microRNA-mediated intercellular communication in the primary breast tumour microenvironment

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This thesis is dedicated to my parents,

Tom and Briege Glynn,

as I owe all that I am to them.

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Abbreviations

°C	Degrees centigrade
µg	Microgram
µl	Microlitre
µM	Micromolar
AGO2	Argonaute 2
ANOVA	Analysis of variance
ATCC	Amercican Type Culture Collection
cDNA	Complementary DNA
cm	centimetre
CT	Cycle Threshold
ddH_2o	Ultra-pure water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides
DPX	Distrene, Plasticiser, Xylene
EC	Endogenous control
EDTA	Ethylenediaminetetracetic acid
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen receptor
FBS	Fetal Bovine Serum
Forkhead Box P1	FOXP1
g	Gram
HDL	High Density Lipoprotein
HER2	Human Epidermal growth factor Receptor 2
M	Molar
mg	Milligram
min	Minute
miRNA	MicroRNA
mM	Minimolar
mRNA	messengerRNA
MRPL19	Mitochondrial ribosomal protein L19
MSC	Mesenchymal Stem Cell

MVB	Multivesicular body
NaOH	Sodium hydroxide
nm	nanometres
NPM1	Nucleophosmin1
nSMase2	Neutral sphingomyelinase 2
NTC	No Target Control
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PPIA	Peptidylprolyl isomerise A
PR	Progesterone receptor
RFP	Red Fluorescent Protein
RIN	RNA Integrity Number
RISC	RNA-induced silencing complex
RNA	Ribonucleotide acid
RPMI	Roswell Park Memorial Institute
RQ-PCR	Relative quantitative PCR
RT	Reverse Transcription
SAGE	Serial analysis of gene expression
TAN	Tumour associated normal
TEM	Transmission Electron Microscopy
U	Units
UTR	Un-Translated Redion

Communications originating from this work

Manuscripts

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Waters PS, Dwyer RM, Brougham C, **Glynn CL**, Wall D, Hyland P, Duignan M, McLoughlin M, Newell J, Kerin MJ. Impact of tumour epithelial subtype on circulating microRNAs in breast cancer patients. PloS one. 2014;9(3):e90605. Epub 2014/03/15.

Glynn CL, Clancy C, Joyce DP, Dockery P, Kerin MJ, Dwyer RM. The exosome-encapsulated microRNA profile secreted from breast cancer cells. **Manuscript in progress**.

Glynn CL, Khan S, Kerin MJ, Dwyer RM. miR-504 in breast cancer and the impact of overexpression *in vitro* and *in vivo*. **Manuscript in progress.**

Communications/Abstracts

Exosomal transfer of microRNAs as a potential vector for gene therapy.
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Investigation of exosome-encapsulated miRNA secretion in breast cancer
Claire L Glynn, Sonja Khan, Cillian Clancy, Doireann Joyce, Peter Dockery, Michael J. Kerin, and Roisin M. Dwyer.
American Association of Cancer Research, San Diego, April 2014

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Elevated expression of miR-106 and miR-202 in breast cancer
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Identification of an optimal method for isolation of microRNAs secreted by cells
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Abstract

microRNAs (miRNA) have been highlighted as potential circulating biomarkers and as therapeutic targets for breast cancer. Recent evidence suggests that miRNAs are transported in protective microvesicles called exosomes, which play an important role in intercellular communication. This study aimed to validate the expression of miRNAs in patient breast tumours, and to investigate their secretion by breast cancer cells, identifying a specific profile of miRNAs packaged into exosomes.

Two miRNAs, miR-106a and miR-191, were shown to be significantly elevated in patient breast tumours and may represent oncomiRs, while one miRNA, miR-504, was found to be significantly decreased and may represent a tumour suppressor. The relationship between circulating miRNA expression levels and menstrual cycle hormones was investigated, where miRNA expression was found to remain stable, with the exception of one miRNA. miRNAs were found to be secreted by breast cancer cells into conditioned media, where a specific miRNA profile was actively and selectively packaged into exosomes. Approximately 390 miRNAs were detectable in exosomes collected from a range of breast cancer cell lines, with four miRNAs, miR-10b/miR-145/miR-492/miR-498, further validated by RQ-PCR. The ability to enrich exosomes with a particular miRNA, miR-504 (previously identified as a potential tumour suppressor in breast cancer), was achieved by transducing a breast cancer cell line to overexpress the miRNA. The impact of overexpressing miR-504 was investigated *in vitro*, with no significant alterations observed in cell proliferation or migration. Furthermore, the impact was also investigated *in vivo*, where miR-504 overexpression was not found to suppress tumour formation. Exosome transfer between cell populations was visualised using confocal microscopy and the impact of transfer on the recipient cells was investigated, where a trend towards increased miR-504 expression was observed. In addition, a significant increase in cell proliferation as a result of the whole exosome was observed, with no significant impact on cell migration identified.

The data presented here is a contribution to the ongoing research of exosome-encapsulated miRNAs in the cancer research field. Identification and functional characterisation of exosome-encapsulated miRNAs in cancer may be central to the generation of novel biomarkers and therapeutic strategies for this disease.

Chapter 1

Introduction

1 Chapter 1: Introduction

1.1 Breast cancer

1.1.1 Introduction

Breast cancer is the second most commonly diagnosed invasive malignancy among women in Ireland, with approximately 2,700 new cases diagnosed each year. Although the incidence of breast cancer has increased significantly, fortunately, a considerable decline in mortality rates has been observed (1). Between 1994 and 2010, there was an annual increase of breast cancer incidence in Ireland of ~ 4%. Some deviation from this trend can be observed at the time of the introduction of BreastCheck, the national breast cancer screening programme, in 2001, and again at the time of its introduction to the South and West of Ireland in 2007 (2). Breast Check promotes the importance of screening and detecting the disease in its earliest stages, which will ultimately reduce the need for extensive treatment and significantly improve patient outcome. It is widely known that early detection can have an immense impact on a patient's survival and this is particularly true with regards to breast cancer (3). Patients that present with Stage 1 disease at time of diagnosis, have a 5 year survival of over 96%. Whereas, patients that present with Stage 4 disease at time of diagnosis, have a 5 year survival of only 28% (Table 1.1).

5- Year Relative Survival (2004-2008)	
Stage 1	96.1%
Stage 2	89.5%
Stage 3	66.4%
Stage IV	28.1%

Table 1.1 Percentage survival for female breast cancer (Adapted from NCRI Annual Report 2014 (1)).

This highlights the importance of regular breast screening and early detection of the disease. Breast cancer is a complex, molecularly heterogenous disease which is a challenge in terms of management and in predicting progression. The National Cancer Registry of Ireland (NCRI) predicts that by 2020 there

will be approximately 5000 new cases per year in Ireland (1). One of the key challenges facing scientists in this field is to unravel the complexities of the molecular interactions which lead to breast cancer initiation and progression.

1.1.2 Breast cancer pathology

The term 'breast carcinoma' encompasses a diverse group of lesions which differ pathologically and biologically. Breast carcinomas can be divided into two major groups; *in situ* carcinomas and invasive carcinomas (4). *In situ* carcinomas are defined by the presence of tumour cells in the breast ducts or lobules, with no evidence of invasion into the surrounding breast stroma. Depending on the cell type from which the tumour arises, *in situ* carcinomas can either be ductal or lobular. Invasive carcinomas are defined by evidence of invasion outside of the normal breast lobules and ducts to grow in to the surrounding breast stroma/connective tissue, and also the metastatic potential of the tumour (5). Approximately 80% of breast carcinomas are invasive ductal carcinoma, followed by invasive lobular carcinomas (~10-15%) (6). Each has its own distinct pathological features. Lobular carcinomas tend to grow as single cells arranged individually and exhibit different molecular and genetic aberrations that distinguish them from ductal carcinomas (7).

1.1.3 Breast tumour classification

In a clinical setting, breast tumours are classified based on their pathological features; stage, grade, and hormone receptor status.

Tumour Stage:

Tumour progression is categorised based on the TNM classification system: Tumour size (T), if lymph nodes are affected (N), and presence or absence of metastatic tumours at other sites (M). The numerical severity of each of these factors then determines the stage of breast cancer (I-IV), assigned by the American Joint Committee on Cancer (AJCC) staging system (8).

Tumour Grade:

The histological grade of the breast tumour (scale of 1-3) is determined microscopically by evaluating acinar formation, nuclear size and mitotic activity. Tumours which exhibit a well-differentiated normal-like phenotype are assigned Grade 1, while poorly differentiated, aggressive tumours are assigned Grade 3 (9).

Hormone Receptor Status:

Estrogen receptors (ER) and Progesterone receptors (PR) are typically co-expressed in malignant breast cells and provide valuable prognostic information (10). Positive ER and PR status generally infers a good patient prognosis and response to endocrine therapies (11). Amplification of Human Epidermal Growth factor receptor-2 (HER2) leads to increased proliferation and angiogenesis (12, 13).

1.1.4 Epithelial subtypes of breast cancer

Just 13 years ago, our understanding *of* breast cancer heterogeneity was revolutionised by large scale gene expression profiling studies. The pioneering research by Perou and Sorlie *et al.* (14, 15), identified several distinct molecular subtypes of breast cancer (Figure 1.1).

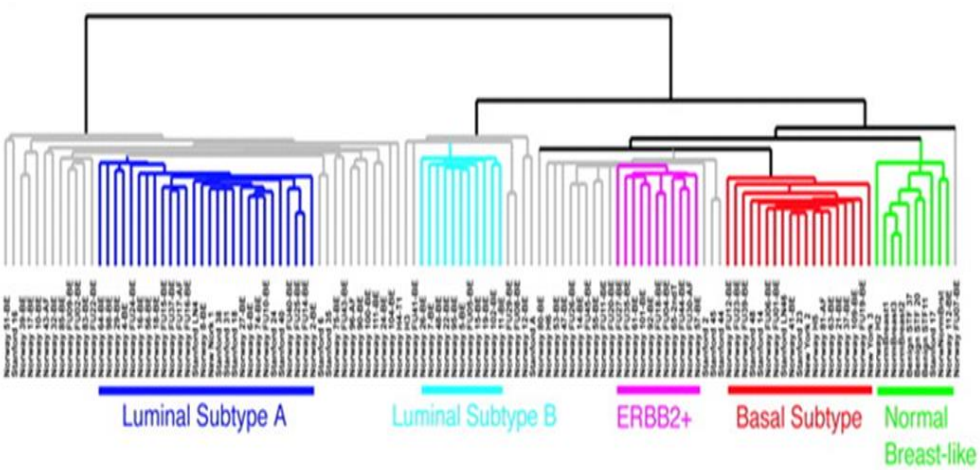


Figure 1.1 Identification of intrinsic breast tumour subtypes. (Adapted from Sorlie *et al.* (16))

As a result, in clinical practice today, breast cancer is additionally classified according to differences in the molecular signature of five major subtypes (Table 1.2).

Subtype	Receptor Status
Luminal A	ER+ PR+ HER2-
Luminal B	ER+/- PR+/- HER2-/+
HER2+	ER- PR- HER+
Basal-like	ER- PR- HER2-
Normal-like	ER+/- PR+/- HER2-

Table 1.2 Molecular subtypes of breast cancer and their receptor status.

These being; Luminal (A and B), HER2 overexpressing, Basal-like and Normal-like. Furthermore, it was also identified that with each subtype of disease, different patient outcomes could be expected relating to survival and recurrence rates. This molecular classification system has been shown to have prognostic value and its usefulness has been realised particularly with regards to predicting reponse to chemotherapy in the breast cancer setting (17).

Luminal A

The term 'luminal' derives from similarity in expression between these tumours and the luminal epithelium of the breast. Luminal A breast cancers are ER+, PR+ and HER2 negative, resulting in their particular utility with endocrine based therapies (14). Luminal A cancers account for approximately 40% of all breast cancers diagnosed and infer the best prognosis of all breast cancer subtypes (18). Estrogen receptor α (ERα) is present on ~70-80% of all breast cancers and a successful treatment strategy for this subtype involves blocking ERα. Tamoxifen is a Selective Estrogen Receptor Modulator (SERM) which is widely used in the treatment of Luminal A tumours. SERMs function by attaching to the estrogen receptors in breast cells thereby blocking the ability of estrogen to bind to the cell (19). Alternatively, aromatase inhibitors can be employed to block the synthesis of estrogen, for example anastrozole. Aromatase inhibitors function by blocking the aromatase

enzyme, which converts androgen into estrogen, thereby limiting the available estrogen to stimulate the growth of hormone-receptor positive breast cancer cells (20). Studies comparing the effectiveness of SERMs (e.g. tamoxifen) and aromatase inhibitors have revealed aromatase inhibitors are preffered for treatment of post menopausal breast cancer patients, with fewer serious side effects than tamoxifen (21, 22). In addition, switching to an aromatase inhibitor after taking tamoxifen for 2 to 3 years offers more benefits than taking tamoxifen alone for 5 years (23).

Luminal B

Luminal B tumours are less common than Luminal A tumours (~20%) and have a significantly lower expression of the ER related genes, although still present. Luminal B tumours infer poor prognosis and have been noted to have high recurrence scores (18). Luminal B tumours are not known to be particularly responsive to tamoxifen and aromatase inhibitors, although they do generally respond well to neoadjuvant chemotherapy (24).

HER2-Overexpressing

HER2 tumours are typically characterised by overexpression of the HER2 receptor, generally through amplification of the HER2 gene, and low expression of the luminal clusters, resulting in their ER-, PR-, HER2 positive status (14). This alteration of the HER2 gene has been associated with increased tumour invasiveness by the down regulation if α-4 integrin via the p38MAPK pathway (25). As a result, HER2 tumours are typically highly aggressive and had traditionally inferred a poor prognosis prior to the introduction of HER2 targeted therapies, such as trastuzumab. However, there has been a marked increase in patient survival since the introduction of HER2 targeted therapies (26, 27).

Basal-like

The term ‘basal’ derives from similarity in expression between these tumours and the basal epithelial cells. This subtype is characterised by low expression in the luminal and HER2 gene clusters, resulting in the term ‘triple-negative’ being ascribed to this subtype (28). However, ‘Basal-like’ and ‘triple-

negative' are terms which should not be used interchangeably as they are two distinct types of tumour. There are some Basal-like tumours which are not triple-negative, they are defined by other characteristics such as cytokeratin expression and mitotic index, while triple-negative tumours are ER/PR negative and HER2 negative (29). Basal-like tumours typically harbour mutations in the p53 and BRCA1 genes, thus explaining their aggressiveness (30). Basal tumours account for approximately 15% of all breast cancers classified, and are known to be prevalent in women of a younger age (14). Due to the aggressive nature of these tumours, current therapies rely on harsh chemotherapy regimens.

