-5p, miR-143-5p and miR-378-5p. There are, however, multiple studies with conflicting findings. Studies vary significantly in ethnicity of populations, use of endogenous controls, source of miRNAs (whole blood, serum and plasma) and methods of detection.
CONCLUSIONS: Circulating miRNAs are promising diagnostic biomarkers in colorectal cancer. Further studies identifying the source of tumour derived miRNAs in circulation, including identification of exosomal miRNA content, are required. Identifying pre-profiling factors affecting miRNA expression and determining stable endogenous controls will expedite the incorporation of miRNAs into clinical practice.

Keywords: Colorectal, microRNA, circulating

1. Introduction

Colorectal cancer accounts for over 9% of all cancer incidence worldwide and is the 4th most common cause of cancer related mortality [1]. Up to 25% of patients newly diagnosed with colorectal cancer will have distant metastases at first presentation, termed stage IV disease according to the American Joint Committee on Cancer (AJCC) staging criteria [2]. The 5 year survival for stage IV disease is 8.1% compared to over 90% for stage 1 disease highlighting a need for early detection methods [3].

Over 20 years ago Fearon and Vogelstein [4] developed the principle of the adenoma carcinoma sequence which describes a step-wise progression of colorectal cancer resulting from a series of genetic alterations. Each progressive step is related to specific genetic abnormalities in oncogenes and tumour suppressor genes. Genetic mutations accumulated during tumour development cause dysregulation of critical signal transduction pathways such as Wnt and TGF Beta which leads to uncontrolled cell growth and inhibits apoptosis [5]. Knowledge of the natural history of colorectal cancer and the ability to recognise histological progression allows screening and early interven-

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tion. Although population based screening combining faecal occult blood tests and colonoscopy has been implemented with significant success there is a drive towards use of biomarkers and novel screening tools as adjuncts to improve detection rates [6].

Until recently RNA was considered to play a relatively passive role in tumorigenesis as it acts as an intermediate to transfer genetic code from DNA to protein or as an intrinsic component of the protein translation machine [7]. However novel involvement of a number of different non-coding RNA classes has been demonstrated in cancer including antisense RNA [8], small nucleolar RNA [9] and microRNA (miRNA) [10]. miRNAs have been shown to regulate almost every cellular process [10,11] and are frequently dysregulated in cancer. Their discovery has begun a new era in cancer research as they show great potential as biomarkers of disease.

miRNAs are a class of small non-coding RNA molecule 19–25 nucleotides in length. They regulate gene expression at a post transcriptional level by binding to the 3' untranslated regions (UTR), coding sequences or 5' UTR of target messenger RNAs. This leads to the inhibition of translation or mRNA degradation [12–14]. Functional studies have shown miRNAs to participate in almost every cellular process including apoptosis, proliferation and differentiation [15]. In fact single miRNAs may regulate multiple target genes acting as a master control of gene expression [7]. Although miRNAs constitute only 1–3% of the human genome, it is suggested that they regulate up to 30% of human genes [16] The first study suggesting miRNA involvement in cancer was published just over a decade ago [17]. Calin et al. identified loss of miR-15a and miR-16-1 to occur frequently in association with a deletion at chromosome 13q14, a region frequently deleted in human B-Cell Chronic Lymphocytic Leukemia [18]. A large number of miRNAs were subsequently found to be dysregulated in a broad spectrum of cancers. Disease specific expression-patterns reflect mechanisms of cellular transformation and support the hypothesis that miRNA expression patterns encode the developmental history of disease [19]. The first reports of miRNAs associated with colorectal cancer identified miR-143-5p and miR-145-5p as novel dysregulated miRNAs and demonstrated they were down regulated in colorectal tumours compared to normal tissue [20]. A significant number of studies have since identified differences in miRNA expression in the tissues of colorectal cancer patients [21]. Only a small number of these, however, may be of use as prognos-

tic markers. Ma et al have previously published an extensive study of candidate miRNA biomarkers in the tissues of colorectal cancer patients [21]. Levels of tumour specific miRNAs have also been found in the blood or its components in a number of different cancers [22]. In addition to being highly abundant in circulation, miRNAs show remarkable stability in both plasma and serum [23]. These properties make miRNAs ideal tumour markers for early detection in colorectal cancer. Several miRNAs have been identified as potential circulating biomarkers in colorectal cancer although none have been incorporated into clinical practice. This review discusses the current literature on the use of circulating miRNAs as diagnostic biomarkers in colorectal cancer and identifies potential factors leading to differing reports of diagnostic significance associated with individual miRNAs.