Normal-like

This subtype of breast cancer has been very poorly characterised to date due to the rarity of such tumours, and is therefore not utilised in clinical practice. It is suggested that their gene expression is similar to adipose tissue, although they are triple negative for all hormone receptors, resulting in no response to neoadjuvant chemotherapy (28).

In 2012 a groundbreaking study (31) revealed novel subgroups of breast cancer which had previously not been recognised. This study found that patients from a large breast cancer cohort (n ~ 2000) could be clustered into 10 molecularly defined subgroups. The authors found these subgroups to hold distinct biology and disease-specific survival characteristics, based on multiple genomic views. This novel subgroup analysis is not currently used in clinical practice and is a target for further investigation. Their identification, however, re-inforces the idea that breast cancer is not one disease. Rather, breast cancer is a multitude of diseases with distinct mutations and gene expression patterns, whereby the molecular phenotypes cluster into diagnostic and prognostic groups.

1.1.5 Breast cancer diagnostic and prognostic tools

The current gold standard for breast cancer diagnosis is Mammography. A mammogram is an x-ray that is capable of detecting the presence or absence of a mass and/or microcalcifications, changes in the breast, or risk factors assocociated with breast caner development (32). Mammography has a reported sensitivity between 62.9% and 87% (33-35). Following detection of a mass, ultrasound is employed to determine if it is solid or a cyst. Although not used routinely, Magnetic Resonance Imaging (MRI) can also be employed as a useful diagnostic tool for the detection of breast cancer. Current clinical recommendations suggest that patients who have a high risk of developing breast cancer should routinely undergo MRI screening (32). Following determination of the solid or cyst state of a mass, a fine needle biopsy is performed to determine if a cancer is present.

Tumour markers, such as ER/PR/HER2 status, influence clinical management as they are are linked to prognosis and treatment choice as previously outlined. CA15-3 is a tumour marker used to monitor cancers, in particular breast cancer. It is a cancer antigen which is found on many types of cancer cells, which are then shed in to the blood stream. It is primarily used to monitor advanced or metastatic cancers as it has limited use in early stage disease (36). In conjunction with Alkaline Phosphatase (ALP), elevated levels of CA15-3 are associated with an increased chance of early recurrence of breast cancer (37).

It is well established that early detection can have a huge impact on a patient's survival. Limitations in screening methods can greatly impact a patient's prognosis. Therefore more rapid, sensitive, specific and minimially invasive methods of detections (i.e. biomakrers) are greatly needed. Furthermore, the presence of metastasis is an additional factor that can have a huge impact on a patien'ts survival and greatly contributes to breast cancer mortality. In addition, the development of resistance to chemotherapeutics severely impacts the treatment options available to certain breast cancer patients. As a result, the development of biomarkers which can provide information relating to the risk of metastasis development and resistance to chemotherapeutics would be immensely beneficial. This allows an individualised therapeutic regime to be designed for the patient to meet his/her requirements. Despite

the significant increase in the volume of research in the breast cancer biomarker field worldwide, there remain challenges that must be overcome, and gaps that must be filled in order to translate the research into clinical practice. Thus, extensive research is required to develop new biomarkers and further validate those on the cusp of implementation into clinical practice. This will greatly enhance the diagnostic, prognostic and predictive tools available in the breast cancer setting.

1.1.6 Breast Cancer Treatment

With major advances in clinical biochemistry and molecular medicine in recent years, a more targeted, individualised therapeutic regime is much sought after. The molecular characteristics of a cancer can provide a wealth of information allowing treatment and dosage to be tailored to a patient's particular tumour characteristics. This in turn will improve response to treatment and reduce the need for unnecessary therapies which can have significant long term side effects. Currently in clinical practice, an array of traditional treatment strategies exist, allowing somewhat of an individualised approach. These include: surgery, chemotherapy, biological therapy, and radiotherapy.

Surgery

Radical mastectomy was first introduced in 1880 by W.S. Halsted as a surgical treatment for breast cancer (38). Surgical interventions have evolved considerably since and less destructive treatments are now utilised. Breast sparing surgery, known as lumpectomy, involves removal of only the tumour tissues and some surrounding tissue to ensure elimination of all cancer/abnormal cells. Mastectomy involves the complete removal of all breast tissue and is typically used on large or multicentric tumours. In recent years, however, mastectomy has become more refined and less intrusive as typically the muscles under the breast are no longer removed (39). Breast re-construction is increasingly being carried out at the time of mastectomy, and sometimes lumpectomy, which not only has the advantage of eliminating the need for more surgery in the future, but also the chest tissues are not damaged by radiation or scarring, so that the end result is often more aesthetically pleasing (40).

Chemotherapy

Chemotherapy is the use of cytotoxic drugs designed to impair mitosis in cancer cells, thereby resulting in cell death, and is currently the mainstay for treatment of many advanced cancers (41). Prior to surgery, neoadjuvent chemotherapy can be employed with the aim to shrink the tumour. Alkylating agents can be employed which damage cellular DNA through the addition of an alkyl group e.g. cyclophosphamide. Alternatively, platinum agents function by damaging DNA through the addition of a platinum adduct e.g. cisplatin (42). Antimetabolites, such as methotrexate and 5-fluorouracil, inhibit pyrimidine and purine production required for DNA and RNA synthesis. Combination therapies such as CMF (cyclophosphamide, mexotrexate, 5-fluorouracil) are frequently administered to patients with node positive cancer (41).

Treatment given in addition to the primary treatment, known as adjuvant chemotherapy, utilises a number of chemotherapy medicines, given alone or in combination, with the aim to eliminate any cancer cells that may have spread from the primary tumour site. This includes a class of medicines called taxanes and a class of medicines called anthracyclines. Taxanes or anti-mitotic agents function by interfering with the ability of cancer cells to divide and include paclitaxel and docetaxel. Taxanes target spindle formation in mitosis thereby impacting tumour cell growth. Anthracyclines or anti-tumour antibiotics function by damaging cancer cell genes and interfering with their reproduction, and include doxorubicin and epirubicin (43).

Chemotherapy however, is not without its drawbacks. Disadvantages include the associated toxicity to normal cells and the development of resistance to treatment. As it is not a targeted therapy, many normal cells with a high proliferation capacity can be affected by the therapeutic agents. As a result, adverse side effects are experienced such as reduced haematopoiesis and sterility. Also, resistance of cancer cells to chemotherapy continues to be a major clinical challenge (44).

Biological Therapy

Biological therapies were introduced to overcome some of the side effects observed with chemotherapy. This type of therapy aims to identify and attack specific cancer cells without harming normal cells. Types of biological therapy include: monoclonal antibodies, hormonal therapy and aromatase inhibitors. In the breast cancer setting, trastuzumab was introduced for the targeted treatment of breast cancers belonging to the HER2 overexpressing subtype. trastuzumab is a monoclonal antibody that blocks the HER2 protein which sends growth signals to breast cancer cells. Preliminary studies revealed increased survival when trastuzumab was used in combination with chemotherapy (45). Hormonal therapy removes hormones or blocks their mechanism of action and stops cancer cells from growing. Tamoxifen is a widely used non-steroidal anti-estrogen drug given to patients with early stage breast cancer and also metastatic breast cancers (46). Aromatase inhibitors, such as Anastrozole, can be given to postmenopausal women who have a hormone-dependent cancer, as the aromatase enzyme can block the body's estrogen production (47).

Radiotherapy

Radiotherapy is typically included in multimodal treatment of breast cancer i.e. combinations of surgery with radiation and/or chemotherapy. Radiotherapy uses controlled ionizing radiation to target and treat solid tumours. Radiotherapy fuctions by targeting the nuclear DNA of the cancer cells and also prevents the cancer cells from growing by interrupting the cell cycle process (48). There are currently three types of radiotherapy employed in clinical practice. External radiotherapy sends radiation from outside the body toward the cancer. Internal radiotherapy uses radioactive substances which are delivered directly into or near the cancer. Intraoperative radiotherapy is performed during surgical excision of the tumour (49).

1.1.7 Breast Tumour Microenvironment

The primary breast tumour microenvironment plays a fundamental role in cancer initiation and progression. Within this microenvironment, the predominant cell types are the stromal cells which are known to have play an active role in tumour progression (50). Tumour stromal cells are fundamentally different from the stroma of equivalent normal breast tissue, and have gene expression signatures that show a relationship with tumour grade/stage and poor prognosis (51). The mechanisms by which stromal cells promote tumourigenesis are yet to be fully elucidated, although their potential as novel therapeutic targets and their role in intercellular communication is already apparent. It is known that the stromal-epithelial cross-talk in the tumour microenvironment is very important, and there have been numerous studies performed in this area investigating various paracrine mediators of their growth-promoting signals, such as chemokines and cytokines (51). However, in order for stromal-epithelial interactions to emerge as appropriate targets for novel breast cancer therapies, further characterisation of the molecular crosstalk between these two populations is required. This opens up an incredibly exciting and novel avenue of investigation in terms of secreted microRNAs selectively packaged in exosomes and their delivery and ultimate function in a new cell population.

1.2 microRNAs

1.2.1 Introduction to microRNAs

A little over 2 decades ago, there were some components of the genome which were considered to be non-functional or dormant, until it was realised that they in fact possessed some gene regulatory ability. These components are known as microRNAs (miRNAs). miRNAs are small non-coding, naturally occurring single stranded RNA molecules, typically 19-25 nucleotides in length (52, 53). miRNAs have been implicated as important regulators of a wide range of cellular pathways and play a pivotal role in gene regulation through translational repression of target messengerRNAs (mRNA) (54). It is estimated that miRNAs regulate approximately 30% of the human protein-

coding genome. One miRNA may target hundreds of mRNAs, and in turn, one mRNA may be targeted by multiple miRNAs (55, 56). miRNAs control gene expression in a large number of biological processes, including apoptosis, proliferation, differentiation, and metastasis (57). miRNAs exhibit their function by sequence-specific modulation of gene expression at a post transcriptional level and have two possible mechanisms of action. If the target messenger RNA (mRNA) and microRNA Induced Silencing Complex (miRISC) have perfect base pairing homology, the mRNA is cleaved and degraded through activation of the RNA mediated interference pathway. miRNAs can also modulate their target genes by binding, resulting in repression of protein translation without mRNA degradation (58). miRNAs bind to partially complementary sequences, often positioned in the 3' untranslated region (UTR) of target mRNAs. However, it is also possible for miRNAs to bind to the coding region and 5'UTR of target genes (59).

More than 2000 miRNAs have been identified in humans since their discovery nearly two decades ago (52, 57) and the number of published studies is growing at a rapid rate. The miRBase database, formerly known as the microRNA Registry, is an archive of miRNA sequences which provides a centralised system for recording miRNA sequence names when they have been identified (60). As of July 2014, there are 28,645 reported mature miRNA sequences identified in 206 species recorded in miRBase Version 21. miRBase is also an authoritative source of miRNA nomenclature which regulates the naming of newly identified miRNAs. Prior to submitting novel miRNAs, validation must be performed via cloning, or via expression and processing data (61). Within the human genome, there are currently 2,588 miRNAs registered. The miRNA name is designated by 'miR' (indicating a mature sequence) followed by a sequentially assigned unique number e.g. miR-195 (62). The species of origin is designated by a three letter prefix e.g. hsa-miR-155 (*Homo sapiens*) or mmu-miR-155 (*Mus musculis*). The suffix -5p is added to the miRNA originating from the 5' arm, and -3p is added to the miRNA from the 3' arm, e.g. miR-423-5p and miR-434-3p. When the relative expression levels of two mature miRNAs, that have originated from opposite arms of the same pre-miRNA are known, the miRNA which is

expressed at a lower level is followed by an asterisk e.g. miR-9* and miR-9, with the latter being the predominant miRNA (61). miRNA families, e.g. the let-7 family, are assigned based on sequence similarity across the whole hairpin precursor, which is suggestive of a common ancestor. miRNA expression profiling allows a deeper understanding of the developmental stages of many diseases. Distinct patterns of miRNA expression have been observed in individual tissues and in different disease states which strengthens the idea that the developmental history of human disease can be encoded from miRNA expression patterns (53).

1.2.2 microRNA biogenesis

miRNA biogenesis begins in the nucleus where transcription of miRNA sequences by RNA polymerase II occurs to form large capped and poly-adenylated primary RNA transcripts (pri-miRNA) of approximately 70-90 nucleotides, as depicted in Figure 1.2.

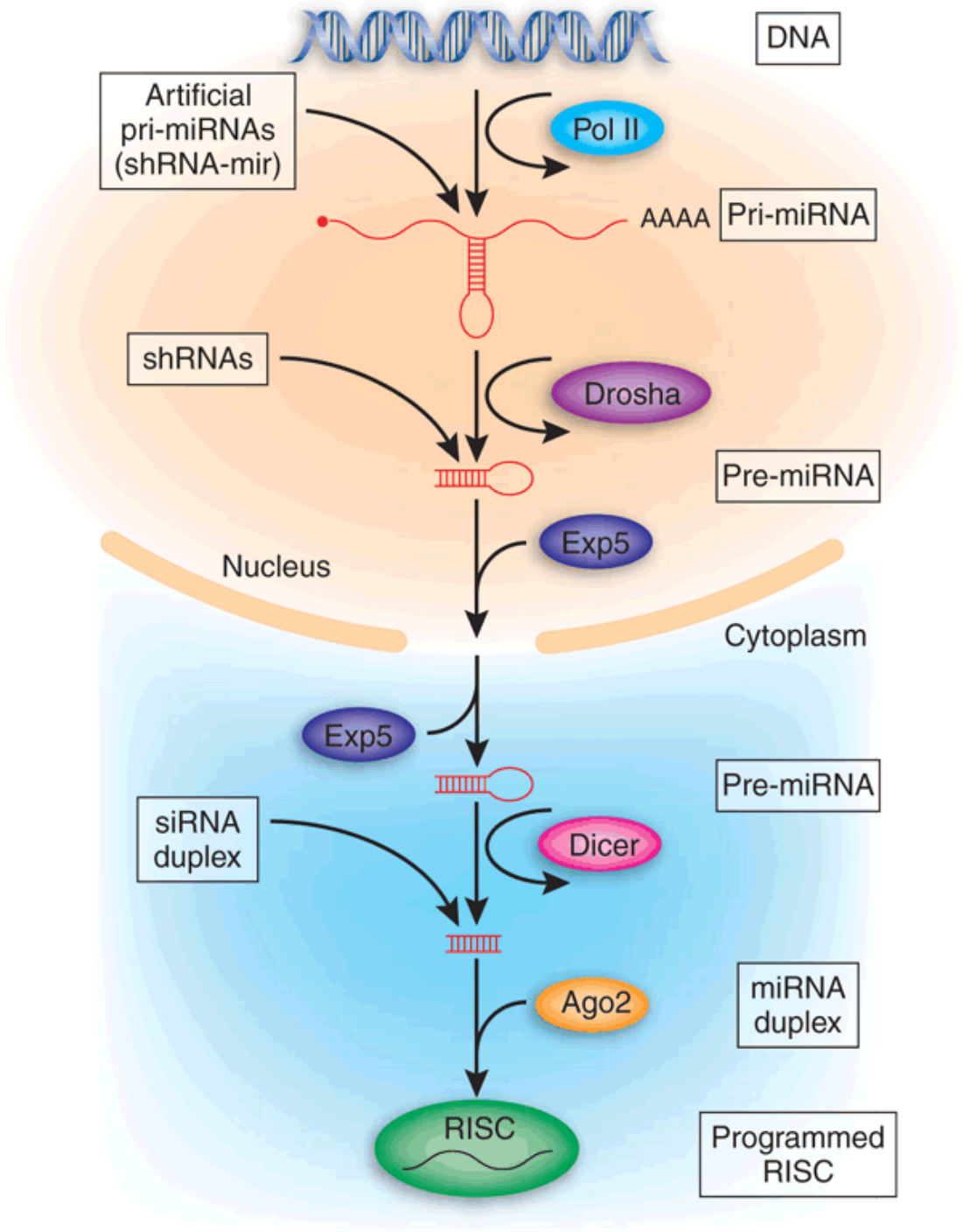


Figure 1.2 Schematic displaying miRNA biogenesis (63). The multistep development commences in the nucleus where a pre-miRNA is produced, and then exported into the cytoplasm by Exportin 5. The hairpin structure is then cropped by Dicer, producing the double stranded duplex. Adapted from Cullen, 2005 (63).

The pri-miRNAs are then cleaved by DROSHA, the Class 2 RNase III enzyme, accompanied by its double stranded binding partner the DiGeorge Syndrome Critical Region 8 (DGCR8), also known as Pasha. This forms a sequence of approximately 70-90 nucleotide precursors (pre-miRNAs) consisting of an imperfect stem loop hairpin structure. The pre-miRNAs are then exported from the nucleus into the cytoplasm by Exportin 5. The hairpin structures are then cleaved in the cytoplasm by an endo-ribonuclease known as Dicer (~200 kDa) and its binding partner Tans-Activator RNA Binding Protein (TRBP), into a small double stranded RNA (dsRNA) duplex (Figure 1.2). This duplex contains the mature miRNA strand together with its complementary strand. The mature strand is preferentially included into a miRNA associated RNA induced silencing complex (miRISC), and directs RISC to seek out mRNAs containing complementary sequences to the mature miRNA and thereby exert its cellular effects (58).

1.2.3 microRNAs and Normal biological processes

Due to the post transcriptional action of miRNAs, the process of gene expression control endures high levels of regulatory complexity. It has been well established from early studies of miRNAs in model organisms that they play major roles in various biological phenomena, which include tissue type determination and also the timing of developmental processes. Subsequent highly focussed studies have unearthed numerous other roles including metabolic control, brain development, and viral defence (64). Despite the overwhelming volume of research being carried out in the area of miRNAs in recent years, and in particular with regards to breast cancer, few have investigated the role of miRNAs in normal breast physiology. One particular landmark study investigated the expression of miRNAs in mouse mammary glands in juveniles and adults and throughout pregnancy, lactation, and involution. This study showed that particular families of miRNAs can be connected to distinct phases of the breast cycle (65). Studies investigating the normal physiology of miRNA function are essential in order to elucidate how miRNA dysregulation influences carcinogenesis.

1.2.4 microRNAs and cancer

miRNAs have been a focal point of research in molecular biology over the last decade. The role and relevance of miRNAs can be associated with numerous normal physiological processes and indeed pathological conditions. With the current prevalence of cancer in society, it is not surprising that the majority portion (~47%) of the research conducted in the miRNA research field focuses on their role in cancer (66).

As cancer is essentially a consequence of disordered genome function, it can be expected that miRNAs, as regulatory molecules, are involved in the development of the disease. In 2002, Calin *et al.* (67) first suggested the role of miRNAs in cancer when they documented a translocation induced deletion at chromosome 13q14 in B-cell chronic lymphocytic leukemia (CLL). It was found that two miRNAs, miR-15a and miR-16-1, were encoded in the smallest minimal common region of deletion. Analysis of expression levels of these two miRNAs in both CCL samples and normal CD5+ lymphocytes revealed the downregulation of miR-15a and miR-16-1, which was consistently related to the deletion at chromosome 13q14. This was the first confirmation of the role of miRNAs as tumour suppressors (68). In the interim, miRNA expression has been associated with almost every cancer and common disease and studies on specific miRNAs have alerted researchers to the overwhelming potential of these short RNA molecules in the context of cancer. There exists great potential for miRNAs to allow a deeper understanding of the initiation and progression of human malignancy. miRNA expression has been documented to be upregulated or downregulated in tumours when compared to normal tissue, which supports the idea of their function in carcinogenesis as either oncomirs or tumour suppressors (69). It has been shown that miRNAs implement their effects at numerous stages in the metastatic cascade by encouraging cancer cell adherence, migration, invasion, motility, and angiogenesis (70). In addition, a number of studies have found miRNA expression to be differentially expressed between molecular subtypes of breast cancer (71, 72).

In terms of global miRNA expression analysis in human tissues, the first report by Liu *et al.* (73), in 2004, utilised a miRNA microarray uncovering tissue specific miRNA profiles, with some even exhibiting distinct fetal

versus adult miRNA expression signatures. A large amount of the original work on miRNAs concentrated on their application in tumour tissue. miRNA expression profiles have been utilized to differentiate tumour tissue from neighbouring normal tissue for classification and prognostication purposes (74). miRNAs have also been shown to remain intact in routinely collected, formalin-fixed, paraffin-embedded clinical tissues. As such, archival samples could potentially be used to examine expression profiles.

miRNAs play a critical role in the cell cycle and their expression has been widely reported to be dysregulated in various pathological conditions, not least in carcinogenesis (75). In terms of diagnosis, miRNAs have been shown to display differential expression in a tissue specific manner. Individual miRNAs have been reported to be disease specific, such as miR-486 in lung cancer (76), miR-29b in liver cancer (77), miR-145 in gastric cancer, and miR-21 in breast cancer (78). In terms of prognosis, it was Calin *et al.* (79) who first suggested a correlation with prognostic factors and disease progression with miRNA signatures in Chronic Lymphocytic Leukaemia. There is also potential for miRNA expression to correctly identify poorly differentiated tumours of uncertain histological origin, thereby aiding treatment planning (58). From a therapeutic standpoint, there is currently a major thrust toward exploiting the miRNAs themselves for therapeutic benefit. The realisation that miRNAs can function as either oncomiRs or tumour suppressors has brought the latter into the spotlight as a novel therapeutic tool. Central to this, there is an ongoing need for novel, minimally invasive, sensitive and specific methods of disease diagnosis and for individualised therapeutic regimens to tailor treatment to achieve maximal benefit for patients.

1.2.5 microRNAs and breast cancer

In recent years, there has been a huge amount of interest in miRNAs in the context of breast cancer, both at a circulating and a tissue level. miRNA dysregulation in breast cancer was first reported by Iorio *et al.* (80) in 2005. This study identified a range of miRNAs to be significantly dysregulated in breast tumour tissues when compared to normal healthy tissues. Mattie *et al.* (81) had previously identified miR-195 overexpression in primary breast

cancer tissue while studies by Zhang *et al.* (82) identified expression levels of miR-195 to be significantly elevated in breast tumours compared with normal breast tissues. Interestingly, it was also observed that certain miRNAs were capable of distinguishing between different epithelial subtypes of breast cancer. This was further reported in studies showing certain miRNAs to be associated with Estrogen and Progesterone Receptor status and HER2/neu receptor status (71, 81). In terms of epithelial subtype specificity, differential expression has been reported between Luminal A and Basal-like tumours (83, 84). miRNAs have immense potential as prognostic indicators with regards to stage and grade of disease. The basal subtype of breast cancer, which is hormone receptor negative, presents a specific therapeutic challenge as there are currently no targeted therapies available. Studies by Iorio *et al.* (85) have shown that manipulation of certain miRNAs, e.g. miR-205, can improve breast tumours response to anticancer agents. It is very important for miRNAs to be functionally characterised thereby allowing an insight into their effect on tumour behaviour. In a recent *in vivo* study conducted within this laboratory (86), a potential relationship between circulating and tissue miRNAs in breast cancer was investigated by assessing if changes in circulating levels of miRNAs were also reflected in tumour tissue. The study highlighted distinct roles for miRNAs in the circulation and in the tissue, which may prove valuable in biomarker development. Indeed, miRNA expression at a circulating level has sparked widespread research as there is a great need for novel, minimally invasive, biomarkers for disease, not least in breast cancer.

1.2.6 Circulating microRNAs

1.2.6.1 Biomarker Discovery

Methods currently used in clinical practice for cancer diagnosis commonly involve cancer tissue biopsy and, for breast cancer, mammography is the current gold standard. These methods, however, can be invasive for the patient and mammography has a false positive rate of 8-10% (87). Therefore, there is a need for the identification of biomarkers present in human body fluids such as blood (whole/serum/plasma), urine, and saliva.

Recognition of the potential of miRNAs as biomarkers of disease is evolving. Many characteristics of miRNAs have fuelled the study of these tiny molecules as potential biomarkers of disease. These characteristics include: their rich genetic information content, their accessibility in a variety of samples, e.g. formalin fixed tissue and plasma/serum and indeed, their potential for highly sensitive quantitation. There is an ongoing quest for sensitive, minimally invasive markers that can be manipulated to uncover early neoplastic changes, thereby allowing the detection of cancer at an early stage. There is also a need for effective biomarkers to be used to monitor the progress of patients and their response to treatments. miRNAs have emerged as a potential ideal biomarker thereby capturing the interest of researchers worldwide in recent years. The unique properties of miRNAs, including their relative stability, tissue specificity and the ease with which they are quantified, highlight these molecules as ideal biomarkers.

To date, a large amount of the research has concentrated on tumour tissues but circulating miRNAs offer unique stability and resilience and allow their detection and quantification to be practical. In 2008, Mitchell *et al.* (88), showed that miRNAs are present in human plasma in an extremely stable form which is protected from RNase activity. The study established that miRNAs detected in plasma or serum can operate as circulating biomarkers for the detection of cancer. Heneghan *et al.* (89) identified miR-195 to be considerably elevated in breast cancer patients and not those with other selected tumour types, which supports the hypothesis that certain miRNAs are disease specific. The study demonstrated the remarkably high sensitivity of miR-195 in combination with the general oncomirs let-7a and miR-155 for discriminating breast cancer cases from controls. This study provides evidence to support circulating miR-195 as a potentially unique, non-invasive, breast cancer specific tumour marker.

More recently, the term ‘liquid biopsy’ has been circulating in the clinical research community. The aim of this approach is to eliminate the need for tumour tissue biopsies and provide much more diagnostic applications from a biological fluid. Circulating miRNAs represent ideal liquid biopsies as the

quantities and sequences of miRNAs communicate critical diagnostic and prognostic information (90, 91).

Barriers to the implementation of miRNAs into clinical practice for diagnosis, prognosis and therapy still remain. It is crucial that the methodologies used are standardised and this includes, but is not limited to, sample collection, sample processing, storage conditions, extraction procedures, the use of endogenous controls and, indeed, statistical tools utilised in data analysis. To implement miRNA sequencing into clinical practice, a simple and standardised workflow must be defined for routine biomarker and diagnostic screening tools.

1.2.6.2 microRNA secretion

Despite the great interest in miRNAs in recent years, the exact mode of miRNA secretion is not yet fully elucidated. Several mechanisms have been suggested, such as passive leakage or release through lysed cells, their release in protective extracellular vesicles including exosomes (92), or via conjugation with RNA binding proteins, e.g. High Density Lipoprotein (HDL), Argonaute2 (AGO2), and nucleophosmin1 (NPM1) (35, 93), as depicted in Figure 1.3.