2. Methods

A literature review was performed using the electronic databases PubMed, Embase and the Cochrane Library. The following term was used to search for relevant studies: (circulating OR blood OR plasma OR serum) AND (microRNA OR miRNA) AND (colon OR rectal OR colorectal) AND (cancer). There were no language restrictions. The latest search was performed on January 21st, 2014. Two authors (CC and MJ) independently examined the title and abstract of citations and the full texts of potentially eligible articles were obtained; disagreements were resolved by discussion. The reference lists of retrieved papers were further screened for additional eligible publications. Studies including data on one or more circulating miRNAs in the diagnosis of colorectal cancer were included. Studies without original data such as reviews, letters and position statements were excluded. All studies relating to miRNAs in colorectal cancer tissues, circulating miRNAs in cancers other than colorectal and circulating miRNAs used as prognostic markers in colorectal cancer were excluded.

3. Results

18 published studies containing data on circulating miRNAs as diagnostic biomarkers in colorectal cancer were identified. The 6 circulating miRNAs most frequently found to be dysregulated in association with colorectal cancer compared to healthy controls

are miR-18a-5p, miR-21-5p, miR-29a-5p, miR-92a-5p, miR-143-5p and miR-378-5p (Table 1). There are, however, several studies which found no diagnostic value associated with some of these miRNAs (Table 2). There are 35 circulating miRNAs identified by single studies found to be associated with colorectal cancer, some of which have been found not to be significant in other studies (Table 3).

3.1. miR-18a-5p

miR-18a-5p belongs to the miR-17-92 cluster located at the chromosome 13q31.1 region [24]. Over expression of this cluster has been associated with accelerated tumour growth and increased cell proliferation [25,26]. Elevated expression of miR-18a-5p has been identified in multiple cancers including bladder cancer and pancreatic cancer tissues [27,28]. In colorectal cancer miR-18a-5p has been found to directly suppress the ATM gene (Ataxia Telangiectasia Mutated) leading to accumulation of DNA damage [24]. miR-18a-5p also inhibits KRAS translation silencing the Mitogen-Activated Protein Kinase (MAPK) inactivation of downstream transcription factors which in turn drives progression of adenomas to carcinomas in colorectal cancer [29]. The role of miR-18a-5p in circulation is unknown.

miR-18a-5p was found to be up-regulated in the circulation of colorectal cancer patients compared to healthy controls in 3 studies [30–32] (Table 1). miR-18a-5p was also found to be up-regulated in the plasma of 60 patients with advanced adenomas compared to healthy controls with a value for the area under the receiver operating characteristic curve of 0.64 (95% confidence interval (CI), 0.52–0.75) [32]. All 3 studies used quantitative reverse-transcription PCR to validate the expression of miR-18a-5p. They differed in use of serum or plasma as the source of miRNAs and differed in reporting of endogenous controls used to identify dysregulation. One further study of a Chinese population investigated the utility of miR-18a-5p as a diagnostic biomarker and found it was not dysregulated in the serum of colorectal cancer patients compared to controls [33]. Quantitative reverse-transcription PCR was used to validate samples and sample groups did not differ significantly in terms of age or tumour stage (Table 2). It has been established that the expression of several miRNAs including the miR-17-92 cluster are altered in Hepatitis B Virus (HBV) carriers and smokers [33]. As China has the largest population of smokers and HBV carriers the authors of this study pos-

tulated that this may have influenced expression levels [33]. The effect of smoking on miRNA expression, however, is disputed [68].

3.2. miR-21-5p

miR-21-5p has been implicated in multiple oncogenic pathways. It is a key regulator of oncogenic Epithelial-Mesenchymal Transition (EMT) targeting proteins such as T-Cell Lymphoma Invasion and Metastases Inducing Protein-1 (TIAM1) which is known to regulate migration and invasion of cancer cells [34,35]. The effect of miR-21-5p on cell migration and invasion has been demonstrated across a number of different colorectal cancer cell lines [35]. It also has a downstream effect on Transforming Growth Factor Beta (TGF-β) and Tumour Necrosis Factor Alpha (TNF-α) signalling in turn affecting proliferation and apoptosis through targeting multiple tumour suppressor genes including Phosphate and Tensin Homolog (PTEN) and Programmed Cell Death Protein 4 (PDCD4) among others [36]. miR-21 has been found to be upregulated in many different tumour tissue types including breast, lung, pancreas, prostate and gastric tumours [37]. Its effect in circulation however, remains undetermined.