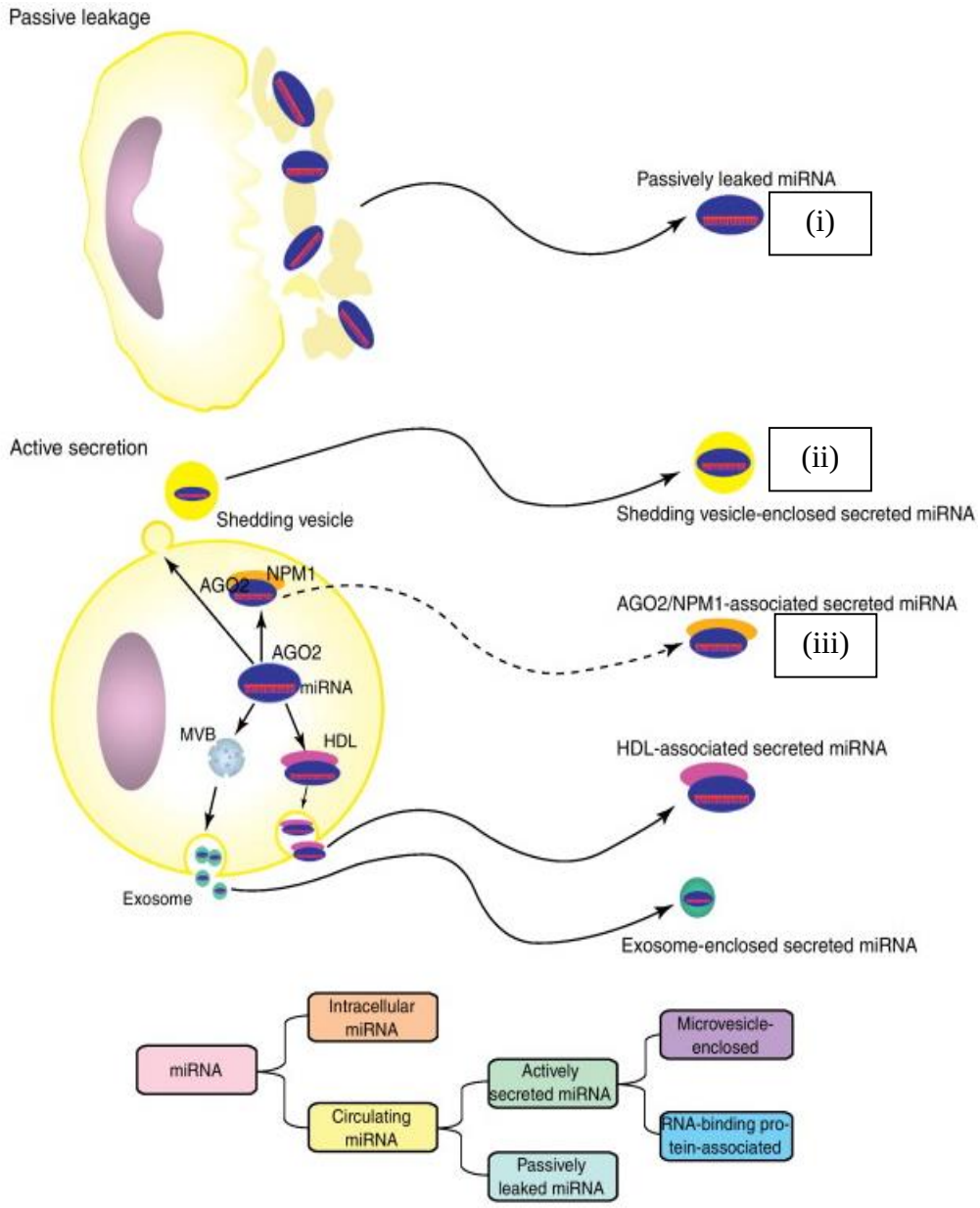


Figure 1.3 miRNAs secretion occers via three mechanisms. (i) passive leakage from lysed cells, (ii) active secretion via extracellular vesicles, and (iii) active secretion bound to various RNA binding proteins e.g. HDL, AGO2 and NPM1 (Adapted from Chen et al (35)).

Following the discovery of miRNAs in the circulation (88), miRNA presence in the serum of patients with diffuse large B-cell lymphoma was described by Lawrie *et al.* (94). Subsequent studies have reported similarly on the presence of miRNAs in the circulation and the potential they exhibit as biomarkers for diseases (75). A study of miR-141 in prostate cancer patients also demonstrated that serum levels of the miRNA could distinguish, with

considerable specificity and sensitivity, patients suffering with cancer, when compared to healthy controls (88). The source of circulating miRNAs is unknown. It is unclear if the miRNAs originate from the diseased tissue itself or as a result of host response to the disease. There are two hypotheses proposed by Slack *et al.* (95):

1. tumour miRNAs are present as a result of tumour cell death and lyses,
2. tumour cells secrete miRNAs into the tumour microenvironment where they enter newly formed blood vessels and, in so doing, make their way into the circulation.

Circulating miRNAs appear to have unlimited possibilities in terms of detecting disease development and recurrence, assessing patient prognosis, and also for predicting response to therapies (96, 97). It has been established that miRNAs are selectively secreted into blood, milk and ductal fluids and that the extracellular and cellular profiles of miRNAs differ, i.e. secreted miRNAs do not necessarily reflect the miRNA content in the cell of origin (98). Secreted miRNAs have been shown to infer stability despite the presence of ubiquitous ribonucleases. This stability can be attributed to two possible mechanisms. First, actively secreted miRNAs via protective vesicles, e.g. exosomes, has been suggested, whereby the membrane structure of the vesicle encapsulates the miRNA providing protection. Secondly, secreted miRNAs associated with RNA binding proteins, such as AGO2 and NPM1, provide a controlled internal microenvironment, thereby protecting the miRNAs from degradation. Regardless of the mechanism of protection, the stability of secreted miRNAs is one of the key characteristics of these tiny molecules which will support their potential introduction as biomarkers of disease. Secreted miRNAs have many other key characteristics: they are a common feature of many cell types, their biological effects can be local or distant, they are delivered without the need for cell-cell contact and, finally, they can deliver multiple messages in one package, thereby regulating numerous target genes simultaneously (99).

1.2.6.3 Non-vesicle associated miRNAs

miRNAs have also been shown to be present in the extra cellular space, although they are not present within protective exosomes, which will be discussed further in this chapter. These miRNAs are termed non-vesicle associated miRNAs. These miRNAs have been shown to bind to a variety of molecules: High Density Lipoprotein (HDL) (100), nucleophosmin 1 (NPM1) (101) and Argonaute2 (Ago2) (102, 103) and, more recently, Ago1 (104). Ago1 and Ago 2 bound miRNAs have been reported to be present in human blood plasma and, further to this, it has been reported that some miRNAs in the plasma do not derive from blood cells under normal conditions (104). In terms of delivery, it has been shown that HDL-associated miRNAs can be delivered to recipient cells whereby they exert their functional targeting capabilities, resulting in altered gene expression (105). HDL cell surface receptors, such as Scavenger Receptor B1 (SR-B1), have been shown to mediate selective uptake of miRNAs to HDL. Furthermore, SR-B1 not only mediates the uptake but also the transfer of these HDL bound miRNAs. SR-B1 mediates transfer by preventing the miRNAs from being delivered into the lysosomal pathway, where the miRNAs would suffer lysosomal degradation. Instead, SR-B1 can divert the miRNAs into the cytoplasm where they will be more stable, resulting in increased functional integrity (105). The specific uptake of miRNAs into particular recipient cells is not yet fully understood. Non-vesicle associated miRNAs, such as protein bound miRNAs, are thought to be taken up via specific receptors on the cell surface, which subsequently bind with cell recognition molecules on recipient cell membranes allowing specificity in uptake. miRNAs associated with vesicles, e.g. exosomal miRNAs, will be discussed in section 1.3.7.

1.2.7 microRNA analysis

Three well established approaches to miRNA profiling are currently in practice. These include: quantitative reverse transcription PCR (RQ-PCR), hybridization based methods (e.g. array platforms) and high throughput next generation sequencing. The most commonly employed approach is RQ-PCR analysis, which was employed in the majority of investigations in this thesis.

This approach involves reverse transcription of miRNA to complementaryDNA (cDNA), followed by real time measurement of the reaction product accumulation. This approach can be scaled up to hundreds of reactions measuring different miRNAs under the same reaction conditions. In order to examine a set of miRNAs of interest, e.g. those associated with metastasis, commercial and customizable plates and cards are available. This approach is widely used as it can be incorporated into relatively small working laboratories and is of a fairly low cost when compared to high throughput next generation sequencing. Hybridization based methods are well established, with high throughput at a fairly low cost. Array based technologies have proved useful in providing 'snapshots' of miRNA profiles from various sample types. This method, however, does not identify novel miRNAs and has been reported to have lower specificity when compared to RQ-PCR and of course RNA sequencing. High throughput next generation sequencing truely represents the 'next generation' of miRNA profiling. This approach allows detection of both novel and known miRNAs with precise identification of miRNA sequences. State-of-the-art systems can readily distinguish between miRNAs that differ by only a single nucleotide. This approach, however, is extremely costly and in addition requires substantial computational support for data analysis.

1.2.8 Therapeutic Potential

It is well established that miRNAs are frequently dysregulated in a wide range of human diseases, including cancers, resulting in their potential as viable targets for the development of miRNA based therapeutics (106-110). As a therapeutic modality, miRNAs confer many advantages. It has been established that most mature miRNA sequences are highly conserved across various vertebrate species. In addition their short sequences infer great stability. As a consequence, these advantages allow miRNAs to be easily targeted therapeutically. As discussed previously, miRNAs typically regulate many targets within a variety of cellular networks, thereby allowing entire pathways in a disease state to be modulated by targeting miRNAs associated with the disease.

A number of major companies in the pharmaceutical industry have been developing methods for miRNA based therapeutics for cancer and other diseases. Depending on whether the aim is up or down regulation, two strategies exist for therapeutic application, which are outlined in Figure 1.4.

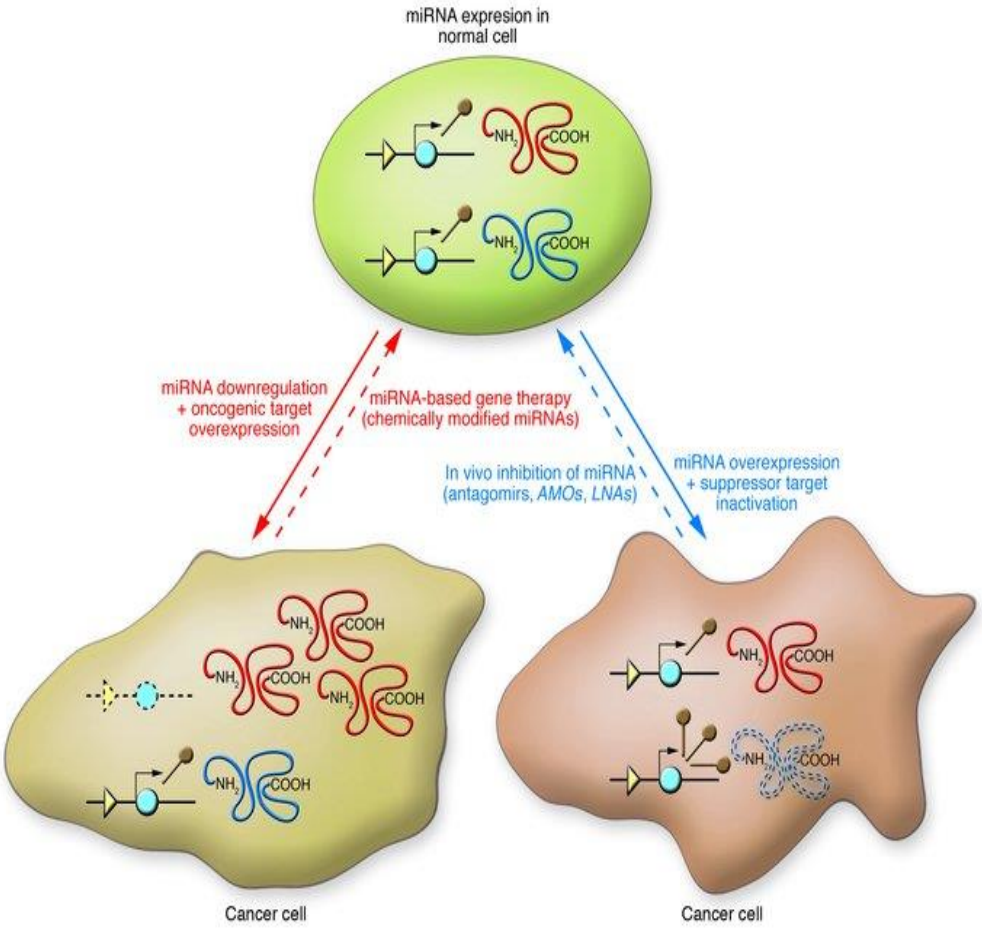


Figure 1.4 miRNA based gene therapy through inhibition or over-expressed miRNAs or by replacement of underexpressed miRNAs (Adapted from Calin and Croce, 2007 (111)).

Through antisense–mediated inhibition of over-expressed miRNAs or by replacement of under-expressed miRNAs with miRNA mimetics or viral vector-encoded miRNAs (53, 112). miRNA function can be inhibited by using antisense oligonucleotides, also known as antimiRs. Mechanisms of miRNA restoration include chemically modified mimics, lenti-viral, adeno-viral and adeno-associated viral methods, which have previously been reported to restore selected miRNAs activity in several studies (113-115).

However, certain challenges are faced with such miRNA replacement methods. One example includes the ability to restore a down-regulated miRNA without introducing supra-physiological levels of the miRNA. Another example includes potential up-take of this miRNA in non-target tissues leading to off-target effects. A major challenge of miRNA-based therapeutics is the safe and efficient delivery of miRNA mimics and antimiRs to the desired cell type or tissue. One suggested approach includes targeted delivery of the miRNA to the specific cell type, which could be achieved via a conjugation mechanism whereby the nucleic acid could link to targeting molecules such as peptides or antibodies. Another approach could enhance cell specific uptake by encapsulation of the miRNA mimic into a lipid based formulation. A novel approach for targeted delivery of miRNAs could employ Mesenchymal Stem Cells (MSCs), which are known for their ability to directly target a tumour site. Therefore, MSCs could potentially be engineered to secrete particular miRNAs, i.e. tumour suppressing miRNAs, which could then be released from the cells via a protective vesicle targeting the tumour site. However, until these mechanisms are refined, device based delivery could be employed, e.g. stents, catheters, or even local injections. If intra-tumoural injections were to be employed, however, perhaps it would be more advantageous to surgically remove the tumour.

As a result of the encouraging miRNA profiling studies conducted worldwide over the last decade, it is not unexpected that miRNAs have begun to slowly filter into human clinical trials. The focus of these studies is in utilising miRNAs signatures as biomarkers for disease with numerous studies currently in progress, such as the ICORG Translational Clinical Trial (ICORG 10-11). In terms of miRNA targeting in the therapeutic setting, an antagonist to miR-122, a miRNA associated with liver cancer, has successfully reached clinical trial as a targeted therapy. Miravirsen, a locked nucleic acid-modified miR-122 antagonist has been successfully investigated in a human phase IIa trial, whereby it was demonstrated to have a safe function (116). Miravirsen is the first miRNA targeting agent to be administered to patients (117).

1.3 Exosomes

1.3.1 Introduction to Exosomes

The first report of nano-sized membraneous vesicles released into biological fluids by viable tumour cells was published over three decades ago in 1979 (118). The term 'Exosome' was coined in 1981 to identify "exfoliated membrane vesicles with 5'-nucleotidase activity' (119). Over the years, these membraneous vesicles have been called by a wide range of terms, resulting in their characterisation to be confusing and subject to disagreement. More recently, however, these vesicles have become defined and standard nomenclature has been accepted. Exosomes are 40-120 nm membranous vesicles derived from the endosome (120). Similarly to miRNAs, exosomes were once thought to serve no function and were considered to be 'cell debris'. It is now, however, realised that these nanosized vesicles play significant signalling roles throughout tumour progression, to include angiogenesis, immune-suppression, invasion and metastasis (121, 122). In addition, extracellular vesicles have been shown to be released by a variety of non-cancerous cells including dendritic cells, macrophages, B cells, T cells and NK cells (123). Exosomes have been shown to contain a vast array of nucleic acids and complex lipid membranes that contain integral proteins, with over 4,000 different proteins identified from purified exosomes (124, 125).

One of the most interesting discoveries was that of Valadi *et al.* (126) in 2007, who reported on the intercellular shuttle of miRNAs via exosomes, whereby the miRNAs are packaged into the protective vesicles and transported to a new cell type. This recently recognised mode of transport allows delivery of genetic information and cell-to-cell communication. This exchange of miRNA is a novel dimension to the regulation of a cell's phenotype and is particularly important in cancer. More recently, the protective function of exosomes has been further realised whereby the miRNAs are protected from hostile ribonuclease-rich environments and are transported into recipient cells to exert their functions (127-129). Exosomes are secreted by most cell types, and are abundantly present in body fluids such as blood, ascites, urine, milk, saliva, and seminal plasma. Exosomes have been suggested to have many

distinct and far-reaching physiological functions, depending on their cellular origin, from immune regulation, to cell migration and differentiation, and other aspects of cell-to-cell communication. Exosomes have also been implicated in the pathogenesis of disease such as tumour development and in cardiovascular and neurodegenerative disease. Exosomes are known to alter the cellular phenotype of recipient cells and, in terms of breast cancer, they play critical roles in growth and metastasis (130, 131). Due to their endosomal origin, exosomes have very unique lipid and protein compositions, including membrane transport and fusion proteins, tetraspannins, heat shock proteins, and lipid related proteins and phospholipases (131-133).

The realization that exosomes may bring about epigenetic alterations by transferring selected RNA molecules between cells has revolutionized our thinking with regards to exosomal signaling. Now that exosomal transfer of miRNAs has been demonstrated, a major future challenge will be to reveal the functional relevance of this process and its impact in the clinical setting. The determination of precisely which miRNAs are being selectively packaged into the exosomes, and what effect they are having in their new location, is poorly understood and requires detailed research.

1.3.1.1 Microvesicles versus Exosomes

A range of membrane vesicles are secreted from almost every cell type and it is therefore crucial to first distinguish smaller extracellular vesicles from other larger membrane vesicles (0.5-3 μm), such as apoptotic bodies. Apoptotic bodies are released when the cell is challenged by apoptotic and death signals but, despite morphological and structural similarities to extracellular vesicles, they can be easily distinguished based on size, cellular origin and composition (134). Moreover, a differentiation must be made within the smaller extracellular vesicles. There are currently at least two distinct types of small extracellular vesicles recognised - namely microvesicles and exosomes (135). These vesicles are distinguished and characterised based on their particle size, composition, and mechanism of biogenesis. Briefly, microvesicles are formed via outward budding of the plasma membrane and are approximately 100 nm to 1 μm in diameter (136),

while exosomes are formed via inward budding of the plasma membrane, and are approximately 40 nm to 120 nm in diameter (137) (Table 1.3).

	Exosomes	Microvesicles
Size	40-100 nm	100 nm-1 µm
Shape	Oval/cup-shaped	Irregular
Membrane Proteins	CD63, CD9, CD61	CD73, Integrins, Selectins
Luminal Proteins	Cytokines, annexins	Caspases, annexins, growth factors
Lipids	Ceramides, cholesterol	Cholesterol
Mechanism of Generation	Fusion and inward budding of plasma membrane	Fusion and outward budding of plasma membrane
Mechanism of Release	Exocytosis of MVB	Plasma membrane budding
Mechanism of sorting	Ceramide dependent	Unknown

Table 1.3 Comparison of composition and biogenesis of exosomes and microvesicles.

To date, however, this nomenclature and certainty of distinction between the two has not been uniformly adhered to by the wider research community (138).

1.3.2 Exosome formation

To date, the exact mechanism of exosome formation remains incompletely understood. The currently accepted model proposes that exosomes are formed within the endocytic pathway and are released via inward budding of the plasma membrane in multivesicular bodies (MVBs) (139). The maturation of early endosomes into late endosomes results in the formation of MVBs (Figure 1.5(i)), which are characterised by the presence of vesicles in the lumen, i.e. exosomes (140). When MVBs are mature, they can go either one

of two ways. They can fuse with the lysosome and their contents will undergo lysosome degradation (Figure 1.5 (iii.b)) or they can fuse with the plasma membrane (Figure 1.5 (iiia)) and their contents can be released into the extracellular environment (Figure 1.5 (iv)).

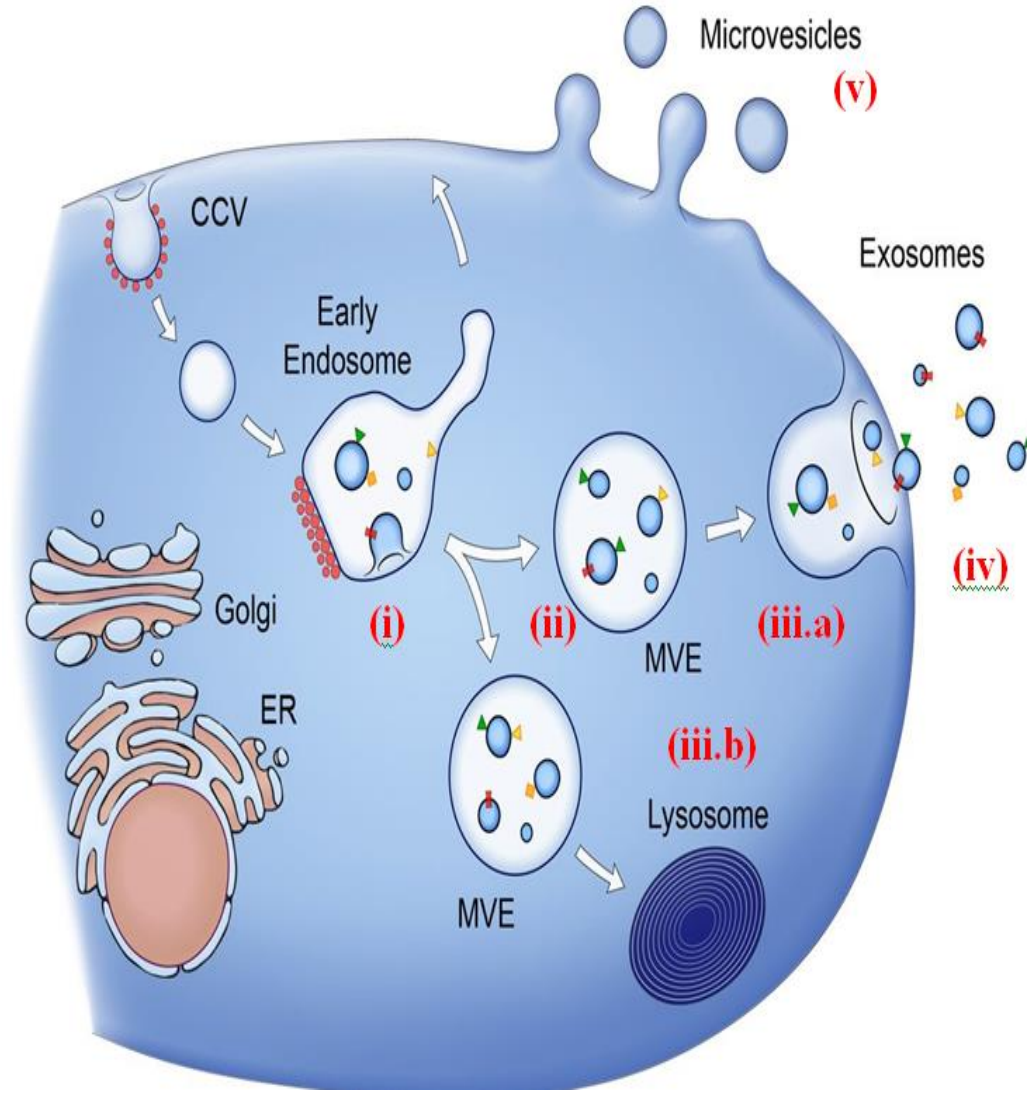


Figure 1.5 The formation of exosomes. (i) via budding into early endosomes and (ii) release of multivesicular bodies (MVEs) followed by (iii.a) fusion with the plasma membrane or by (iii.b) fusion with lysosomes. Exosomes are then released (iv) via inward budding of the plasma membrane, while microvesicles are released (v) via outward budding. (Adapted from Raposo and Stoorvegel, 2013 (120)).

1.3.3 Exosome isolation techniques

Exosomes can be isolated and purified from a wide variety of biological sample types. The most commonly employed methods are Differential Ultracentrifugation and Sucrose Density Gradient Centrifugation (141). Other methods include Ultrafiltration and High Performace Liquid Chromatography (HPLC) based processes (142). For more applied analysis, Free Flow Electrophoresis (FFE) allows isolation of highly purified exosomes in their native state. These methods may support elucidation of the biological functions of specific proteins (143, 144). With the rapid growth in exosome analysis in recent years, it is no surprise that new methods for exosome isolation are being rapidly developed. These include commercially available kits which utilise Affinity Purification methods for the specific isolation of exosomes. These kits employ 96-well plates which contain anti-CD63, CD81 and CD9 antibodies and utilise affinity capture that target specific antibodies on the surface of the exosomes (145). The current gold standard, however, for exosome isolation continues to be the traditional method of differential ultracentrifugation. This method employs multiple sequential centrifugations at increasing speed in order to isolate smaller and less dense components in the subsequent steps. A micron filtration step eliminates the larger vesicles (e.g. apoptotic bodies), while microvesicles can be isolated following centrifugation at ~10-20,000 x g, and exosome isolation is achieved at >100,000 x g (141). Regardless of the method employed, it is important to note that, when isolating exosomes from cell lines, it is vital to avoid contamination from exosomes present in Fetal Bovine Serum (FBS). This can be achieved by either depriving the cells of FBS or by supplementation with exosome-depleted FBS. Exosome-depleted FBS is available to purchase from a variety of commercial providers, although it is very costly. Alternatively, FBS can be depleted of all exosomes by ultracentrifugation overnight (141).

1.3.4 Exosome Characterisation

Analysis of extracellular vesicles requires characterisation of the purified fraction in order to determine the type of extracellular vesicle isolated. Characterisation of extracellular vesicles has faced challenges in the past due to their small size and the inability to characterise at a single particle level. Single particle analysis would reveal a wealth of information based on size, concentration, charge, sub-cellular origin and, moreover, potential function (146). Currently employed methods for characterisation, however, rely heavily on morphological analysis (Transmission Electron Microscopy) and detection of surface protein markers (Western Blot). Other, less widely used methods include flow cytometry, scattering and fluorescence flow cytometry and scanning electron microscopy. TEM is perhaps the most widely used method for morphological analysis. This method transmits a beam of electrons through a thin specimen and then focuses the electrons to create an image of high resolution. As TEM is performed in a vacuum, it is necessary to fix, dehydrate and embed the sample. TEM can also be performed in conjunction with immune-gold labelling which would provide biochemical information regarding the vesicles cell surface. Further validation of purified exosome fractions can be obtained using Western Blot, targeting the exosome-associated proteins. Currently, there are no exosome-specific markers available on the market. Therefore, proteins that are enriched in exosomes arising from all different cellular origins are commonly used to confirm the presence of exosomes. It has also been shown that a majority of proteins present in exosomes are ubiquitinated (147). As a result, it has been suggested that proteins which enter the endocytic network via ubiquitination are preferential protein targets as they are more likely to be present in exosomes than in non-ubiquitinated proteins. A group of membrane bound proteins found on the plasma and endosomal membrane, known as tetraspanins (CD9, CD63, CD81 and CD82), are commonly enriched on exosomes (123, 148).

1.3.5 Exosome Composition

The content of extracellular vesicles, including exosomes, has been extensively investigated using a variety of techniques including Western Blot, fluorescence activated cell sorting, immune-electron microscopy, and mass spectrometry (149). The protein and RNA composition varies depending on the parent cell, however, as conserved proteins exist among extracellular vesicles from different cellular origins (150). Exocarta is a manually curated database of exosomal proteins, RNA, and lipids. As of July 2014, this catalogue lists 13,333 proteins, 2,375 mRNAs, 764 miRNAs and 194 lipids found within or associated with exosomes. It has been well established that the molecular composition of exosomes can be a reflection of its parent cell and their endosomal origin (130, 151). However, there is more to be ascertained from their composition. Specific proteins, lipids and RNAs are found to be enriched in exosomes, although others are absent (152). Other molecular components of extracellular vesicles include: cytoskeleton proteins (e.g. ezrin and actin), proteins associated with MVB biogenesis (e.g. alix and TSG101), membrane transport and fusion proteins (e.g. annexins and Rab proteins), and tetraspanins (e.g. CD9, CD63 and CD81). Using a method of deep sequencing, Nolte *et al.* (153) discovered that cellular and shuttle RNA can contain vastly different small RNAs which have originated from the same non-coding RNA. The unequal distribution between cellular and shuttle RNA, combined with emerging evidence for critical roles in gene regulation, leads to a conclusion that cells selectively release specific RNAs to alter function in the target cell location. This method of sorting the content of exosomes is undoubtedly controlled through a variety of pathways, although the majority still remain unclear and require further research. However, what is known, is that the composition of exosomes will ultimately determine the outcome of the communication, with several recent studies showing small RNA species, including miRNAs, to be functional in their new recipient cell location (154-156).