A significant association between elevated levels of circulating miR-21-5p and colorectal cancer has also been described in multiple studies [30,33,37–39] (Table 1). All studies used quantitative reverse-transcription PCR to validate the expression levels of miR-21-5p in patients of similar tumour stage. Studies differed in the use of serum or plasma as the source of miRNA and differed in the use of endogenous controls/control spike ins (miR-16, RNU6B, cel-miR-39). In addition to differentiating cancers from healthy controls, serum miR-21-5p expression levels demonstrated 81.1% sensitivity and 76.7% specificity for discerning patients with adenomatous polyps from healthy control subjects in one study including 43 advanced adenomas and 53 healthy controls [39].

Three studies have found no dysregulation of miR-21-5p in colorectal cancer (Table 2) [31,40,41]. All studies extracted miRNAs from different sources (whole blood, serum, plasma) and used different endogenous controls. Vega et al. found no dysregulation of miR-21 with colorectal cancer but focused specifically on patients with stage III disease [31]. This patient group only accounted for approximately 30% of the entire patient group in which miR-21-5p was found to be upregulated. Previous studies have reported however that miR-21 is more highly expressed in the serum

Table 1

Circulating microRNAs associated with colorectal cancer in 2 or more studies

Dysregulated circulating miRNAs	Studies	No of CRC patients	No of healthy controls	Median age	TNM stage I/II/III/IV	Endogenous control	Source of miRNA	Method of RNA extraction	Method of miRNA detection	P value
miR-18a-5p (upregulated)	Luo et al. 2013	80	144	68	22/25/26/5	miR-16-5p	Plasma	miRNEasy	PCR (Taqman)	< 0.001
	Vega et al. 2013	30	26	64	0/0/30/0	miR-16-5p/let7a-5p/miR-103-5p	Serum	TRIzol® LS	Agilent microarray	< 0.05
	Giraldez et al. 2013	53	82	63	8/13/16/5	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	< 0.001
miR-21-5p (Upregulated)	Wang et al. 2012	32	39	63	NA	miR-16-5p	Plasma	TRIzol® LS	PCR (SYBR green)	< 0.001
	Kanaan et al. 2012	20	20	57	4/7/4/5	RNU6B	Plasma	TRIzol® LS	PCR (Taqman)	NA
	Liu et al. 2013	200	80	51	18/96/64/22	miR-16-5p	Plasma	TRIzol® LS	PCR (Taqman)	< 0.001
	Luo et al. 2013	80	144	68	22/25/26/5	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	< 0.001
	Toiyama et al. 2013	186	53	67	45/57/43/41	cel-miR-39 (spike in)	Serum	miRNeasy	PCR (Taqman)	< 0.001
miR-29a-5p	Huang et al. 2010	100	59	61	27/25/38/10	miR-16-5p	Plasma	miRVana	PCR (SYBR green)	< 0.001
(Upregulated)	Vega et al. 2013	30	26	64	0/0/30/0	miR-16-5p/let7a-5p/miR-103-5p	Serum	TRIzol® LS	Agilent microarray	< 0.05
	Luo et al. 2013	80	144	68	22/25/26/5	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	< 0.001
	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	NA
	Giraldez et al. 2013	53	82	63	8/13/16/5	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	0.003
miR-92a-5p	Ng et al. 2009	90	50	71	6/34/23/27	RNU6B	Plasma	miRNeasy	PCR (SYBR green)	< 0.001
(Upregulated)	Huang Z et al. 2010	100	59	61	27/25/38/10	miR-16-5p	Plasma	miRVana	PCR (SYBR green)	< 0.001
	Liu et al. 2013	200	80	51	18/96/64/22	miR-16-5p	Serum	TRIzol® LS	PCR (Taqman)	< 0.001
	Luo et al. 2013	80	144	68	22/25/26/5	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	0.004
	Hofsli et al. 2013	70	20	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	NA
miR-143-5p (Downregualted)	Qian et al. 2013	41	10	NA	NA	cel-miR-39 (spike in)	Plasma	TRIzol® LS	PCR (SYBR)	< 0.001
	Luo X et al. 2013	80	144	68	22/25/26/5	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	< 0.001
	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	NA
miR-378-5p (Upregulated)	Hofsli et al. 2013	40	10	70	3/5/7/15	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01
	Zanutto et al. 2014	24	19	NA	NA	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	0.001

CRC – colorectal cancer, NA – not applicable.