1.3.6 Exosomes in cell-to-cell communication

One of the hottest topics in bio-medical research at the moment is the role of exosomes in intercellular communication. Multi-cellular organisms have a complex network of cellular activity and cell-to-cell communication is pivotal to co-ordinating this activity. Traditional methods of cell-to-cell communication are considered to include cell junctions, adhesion contacts, and soluble messengers (157). However, in recent years, a new form of intercellular communication has been identified. Horizontal transfer of secreted miRNAs via exosomes is a recently realised mechanism of intercellular communication (158). Indeed, miRNAs secreted by donor cells can exert their effects and influence gene expression in the recipient cells. The current proposed model for this iontercellular communication is outlined in Figure 1.6, whereby miRNAs are packaged into interluminal vesicles, which then fuse with the plasma membrane, allowing secretion of the vesicles into the extracellular space. Through binding to a target cell and fusion with the plasma membrane, the exosomal mIRNAs are delivered to the cytosol of the target cell, where they may associate with and silence the corresponding mRNA (156).

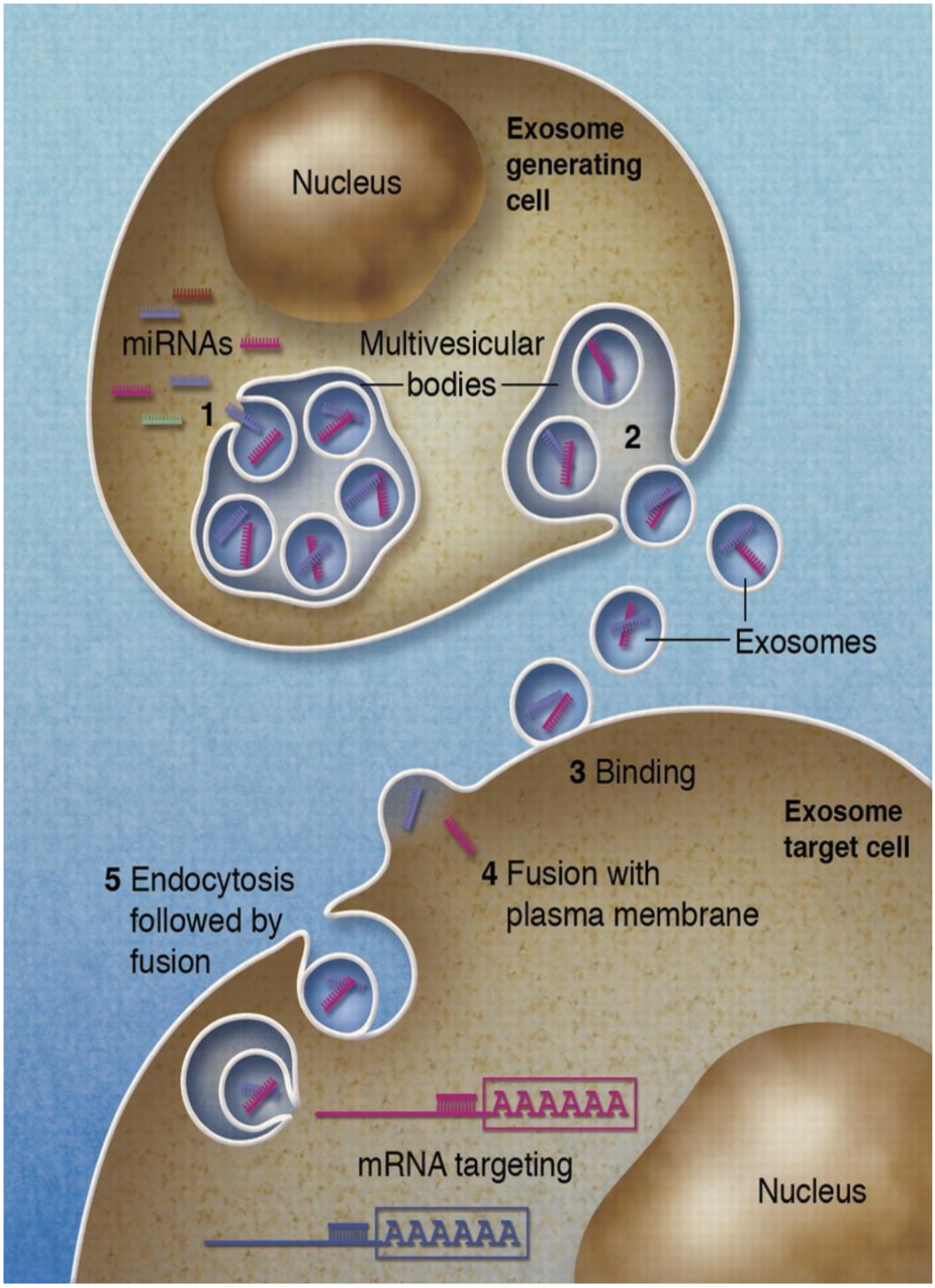


Figure 1.6 Exosome-mediated horizontal transfer of miRNAs between two cell populations (Adapted from Stoovogel *et al.* (159)).

Initially, there was much scepticism surrounding the presence of RNA in exosomes, with some alluding to its presence as merely incidental. However, numerous studies have since demonstrated the specificity of the content of these delivery vehicles (160, 161). Mittelbrunn *et al.* (162) reported that

miRNAs transferred during immune synapsis are able to modulate gene expression in recipient cells. The immune synapse is a method communication used between T cells and antigen-resenting cells (APCs) during antigen recognition. Exosome-mediated transfer of miRNAs between cells allows dissemination of genetically encoded messages, which could alter the function of target cells. The ability of exosomes to affect and modulate gene expression in recipient cells has been realised, although the extent to which the exosomal-miRNAs account for this impact is only just being revealed. A recent study revealed that exosomes from triple negative breast cancer cells can transfer phenotypic traits to recipient cells, which reflect the characteristics of the parent cell (163). Pro-cancer effects of exosome-mediated intercellular communication must also be discussed. One such effect is an exosome's ability to mediate resistance to immunotherapeutic agents. Several studies suggest exosomes interfere with trastuzumab uptake by breast cancer cells, thereby decreasing the drugs efficacy (164-166). HER2 on the surfaces of breast cancer exosomes has been reported to bind and sequester the trastuzumab, in so doing allowing continued tumour cell proliferation (165). As discussed previously, the exact secretory mechanism of miRNAs remains unclear, with several hypotheses proposed varying from passive to active secretion. In addition, the biological impact of this horizontal transfer has not yet been fully elucidated.

1.3.7 Exosomal microRNAs

miRNA packaging into exosomes has been shown to be a non-random, selective process, whereby specific miRNAs are preferentially sorted and packaged (161). In a study by Zhang *et al.* (167), evidence showed that blood cells and cultured THP-1 cells actively and selectively package miRNAs into exosomes which are then secreted in response to various stimuli (167). Interestingly, Trajkovic *et al.* (168), discovered a ceraminde-dependent secretory mechanism which induces endosome sorting in the exocytic MVBs, which in turn, regulate the release of exosomal miRNAs from HEK293 cells (93). The selective nature of packaging particular miRNAs into exosomes is not yet fully understood, although several mechanisms have been proposed. Within exosomes, certain components of the RNA Induced Silencing

Complex (RISC), such as AGO2 and GW182 have been detected (129). As a consequence, it could be proposed that RISC is involved in the packaging process where certain miRNAs are chosen, based on their need to exert functions in distant cells. In addition, an alternative suggestion for the selective packaging could involve the ceramide dependent pathway which controls packaging, but represses sorting in to HDL particles. As a result, nSMase2 may be critical in the sorting of miRNAs in to exosomes (35). Deep sequencing studies have revealed that the relative amounts of secreted miRNAs in exosomes are significantly lower than those of cellular origin, although the effects of secreted miRNAs are far reaching (153). While it has been established that miRNAs are sorted and packaged into exosomes and are then released into the extracellular space, further research is required on what exactly the functions of these secreted miRNAs are and indeed how they execute their functions i.e. their uptake in recipient cells. Only a few published reports have investigated this. In 2009, Keller *et al.* (169), investigated exosomes present in ascites and blood of ovarian carcinoma patients, where systemic application of patient derived exosomes were found to promote growth of human xenografted SKOV3ip carcinoma in mice. In a study by Xiao *et al.* (170), it was shown that THP-1 cell-derived microvesicles contained abundant miR-150, which were then delivered into the human microvascular cell line (HMEC-1). This resulted in suppression of the transcription factor c-Myb in the recipient cells, which in turn enhanced the migration capacity of the cells (167). In another study, exosomes enriched with miR-16 were intratumourally injected into nude mice implanted with PC-3M-Fluc/RLUC-bcl2 3'UTR cells (prostate cancer). The data showed that exosomal miR-16 exerted its inhibitory ability on its target gene in the recipient cells (171). With all of this mounting evidence, it is clear that miRNAs play a pivotal role in the systemic signal network. However, the exact molecular mechanism of how these exosomal miRNAs are taken up in recipient cells and whether they target specific cells, still remains undefined. This is a very important question as the mode of uptake and cell specificity will undoubtedly influence the functional role of the delivered miRNAs. It has been suggested that when exosomal miRNAs reach their recipient cell, they are taken up by endocytosis, phagocytosis or direct fusion with the

plasma membranes (157). Cell recognition molecules on the surface of the exosomes could perhaps recognise specific receptors on recipient cell surfaces, thereby allowing cell specific uptake.

1.3.8 Therapeutic Potential

It has been established that exosomes are efficient mediators in intercellular communication. As a result of their internal cargo and ability to cross biological membranes, exosomes represent ideal delivery systems which can be manipulated for the transfer of miRNAs. Exosomes are naturally taken up by cells, and the potential to engineer the miRNA content of exosomes represents an ideal approach for improved cancer therapy. However, in order for the exosomal miRNAs to be truely effective, a greater understanding of the mechanism of sorting miRNAs into exosomes and, indeed a targeted delivery approach is certainly required. miRNA replacement therapies are emerging as promising therapeutic strategies for cancer, however their clinical implementation has been hampered by a lack of suitable delivery systems. It is well established that Mesenchymal Stem Cells (MSCs) can migrate directly to cancer sites and therefore may represent a suitable mechanism for functional delivery. Munoz *et al.* (172) recently reported on the successful delivery of anti-miR-9 via MSC-derived exosomes to glioblastoma cells, resulting in significantly increased drug efficacy. Further to this, Katakowski *et al.* (173), tested whether MSC exosomes could be used as a vehicle for delivery of anti-tumour miRNAs. The authors engineered MSCs to secrete exosomes containing miR-146b, a known anti-glioma miRNA. Glioma bearing rats were subsequently treated with these miR-146b exosomes which resulted in significantly smaller tumour volumes. The authors further investigated this in an *in vitro* model which revealed slowed glioma growth and also inhibited putative miR-146b targets. However, in a recent study by Xiao *et al.* (174), it was reported that miRNAs delivered via exosomes were potentially responsible for the decreased sensitivity of lung cancer cells to the widely used chemotherapy drug, cisplatin.

With regards to breast cancer, radiotherapy requires oxygen, although ~40% of all breast cancers have hypoxic tumour microenvironments (175). As a result, the tumour cells are significantly more resistant to irradiation. The

ability to assess tumour hypoxia in breast cancer has gained much attention in recent years. Interestingly, it has been shown that the proteomic profiles of tumour-derived exosomes are indicative of the oxygenation status of patient tumours (176). Furthermore, studies have shown breast cancer cells release greater levels of exosomes when exposed to hypoxia, revealing important implications with regards to tumour cells signalling surrounding tissue in the the tumour microenvironment (177).

By exploiting the fact that exosomes are natural carriers of miRNAs, new strategies for the development of novel delivery systems in gene therapy exist. However, further understanding of the complexity of the cargo contained within the exosomes is required as some components may infer unwanted effects. In terms of breast cancer, the true therapeutic potential of exosomal miRNAs has not yet been thoroughly investigated, and further research is necessary before implementation into clinical practice.

1.4 Thesis Aims

The aims of the research in this thesis were as follows:

- To validate the expression of a range of previously identified miRNAS in patient breast tumour tissues and compare them to normal controls.
- To determine the impact of menstrual status on circulating miRNA expression.
- To characterise miRNA secretion from cell-conditioned media and to identify the optimal method for miRNA isolation.
- To isolate and characterise the exosome fraction from within cell-conditioned media and to investigate selective packaging of miRNAs into exosomes.
- To identify the full exosome-encapsulated miRNA secretion profile from a range of breast cancer cell lines and to validate a selection of identified targets using RQ-PCR.
- To investigate enrichment of a particular miRNA into exosomes by engineered overexpression in the secreting cell population.
- To visualise the transfer and uptake of exosomes into a new cell population and to determine any effects once transferred in terms of miRNA expression, cell proliferation and migration.
- To validate miR-504 dysregulation in patient breast tumour tissues and to investigate the impact of miR-504 overexpression *in vitro* and *in vivo.*

Chapter 2

Materials & Methods

2 Chapter 2: Materials and Methods

2.1 Cell Culture

2.1.1 Overview of Cell Culture

Within the Discipline of Surgery research laboratory, there is a dedicated cell culture laboratory located away from the main research laboratory in order to prevent contamination. Upon entering the cell culture laboratory it is essential to use designated red collar laboratory coats which are never worn outside of the culture lab. The cell culture laboratory contains all the necessary equipment required for culture experiments, including 2x Laminar Flow Hoods (LAF) (one dedicated for cell lines and one for primary culture), 4x High Efficiency Particulate Air Filter (HEPA) incubators, bench top centrifuges and microscopes. Cell culture refers to the process by which cells are grown under controlled laboratory conditions i.e. *in vitro*. This refers to the culturing of cells derived from multi-cellular eukaryotes, particularly animal cells. Cell cultures can be described based on two parameters:

1. The origin of the cells (primary, extended or transformed), and
2. The manner of growth (adherent or suspension).

There are numerous cell lines that are well established as representative of particular cell types. The advantages of using cell lines as cancer models are that they are easy to grow, easy to genetically manipulate, and apply functional assays. However, multiple variants of the same cell line exist throughout the scientific community. These have been shown to display distinct phenotypes indicating acquired genetic changes, which would impact results arising from separate studies to be compared (178).

Cells can be cultured in either suspension or adherent methods of culture. Cell lines were originally purchased from the American Type Culture Collection (ATCC). However, any cell lines which were more than 2 years since purchase were subjected to Short Tandem Repeat (STR) DNA profiling (LGC Standards, United Kingdom) to ensure their authenticity. STR profiling identifies hyper-variable regions within genomes of human cell lines derived from the tissue of a single individual, thereby ensuring the cell lines have not been misidentified or cross-contaminated. The adherent method, also known as monolayer cell culture, was used in all experiments. This is the most

commonly used technique which involves cells growing attached to the surface of a culture vessel or flask. The yield of cells is limited by the available surface area to which the cells may attach, i.e. T25cm^3, T75cm^3, and T175cm^3 flasks. A major advantage of adherent cell cultures is the ready accessibility for direct microscopic examination to inspect the morphology of the cells as they grow and also to investigate any potential contamination or mutations. Disadvantages of adherent cell cultures include the requriemnt of periodic passaging, in addition cell growth is limited by cell surface area which may limit product yields. Cell line models have been widely used for research on the molecular biology of breast cancer and for identification of novel biomarkers and drug targets prior to clinical studies.

2.1.2 Asepsis in Cell Culture

Asepsis refers to the state of being free from disease-causing contaminants, for example bacteria, viruses and parasites. The term asepsis is often used in reference to the practices used to promote or induce asepsis in cell culture laboratories. It is crucial to observe and maintain strict aseptic technique when performing cell culture procedures. This includes spraying all surfaces, including gloves, equipment and consumables with 70% Industrial Methylated Spirits (IMS), before and after use. Prior to using the LAF hoods, they are thoroughly cleaned with 70% IMS and left to air dry for at least 20 minutes. When undertaking cell culture procedures in the LAF hoods, as a general rule clean consumables are kept to the right hand side, while used equipment and waste are kept to the left. Biological waste including spent media was treated by adding Virkon (Fisher Scientific) powder directly to the spent media for a minimum of 3 hours and then disposed of by pouring down the drain with flowing water. The cell culture laboratory was thoroughly cleaned on a weekly basis including replacing water baths with sterile water, a full clean of inside of LAF hoods, and full re-stock of all consumables.

2.1.3 Immortalised Breast Cancer Cell Lines

The following human breast cancer cell lines were purchased from the American Type Culture Collection (ATCC): T47D, SKBR-3, MDA-MB-231, BT-20, MCF-7, MCF10-2A, and MCF12A (Table 2.1). The cells were routinely maintained in RPMI-160 (T47D), Mc Coys 5A (SKBR-3), Leibovitz-15 (MDA-MB-231), and EMEM (BT-20, MCF-7) media. Each media type was supplemented with 10% FBS and 100 IU/Ml Penicillin G/100 mg/mL Streptomycin sulphate (Pen/Strep). MCF10-2A and MCF12A were cultured in DMEM + HamsF12 medium supplemented with 5% Horse Serum, 20 ng/mL Epidermal Growth Factor, 100 ng/mL Cholera Toxin, 0.01 mg/mL Bovine Insulin and 500 ng/mL Hydrocortisone. All media and supplements were purchased from Gibco®. All cells were maintained at 37°C, 5% CO_2, with a media change three times weekly and passage every seven days.

Cell Line	Characteristics	Breast Cancer Subtype
T47D	ER+, PR+, Her2-	Luminal A
SKBR-3	ER-, PR-, Her2+	Her2 Overexpressing
MDA-MB-231	ER-, PR-, Her2-	Basal
BT-20	ER-, PR-, Her2-	Basal
MCF-7	ER+, PR+, Her2-	Luminal A
MCF10-2A	Non-tumourigenic cells	Normal breast-like
MCF12A	Non-tumourigenic cells	Normal breast-like

Table 2.1 Breast cancer cell lines used, with details of receptor characteristics and subtype classification (table prepared by the author).

2.1.4 Feeding cells

Spent media was removed every 2-3 days and replaced with fresh complete media. It was essential to preheat the media to 37°C prior to feeding the cells. A consistent media composition was maintained with no rapid protocol changes as this could potentially introduce phenotypic changes in the cells. As cells require moisture, it was therefore important not to leave the cells uncovered for long periods of time when exchanging liquids, i.e. feeding the

cells. Depending on the size of culture flask used, varying amounts of culture media were added, e.g. 8 mL of media for T25cm^3 flasks, 12 mL of media for T75cm^3 flasks, and 25mL of media for T175cm^3 flasks. All flasks used in this research had vent caps to allow efficient gas exchange and were purchased from Sarstedt.

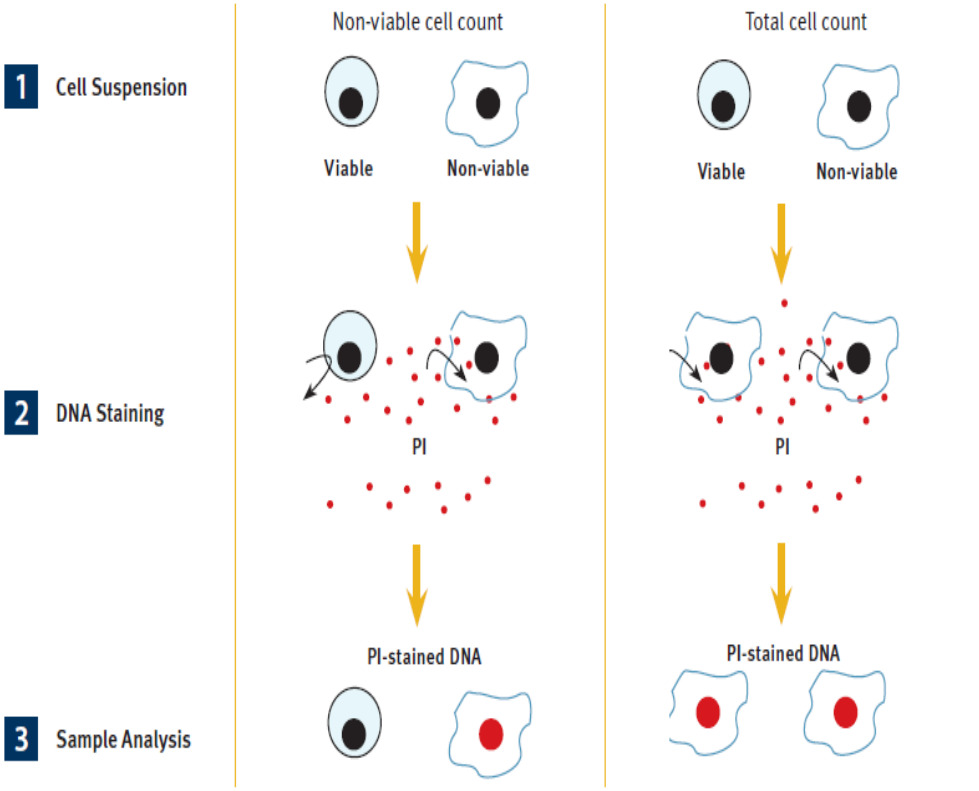
2.1.5 Subculturing

Subculturing, also known as passaging, involves the dissociation of the cells from each other and from the substrate, to generate a single cell suspension which can then be quantified. This process of cell culturing involves the removal of the medium and transfer of cells from a previous culture into a fresh growth medium to enable further propagation of the cell line. When the cultures occupy all the available substrate, i.e. 100% confluency, there is no room left for expansion or growth. Therefore, cell proliferation is greatly reduced or ceases entirely. To keep them at an optimal density for continued growth and to stimulate further proliferation, the cultures were divided and fresh medium supplied. Trypsin in the presence of EDTA (Gibco®) (0.25%, 1mM EDTA) is used to subculture cells. Trypsin is a highly active, relatively non-specific, economic protease derived from the pancreas. Trypsin cleaves the proteins on the cell surface and extracellular matrix, thereby removing any adhesion molecules. EDTA is used to enhance the activity of the trypsin. Prior to treating the cells with trypsin, the cells were washed with sterile Phospate Buffered Saline (PBS) (Gibco®) which removed any traces of serum, which is a trypsin inhibitor. The PBS used was Ca^{2+}/Mg^{2+} free as this reduces the concentration of divalent cations and proteins that inhibit trypsin action.

2.1.6 Cell Counting

Conventional methods of cell counting involve the use of a hemacytometer and microscope, which can be time consuming, labour intensive, and sometimes unreliable due to operator subjectivity. The cell culture laboratory at the Discipline of Surgery is equipped with an automated Nucleocounter® (Chemometec) which adds precision and reliability to all cell counts. The cell counter incorporates a miniaturized low-power (1x) transmitted light

fluorescence microscope illuminated by 8 green LEDs and a CCD camera for determining cell viability and number. The counter uses disposable cartridges which are internally coated with propidium iodide (PI). A lysis buffer is mixed with the cell suspension to produce free nuclei which is then loaded into the nucleocasette (Figure 2.1).



Plasma membrane	Intact	Disrupted	Disrupted	Disrupted
Cell count	1		2	
Characteristics	Non-viable cell count		Total cell count	

Figure 2.1 Staining of cell DNA with Propidium Iodide. Adapted from the NucleoCounter™ NC-100® Operator Manual.

Using automated software, the system calculates the total cell number. The total number of dead or non-viable cells is counted by directly loading a sample of the cell suspension without the addition of the lysis buffer. The non-viable cell count is then subtracted from the total cell count which provides the viable cell number in the cell suspension. This method of cell

counting is very quick and reliable thereby increasing the accuracy of the count.

2.1.7 Cryopreservation of Cells

Cells were cryopreserved to maintain stocks, to avoid loss by contamination, to minimize genetic change in continuous cell lines and to avoid aging and transformation in finite cell lines. Before cryopreservation, cells were characterized and checked for contamination microscopically. Cultured cells were washed with sterile PBS, trypsinised into a single cell suspension and counted. A cryopreservative, 5% dimethylated sulphoxide (DMSO) (Sigma-Aldrich®), was used to protect the cells from the stress of the freeze-thaw process, to reduce crystal formation and resulting cell lysis. 2 mL Nunc Cryovials (Sarstedt) were appropriately labelled and were placed on ice prior to addition of the cell suspension as DMSO is toxic to cells at 37°C. The required number of cells in the presence of DMSO was immediately transferred to a 'Mr Frosty' (Nalgene) container and placed in the -80°C freezer for a minimum of 3 hours. The Mr Frosty freezing container consists of an isopropyl alcohol bath which controls the freezing of biological samples at the recommended controlled rate of cooling (-1°C per minute) when placed in a -80°C freezer.

2.1.8 Recovery of Cryopreserved Cells

Required cells were recovered by rapid thawing of cryovials in a 37°C water bath and immediate re-suspension in pre-warmed appropriate culture medium in the required size flask. During recovery from a cryopreserved state, cells are especially sensitive to mechanical disruption. Therefore, vigorous trituration, centrifugation, and other physical stresses were avoided/minimized.

2.2 Primary Cell Culture

2.2.1 Primary Cell Culture Overview

Primary cultures are derived directly from patient tumour samples. It has been widely accepted that this is a viable alternative or complementary route to be used alongside the culture of immortalised breast cancer cell lines. Primary culture has a number of advantages over traditional cell line cultures as it offers a more relevant clinical model of the disease, which will ultimately provide more meaningful data. Also, a detailed pathology is available of the original tumour allowing comparison to the characteristics of the culture. The most commonly used technique for isolating cell populations, and the method employed by the Discipline of Surgery, NUI Galway, relies upon the differing sedimentation rates for cells of different size and uses a differential centrifugation technique for cell separation. Prior to this, Collagenase III (Sigma-Aldrich®) is used as a digestion tool for enzymatic dispersal of tissue fragments, which was first described by Emerman and Wilkinson in 1990 (179).

An Organoid fraction often arises from outgrowths of cells from small fragments of partially digested tissue. This is a heterogenous population, therefore precautions must be taken to ensure that fibroblasts do not outgrow the epithelial cells. In order to avoid this, a well defined basal media which is not supplemented with serum is used. The organoid fraction is not used as a source of epithelial cells as it has been reported that tumour organoids give rise to rapidly proliferating epithelial cells which are mostly genetically normal (180, 181). The Epithelial fraction is made up of mostly single cells. These cells display the classical cobblestone morphology observed in epithelial cells and can be expanded preferentially and then characterised using a variety of techniques. The Stromal fraction mostly consists of fibroblasts which have a bipolar spindle shape and can also be characterised by a number of immunohistochemical, biochemical and molecular biology techniques. An overview of of the primary cell culture protocol is presented in Figure 2.2.

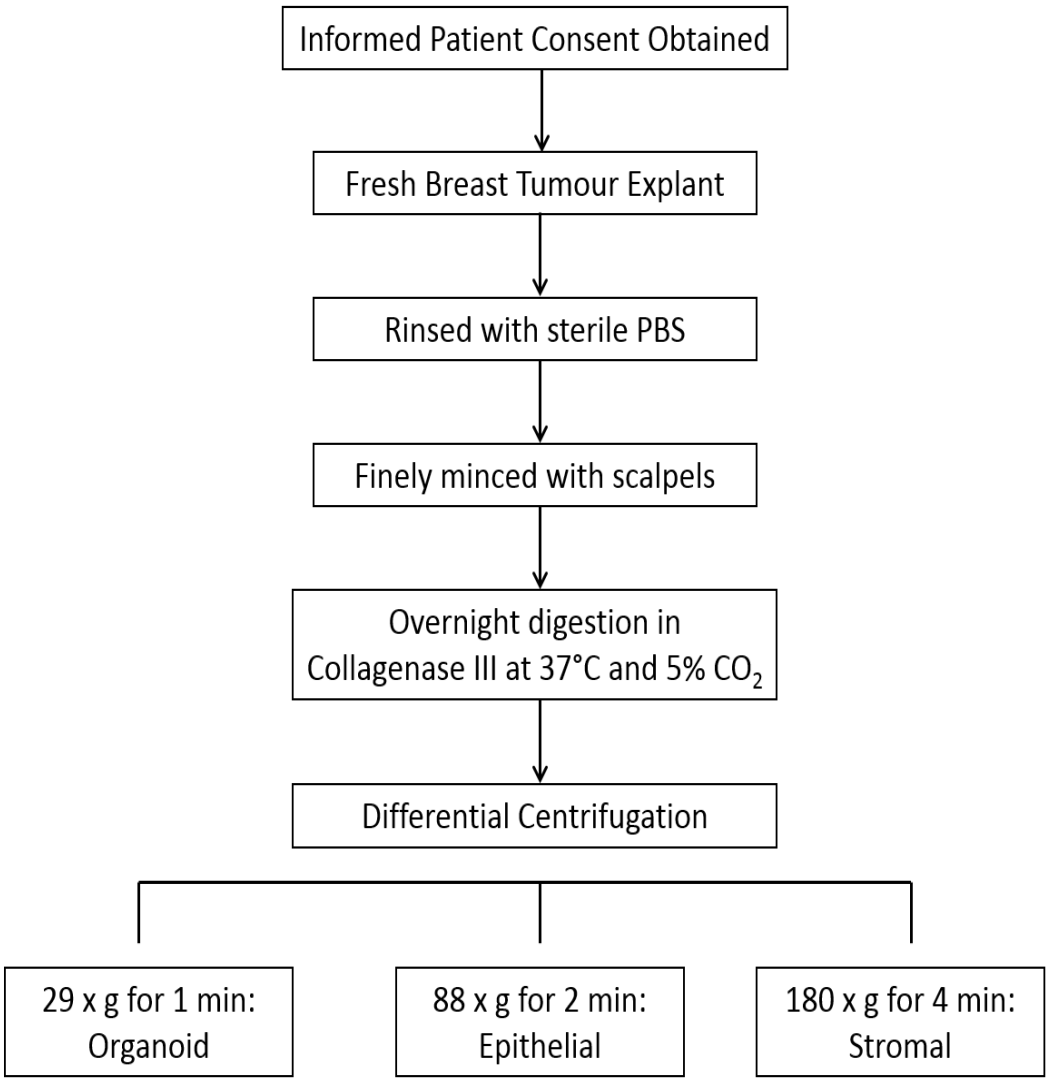


Figure 2.2 Overview of workflow for Primary Cell Culture Protocol. (Adapted from Speirs *et al.* (180)).