Table 2

Studies finding no diagnostic value of commonly reported miRNAs

Circulating microRNA	Studies	No of CRC patients	No of healthy controls	Median age	TNM stage	Endogenous control	Source of microRNAs	Method of RNA extraction	Method of miRNA detection	P value
miR-18a-5p	Liu et al. 2013	200	80	51	18/96/64/22	miR-16-5p	Serum	TRIzol® LS	PCR(Taqman)	0.093
miR-21-5p	Pu et al. 2010	103	37	58	7/38/40/18	NA	Plasma	NA	PCR (SYBR green)	NA
	Nugent et al. 2012	63	45	71	5/22/19/16	miR-425-5p	Whole blood	TRIzol® LS	PCR (Taqman)	0.136
	Vega et al. 2013	30	26	64	0/0/30/0	miR-16-5p/let7a-5p/miR-103-5p	Serum	TRIzol® LS	Agilent microarray	0.087
miR-29a-5p	Zanutto et al. 2014	36	42	NA	NA	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	NA
	Faltejskova et al. 2012	100	30	67	16/24/34/26	miR-16-5p	Serum	miRNeasy	PCR (Taqman)	0.14
miR-92a-5p	Nugent et al. 2012	63	45	71	5/22/19/16	miR-425-5p	Whole blood	TRIzol® LS	PCR (Taqman)	0.113
	Wang et al. 2013	55	57	58	NA	miR-1228-5p	Plasma	miRVana	PCR (Taqman)	NA
	Vega et al. 2013	30	26	64	0/0/30/0	miR-16-5p/let7a-5p/miR-103-5p	Serum	TRIzol® LS	Agilent microarray	0.087
	Faltejskova et al. 2012	100	30	67	16/24/34/26	miR-16-5p	Serum	miRNeasy	PCR (Taqman)	0.6

TNM – Tumour, Nodes, Metastases staging criteria, NA – not applicable.

Table 3
Circulating microRNAs associated with colorectal cancer in 1 study

Dysregulated circulating microRNAs	Study	No of CRC patients	No of healthy controls	Median age	TNM stage (I/II/III/IV)	Endogenous control	Source of microRNAs	Method of RNA extraction	Method of miRNA detection	P value	Studies finding no significance
miR-7-5p (Down)	Wang et al. 2013	55	57	58	NA	miR-1228-5p	Plasma	miRVana	PCR (Taqman)	0.02	Nil
miR-15b-5p (Up)	Giraldez et al. 2013	53	82	63	8/13/16/5	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	0.002	Nil
miR-19a-5p (Up)	Giraldez et al. 2013	53	82	63	8/13/16/5	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	< 0.001	Wang et al. 2012
miR-19b-5p (Up)	Giraldez et al. 2013	53	82	63	8/13/16/5	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	< 0.001	Nil
miR-20a-5p (Up)	Luo et al. 2013	80	144	68	22/25/26/5	miR-16-5p	Plasma	miRNeasy	PCR(Taqman)	0.001	Vega et al. 2013
miR-23a-5p (Up)	Yong et al. 2013	112	50	64	25/32/31/24	RNU48	Whole blood	miRNeasy	PCR (Taqman)	NA	Nil
miR-34a-5p (Down)	Nugent et al. 2012	63	45	71	5/22/19/16	miR-425-5p	Whole blood	TRIzol® LS	PCR (Taqman)	0.004	Vega et al. 2013
miR-93-5p (Down)	Wang et al. 2013	55	57	58	NA	miR-1228-5p	Plasma	miRVana	PCR (Taqman)	< 0.001	Zanutto et al. 2014
miR-103-5p (Down)	Hofsli et al 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Nil
miR-106a-5p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	NA	Nil
miR-106b-5p (Up)	Luo et al. 2013	80	144	68	22/25/26/5	miR-16-5p	Plasma	miRNeasy	PCR(Taqman)	0.03	Nil
miR-107-5p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Nil
miR-133a-5p (Up)	Luo et al. 2013	80	144	68	22/25/26/5	miR-16-5p	Plasma	miRNeasy	PCR(Taqman)	< 0.001	Zanutto et al. 2014
miR-145-5p (Up)	Luo et al. 2013	80	144	68	22/25/26/5	miR-16-5p	Plasma	miRNeasy	PCR(Taqman)	< 0.001	Nil
miR-151-5p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Nil
miR-155-5p (Up)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Nil
miR-191-5p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	NA	Nil
miR-193a-3p (Up)	Yong et al. 2013	112	50	64	25/32/31/24	RNU48	Whole blood	miRNeasy	PCR (Taqman)	NA	Nil
miR-199a-3p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Nil
miR-210-5p (Up)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Wang et al. 2012
miR-221-5p (Up)	Pu et al. 2010	103	37	58	7/38/40/18	NA	Plasma	NA	PCR (SYBR green)	0.002	Huang et al. 2010
miR-221-5p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Huang et al. 2010
miR-320a-5p (Up)	Hofsli et al 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Nil
miR-335-5p (Up)	Giraldez et al. 2013	53	82	63	8/13/16/5	miR-16-5p	Plasma	miRNeasy	PCR (Taqman)	< 0.001	Nil
miR-338-5p (Up)	Yong et al. 2013	112	50	64	25/32/31/24	RNU48	Whole blood	miRNeasy	PCR (Taqman)	NA	Nil
miR-382-5p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Nil
miR-409-3p (Up)	Wang et al. 2013	55	57	58	NA	miR-1228-5p	Plasma	miRVana	PCR (Taqman)	0.04	Nil
miR-409-3p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Nil
miR-423-3p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	NA	Nil
miR-423-5p (Up)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	NA	Nil
miR-601-5p (Down)	Wang et al. 2012	90	58	60	26/25/29/10	cel-miR-39(spike in)	Plasma	miRVana	PCR (Taqman)	< 0.001	Nil
miR-652-5p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Nil
miR-720-5p (Up)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Wang et al. 2012
miR-760-5p (Down)	Wang et al. 2012	90	58	60	26/25/29/10	cel-miR-39 (spike in)	Plasma	miRVana	PCR (Taqman)	< 0.001	Nil
let7D-5p (Down)	Hofsli et al. 2013	40	10	70	3/5/47/14	NA	Serum	miRNeasy	PCR (miRCURY)	< 0.01	Nil

NA – not applicable.

in stage IV colorectal cancer [39]. Importantly miR-21-5p has been found to be strongly expressed by red blood cells and was found to be highly expressed in plasma samples of colorectal cancer patients with high haemolysis ratios by Zanutto et al. [46]. When samples with low haemolysis ratios were examined miR-21-5p was not found to be dysregulated [46,59].

3.3. miR-29a-5p

miR-29a-5p function has recently been analysed by invasion assays and orthotopic transplantation in a mouse model [42]. miR-29a-5p directly targets Kruppel-like Factor 4 (KLF4), a tumour suppressor which, when silenced, is critical for the pathogenesis and progression of digestive system tumours. Through direct targeting of KLF4, miR29a promotes colorectal cancer metastases by regulating Matrix-Metalloproteinase 2 (MMP2) and E-Cadherin [42].

Five studies found miR-29a-5p to be upregulated in colorectal cancer compared to healthy controls (Table 1) [30–32,43,44]. Studies differed in the sources of miRNA used (serum and plasma) and differed in the use of endogenous controls (miR-16, let7a, miR-103). When evaluated independently miR-29a-5p was found to yield an AUC of 0.844 [43]. When tested in conjunction with miR-92a-5p it could differentiate advanced adenomas from healthy controls with 73% sensitivity and 79.9% specificity [43].

2 studies found no association or found contrary findings with previously reported associations of miR-29a-5p and colorectal cancer (Table 2) [45,46]. Faltejskova et al. found miR-29a-5p was downregulated in the serum of advanced colorectal cancer patients. No significance was found in differentiating any stage colon cancer and healthy controls. miR-16-5p was used as an endogenous control and they could not identify any factors leading to contrasting findings in the literature. One stipulation was that several previous studies had been performed in Asian populations and this may lead to potential variances as this study was performed on an Eastern European population [45]. Zanutto et al. also postulated that differing endogenous controls and genetic variations in ethnic groups may contribute to contrasting results [46].

3.4. miR-92a-5p

miR-92a-5p, along with miR-18a-5p is derived from the miR-17-92 cluster located at the chromosome 13q31.1 region. As previously discussed, the miR-17-92 cluster can promote cell proliferation, suppress apoptosis, induce angiogenesis and promote tumour progression [33]. miR-92a-5p has recently been shown to target the anti-apoptotic molecule BCL-2 interacting mediator (BIM) of cell death in colon cancer tissue highlighting its pivotal role as a key oncogenic component of the miR-17-92 cluster. Its action in circulation is unknown.

miR-92a-5p was found to be upregulated in colorectal cancer compared to healthy controls in 5 studies (Table 1) [30,33,43,44,47]. Studies again differed in their use of endogenous controls and source of miR-NAs. Both Liu et al. and Huang et al. used miR-16-5p as an endogenous control, had similar patient groups from homogenous ethnic backgrounds and found significant upregulation of miR-92a-5p in colorectal cancer. Sensitivity for miR-92a-5p ranged from 65%–89% and specificity from 70%–85%. As previously discussed, miR-92a-5p, when combined with miR-29a-5p was capable of discriminating advanced adenomas from controls yielding an AUC of 0.773 [43].

4 studies found no association with miR-92a-5p and colorectal cancer (Table 2) [31,41,45,48]. Wang et al., examined a similar population to previous studies identifying a positive correlation with miR-92a-5p and colorectal cancer. Patients were classified by Dukes Criteria rather than TNM stage and miR-1228 was used as an endogenous control. They found a lower expression of miR-92a-5p in colorectal cancers compared to controls in micro-array but could not validate this by RQ-PCR in an independant cohort of 112 plasma samples [48]. Nugent et al. extracted RNA from whole blood and used miR-425-5p as an endogenous control [41]. Faltejskova et al. found no association with miR-92a-5p and colorectal cancer and again the only difference between their study and those with positive findings was the ethnicity of the patient cohort [45].

3.5. miR-143-5p

Downregulation of the miR-143-145 cluster has been identified in colorectal tumours compared to normal colon epithelium [49]. miR-143-5p regulates major mediators of oncosuppression belonging to the growth factor receptor-mitogen-activated protein kinase network and to the p53 signalling pathway. Previously validated targets of miR-143-5p include KRAS and Kruppel-like Factor 5 (KLF5), an important mediator which acts synergistically with KRAS to mediate colorectal transformation. miR-143-5p also has an effect on CD44, a receptor which regulates apoptosis [49].

Three studies found miR-143-5p to be down regulated in association with colorectal cancer compared to healthy controls [30,44,50]. All 3 studies used different endogenous controls and extracted RNA from plasma and serum. Qian et al. had relatively small numbers, specifically in the healthy control group which consisted of only 10 patients and it was unclear what disease stages the colorectal cancer group was composed of [50]. Hofsli et al. also contained only a small number of controls (10) [44]. To date there are no studies finding contradictory evidence of miR-143-5p expression in circulation of colorectal cancer patients although current data is not robust.

3.6. miR-378-5p

Upregulation of miR-378-5p has been shown to induce malignant transformation in human breast cancer cells and promote migration, invasion and angiogenesis in human lung cancer cells [51,52]. Recent studies identifying expression levels of miR-378-5p in colon cancer tissues found it is downregulated in cancer and may act both as a tumour suppressor and regulator of EMT through targeting of Vimentin [53]. Yet another recent study of miR-378-5p in colorectal cancer showed over-expression of miR-378-5p inhibited cell proliferation and colony formation and suggested the miR-378a-3p/IGFIR/ERK signalling pathway is involved in the carcinogenesis of colorectal cancer [54]. The role of miR-378-5p in colorectal cancer appears complex and will require further evaluation.

miR-378-5p was found to be over expressed in the plasma of colorectal cancer patients in 2 studies (Table 1) [44,46]. Zanutto et al. identified miR-378-5p as a haemolysis independant biomarker in a validation set of plasma samples [46]. Initially miR-21-5p had appeared to be upregulated in cancer samples but in samples with significantly low haemolysis ratios this was not the case. In addition Zanutto et al. found miR-378-5p levels were significantly reduced after resection of the primary tumour [46]. Hofsli et al. also found circulating miR-378-5p to be upregulated in colorectal cancer patients [44]. Both studies differed in their use of endogenous controls and extracted miRNAs from different sources. There are currently no studies which find differing results for circulating levels of miR-378-5p in colorectal cancer.

3.7. miRNAs identified in single studies

There are 35 miRNAs found to be dysregulated in colorectal cancer by 8 studies however conflicting results have been found in several studies (Table 3) [30–32,40,41,43,44,46,48,55,56]. Studies extracted miRNA from whole blood, serum and plasma and used several different endogenous controls including miR-16-5p, miR-425-5p, miR-1228-5p, RNU48, dilution techniques and spike ins such as cel-miR-39 . Studies differed in their inclusion of colonic and rectal cancer primary tumours and TNM stages varied significantly across studies. There was significant heterogeneity present in terms of ethnicity of patient populations.

3.8. miRNA expression levels in tissue and serum

A number of studies have found corresponding dysregulation of miRNA expression in tissue and serum [31,38,39,47,50]. Two studies have found corresponding increased levels of miR-21-5p in tumour samples and serum or plasma [38,39]. Toiyama et al. demonstrated increased serum and tissue miR-21-5p expression in colorectal cancer patients and found a corresponding decrease in serum expression following the removal of the primary tumour [39]. In addition, they found miR-21-5p expression levels in the serum correlated with the size of the tumour [39]. Kanaan et al. also found increased levels of miR-21-5p in both tumour tissue and plasma of CRC patients [38]. Increased circulating levels of miR-18a-5p, miR-29a-5p, miR-92a-5p and reduced levels of miR143-5p were also demonstrated in single studies to correspond with expression levels identified in tumour samples [31,47, 50].

4. Discussion

Significant heterogeneity exists among current studies regarding the source used to detect miRNAs in circulation. Reducing pre-analysis variables between studies may significantly improve reproducibility of results. Patient ethnicities, the presence of disease and smoking status have been identified as pre-analysis factors which may contribute to differeing results in previously discussed studies [33,45,46]. Other pre-analysis variables such as fasting and non-fasting patients and timing of sampling due to potential diurnal variation of miRNA presence in circulation may

further add to this [57]. Disagreement exists regarding some of these factors however as a recent study by MacLellan et al. has found smoking and fasting do not significantly effect miRNA profiles in serum and identified haemolysis to be the most significant pre-profiling factor affecting subsequent results [68]. Studies have reported a large number of miRNAs believed to be released by tumours as circulating markers of disease may be present in the plasma and serum secondary to haemolysis due to collecting procedures or storage methods [59]. miR-21-5p, miR-29a-5p and miR-92a-5p have been shown to be expressed by red blood cells and are strongly affected by haemolysis [46,59].

The differing use of serum, plasma and whole blood among studies has potential to cause varying results. Plasma is the cell free supernatant present following centrifugation of blood collected in the presence of an anti-coagulant. Serum is collected without an anti-coagulant and is the cell free supernatant present following centrifugation of blood that has been allowed to spontaneously clot [57]. Collection methods of serum and plasma and the use of anti-coagulants present in collecting tubes can significantly alter the resultant miRNA content of samples. In addition, it is unknown if the duration of time between specimen collection and miRNA expression analysis effects miRNA expression levels in samples [57].

There are multiple commercially available kits to determine miRNA expression levels in biological samples. A study by Mestdagh et al. [58] has shown substantial differences in miRNA expression levels of the same samples between commercially available kits. Different kits suggest different methods of data normalisation, data analysis and processing which can further add to differences in end results of miRNA expression levels [58].

Pre-profiling factors and the impact of haemolysis on miRNA expression highlight a need to identify the mechanism by which cancer cells secrete miRNAs into the circulation. Some studies have identified corresponding dysregulation of miRNAs in tissue and circulation and one potential source of tumour derived miRNAs is cancer cell secreted exosomes [31,38,39, 47,50].

Exosomes are a class of secreted lipid membrane vesicles known to be produced by many cell types. Exosomes are well characterised in comparison to other secreted lipid vesicles. They have a diameter of 40–100 nm, have a bi-lipid membrane and are known to contain proteins, RNA and more recently have been shown to contain miRNA [60]. Exosome mem-

branes are highly stable due to their cholesterol, sphingomyelin, ceramide and lipid-raft associated protein content [61]. This facilitates collection from various body fluids as well as cell culture mediums. Exosomes were originally thought to function as a method of waste disposal to discard unwanted transferrin receptors but are now recognised as a vehicle to facilitate intercellular communication through transfer of their contents which includes miRNAs [62,63].

Exosomes secreted by cancer cells are generally considered to promote tumour growth and metastasis [62]. The fraction of exosomes in circulating plasma in colorectal cancer patients has previously been demonstrated to be higher than in healthy controls and the level of circulating exosomes was reported to correlate with poor prognosis [62]. Only a small number of studies have identified miRNAs contained within colorectal cancer cell secreted exosomes thus far. Chiba et al. [63] grew three colorectal cancer cell lines (HCT-15, SW480 and WiDr) and harvested their exosomes. Exosomes secreted by colorectal cancer cells contained miRNAs which have been shown to be dysregulated in association with colorectal cancer in patients (miR-21-5p, miR-34a-5p, miR-143-5p, miR-192-5p, miR-215-5p, miR-221-5p). Ogata-Kawata H et al. isolated exosomes from the serum of 88 colorectal cancer patients and 11 controls and performed microarray analysis of miRNAs contained within the isolated exosomes [64]. Expression levels of miR-21-5p and several other miRNAs were found to be consistently higher in exosomes isolated from cancer patients compared to controls [64]. As disease specific expression-patterns of miRNAs reflect mechanisms of cellular transformation, exosomes in the circulation of colorectal cancer patients may contain specific miRNAs identifying disease presence and stage. Uptake of exosomes containing miRNAs which affect tumour suppressor genes and have well described roles in the pathogenesis of cancer may explain the associations identified with certain miRNAs and advanced disease. miRNAs in exosomes may influence donor cells creating a metastatic niche encouraging disease progression.

The use of endogenous controls is a contentious issue in the search for miRNAs predicting disease. The most commonly used endogenous control is miR-16-5p. There are significant caveats which must be taken into account when using miR-16-5p. Levels of miR-16-5p are affected by haemolysis as it is expressed in red blood cells [59]. When samples with significant haemolysis ratios are excluded however, miR-16-5p re-

mains consistent [65]. Recently Hu et al. published a study identifying miR-1228-5p as a stable endogenous control for quantifying circulating miRNAs in cancer patients [66]. In a cohort of 168 controls and 376 cancer patients miR-1228-5p was found to be more stably expressed in circulation than a number of other miRNAs including miR-16-5p, let7a-5p and RNU6B. Another recently published study by McDermott et al. suggested the combined usage of miR-16-5p and miR-425-5p in breast cancer to be the most accurate endogenous controls [67]. It is likely endogenous controls specific to cancer and patient types may be required. Further studies examining large patient cohorts and identifying variances in potential endogenous controls according to patient demographics, cancer type and stage and any pre-existing disease could significantly improve the potential for circulating miRNAs to be incorporated into clinical practice as biomarkers.

The discussed studies demonstrate that circulating miRNAs hold significant potential as biomarkers in colorectal cancer. There are a number of factors leading to difficulty in interpreting and comparing results but this rapidly expanding area of research is still in its infancy. Pre-profiling factors which could potentially affect miRNA expression levels require further study. In recent times, there has been a focus on finding appropriate endogenous controls for RQ-PCR to adequately assess dysregulation in cancer patients. Uniformity of endogenous controls may significantly decrease variance in results. Exosome-encapsulated miRNAs represent a previously unexplored area which firstly could eliminate concerns regarding haemolysis in blood samples or derivatives and secondly provide valuable insight into the functional role of circulating miRNAs. While significant promise has been shown in a number of studies for the identification of a circulating miRNA biomarker in colorectal cancer and other cancers there is a requirement for large scale studies using standardised protocols to achieve clarity and consensus.

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