With the increasing ethical pressure placed upon scientists to reduce or eliminate the use of animals in experimental research, the use of primary cell culture certainly provides a partial substitute as a clinically relevant model of aspects of the disease. However, it is not without its drawbacks and limitations. These include slow doubling times, finite lifespans, and possible contamination by normal epithelial cells due to the heterogeneity of the sample.

2.2.2 Primary Cell Culture Protocol

Following informed patient consent and approval from the relevant ethics committee (see appendix 9.1), fresh breast tissue samples from surgical tumour resection were harvested in theatre and placed into sterile tubes containing 2 mL of fresh Dulbeccos's Modified Eagle's Medium (DMEM) supplemented with 200 IU/mL Penicillin/200 mg/mL Steptomycin (Pen/Strep). Breast tumour tissues were obtained during surgical resection, while Tumour Associated Normal (TAN) tissues were taken at least 2 cm away from the primary tumour site. The tissues were brought to the cell culture laboratory and placed in the incubators with the caps loose to allow gaseous exchange for a period no more than 2 hours.

The media was removed using a transfer pipette and the tissue was rinsed twice in sterile Phosphate Buffered Saline (PBS) supplemented with 100 IU/mL Pen/Strep. Each tissue was minced on a sterile 10 cm^2 petri dish using individually packaged scalpels. The tissues were minced into cubes approximately 1mm^3 in size. Several drops of 0.1% Collagenase III was added to the petri dish to promote digestion. This sample was then transferred to a 15 mL falcon and the total volume was made up to 2 mL with 0.1% Collagenase III. The sample was then allowed to incubate at 37°C, 5% CO_2, with the lid ajar overnight to allow digestion.

Using a process of differential centrifugation, individual cell populations were isolated based on the different densities of cells. Following overnight digestion, samples were centrifuged at 29 x g for 1 minute. The resulting pellet containing mixed cell fragments is then designated the Organoid Cell Fraction. The supernatant was transferred to a fresh 15 mL falcon. This supernatant was then centrifuged at 88 x g for 2 minutes. The resulting pellet, containing epithelial cells, is then designated the Epithelial Cell Fraction. The supernatant was transferred to a fresh 15 mL falcon. This supernatant was then centrifuged at 180 x g for 4 minutes. The resulting pellet, containing the stromal cells, was then designated the Stromal Cell Fraction. The supernatant was discarded to waste. All pelleted cell fractions were re-suspended in 2 mL of fresh stromal media. Complete Stromal media consists of DMEM supplemented with heat inactivated Foetal Bovine Serum and 100 IU/mL Penicillin G/100 mg/mL Streptomycin sulphate (Pen/Strep). The cell

suspensions were then transferred to T25 cm^3 tissue culture flasks and the media volume was made up to 8 mL. Cells were maintained at 37°C with 5% CO_2. Following 24 hour incubation, the media on the organoid and epithelial cells was replaced with complete Organoid Medium to positively promote growth. Complete Organoid Medium constituents include: 0.5 µg/mL Hydrocortisone, 0.075% Bovine Serum Albumin (BSA), 10 ng/mL Cholera Toxin, 5 ng/mL Epidermal Growth Factor, 100 IU/mL Penicillin G/100 mg/mL Streptomycin sulphate (Pen/Strep), 0.01mg/mL Bovine Insulin in DMEM media. Media was changed on Organoid, Epithelial and Stromal Cell populations twice weekly and were passaged/subcultured every 7-10 days.

2.3 microRNA Expression Analysis

2.3.1 RNase Contamination

RNA integrity and purity is critical for full-length cDNA synthesis, which results in high quality RQ-PCR results. For this reason, preventing RNase contamination throughout the entire process must be considered crucial. RNase A is a highly stable contaminant of a laboratory and can be detrimental to RNA quality. As a result, all precautions must be taken to eliminate RNases and prevent contamination both in the laboratory environment and also in all prepared solutions. The following precautions were observed during experimentation:

- All stages of the extraction process were carried out in Class II Safety Cabinets specifically designated solely for RNA extraction.
- Equipment used e.g. Gilson pipettes, barrier pipette tips, racks, tubes, etc, were designated for RNA extraction only.
- Disposable non-latex gloves were worn at all times, regularly sprayed with 70% IMS and were changed frequently.
- Optional DNase digestion steps were included in procedures to remove all traces of genomic DNA.

2.3.2 RNA Extraction Overview

Prior to performing molecular techniques such as Relative Quantification-PCR, it is critical to first obtain high-quality RNA. To generate the most sensitive and biologically relevant results, the RNA isolation procedure must be appropriate for the sample type. It is also critical to consider several aspects of the process in order to optimise the extraction, such as the treatment and handling of samples prior to RNA isolation, the choice of technologies used to prepare the RNA, and finally the storage of the prepared RNA samples. Different sample types require specific methods for RNA extraction. This research utilised a number of different sample types including:

- Breast cancer cell lines
- Cell-conditioned media exposed to primary tissue samples, primary culture cell populations, and cell lines
- Exosomes
- Tumour and Normal breast tissue
- Blood (Whole/Serum/Plasma)

The RNA extraction methods employed varied from Tri Reagent adaptations to commercially available on-column purification methods (Table 2.2). All biological waste was disposed of in yellow biohazard bags as per the laboratory protocol. All chemical waste was disposed of appropriately.

Method	Sample Type	Source	Design Specification
Blood Protocol (182)	Blood (Whole Blood, plasma, serum)	Discipline of Surgery adaption of the TRI Reagent® technique	Utilizes Trizol® reagent and Bromoanisole.
RNeasy®	Tissue, Cells.	Qiagen	On-column method designed to isolate total RNA (i.e. small and large)
miRNeasy®	Tissue, Cells.	Qiagen	On-column method designed to specifically isolate microRNA
mirVana™	Conditioned media. Exosomes	Ambion	Double column method designed to isolate both small and large RNA
RNAqueous®	Cells, Conditioned media.	Ambion	Phenol free method designed for total RNA isolation

Table 2.2 Methods of extraction used for isolation of miRNAs from cell-conditioned media.

2.3.3 RNA Extraction Procedures

The mirVana™ miRNA Isolation Kit (Ambion®) employs organic extraction, as described previously, followed by purification on two sequential Glass Fibre Filters (GFF) under specialized binding and wash conditions. Unlike other methods, the mirVana™ kit utilizes two sequential GFFs, as it has been suggested that the small RNAs are essentially lost in the first filtration through the column and therefore a second filtration is required to capture the tiny microRNAs (Figure 2.3).

The RNAqueous® Kit (Ambion®) is designed for phenol-free total RNA isolation using a guanidinium-based lysis⁄denaturant and Glass Fiber Filter (GFF) separation technology. The RNAqueous method is based on the ability of glass fibers to bind nucleic acids in concentrated chaotropic salt solutions. Following the steps described below, the lysate was diluted with an ethanol solution to make the RNA competent for binding to the GFF in the filter

cartridge. This solution was passed through the filter pad where RNA binds and most other cellular contents flow through. The filter cartridge was washed 3 times to remove contaminants and the RNA was eluted in RNase-free water (Figure 2.3).

The RNeasy and miRNeasy Mini Kit (Qiagen®) both combine phenol/guanidine-based lysis of samples and a silica membrane column based purification. Following the steps described below, the upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) upwards. The sample was then applied to the RNeasy/miRNeasy Mini spin column, where the total RNA binds to the silica membrane and phenol and other contaminants are efficiently washed away. High quality RNA was then eluted in RNase-free water. Figure 2.3 illustrates the various steps involved in all 3 methods.

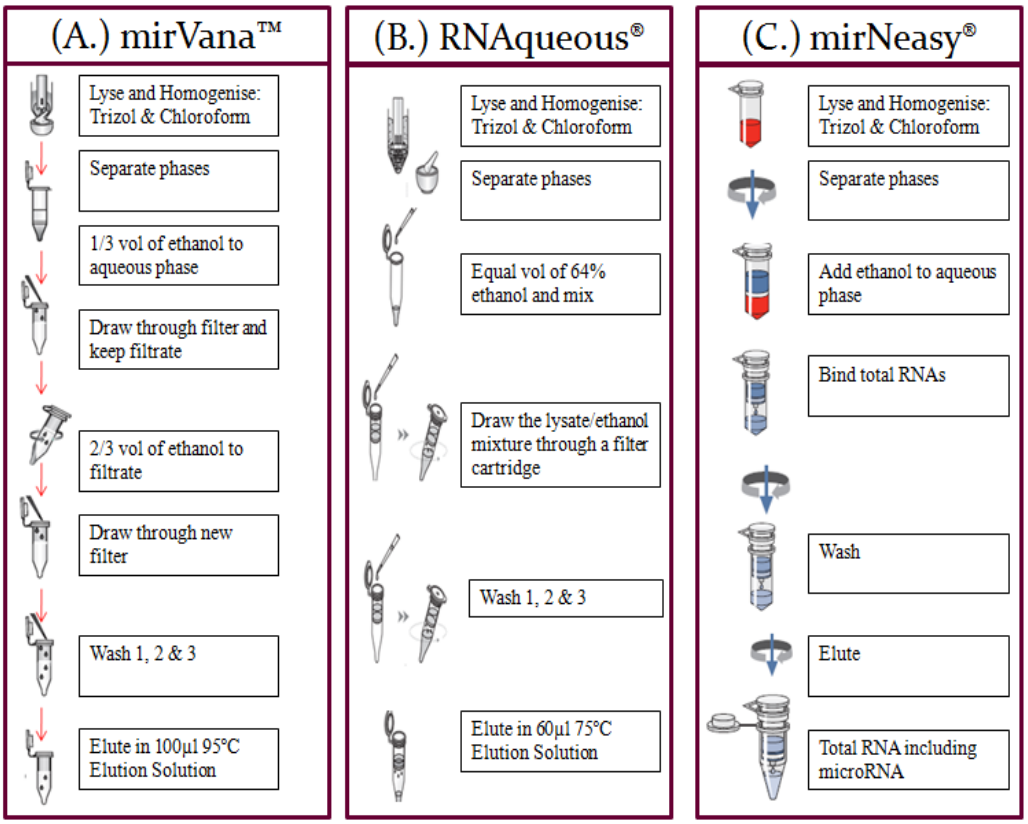


Figure 2.3 Schematic of miRVana™, RNAqueous® and miRNeasy® isolation methods.

2.3.3.1 Cell Line Pellet RNA extraction

Cell pellets were removed from the -80˚C freezer and placed on ice. 700 µL Trizol Lysis Reagent (Invitrogen®) was added to the pellet and vortexed. The lysate was then passed through a syringe to lyse the cells and reduce the viscosity of the sample. The homogenate was then incubated at room temperature (15–25°C) for 5 minutes to permit the complete dissociation of nucleoproteain complexes. 140 µL of chloroform (Merck Millipore) was then added and the sample was shaken vigorously for 15 seconds. Again, the sample was incubated at room temperature for 5 minutes and centrifuged for 15 minutes at 12,000 x *g*, at 4°C. Following centrifugation, the mixture separates into a lower red, phenol/chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase was transferred to a new collection tube carefully avoiding the interphase. 1.5 volumes of 100% ethanol was added to the aqueous phase, and mixed thoroughly by pipetting. 700 µL of the sample was then transferred into an RNeasy® Mini column in a 2 mL collection tube, which was then centrifuged at 12,000 x g for 15 seconds at room temperature. The resultant flow-through was discarded and the remainder of the sample was passed through the column as per the previous step. The spin column was then washed 350 µL of RW1 wash buffer and centrifuged at 12,000 x g for 21 seconds. An optional DNase digest was performed with DNase/Buffer RDD mix. 80 µL of the DNase/RDD Bufffer mix was pipetted onto the column and incubated at room temperature for 15 minutes. The column was then washed again with 350 µL of RW1. 500 µL of RPE buffer was pipetted onto the column and centrifuged at 12,000 x g for 15 seconds. This step was then repeated. The column was placed into a new 2 mL collection tube and centrifuged at 12,000 x g for 1 min to further dry the membrane. The column was transferred again to a new 2 mL collection tube and 30 µL of RNase-free water was pipetted directly onto the column membrane, allowed to stand for 1 minute, and centrifuged at 12,000 x g for 1 minute to elute. The last step was repeated again with 20 µL of RNase-free water (Ambion®), bringing the total eluate volume to 50 µL. The extracted RNA was then stored at -80˚C until required.

2.3.3.2 Cell-Conditioned Media microRNA extraction

Upon commencing this study, isolating quality miRNA from cell conditioned media presented a significant challenge. No standardised or reproducible protocol existed, and there were few successful methods described in the literature in this emerging field of miRNA research. We compared various methods, including separate purification of large and small RNA from cell-conditioned media using column based methodology, and co-purification of total RNA using Trizol based techniques. The use of five available and widely used miRNA isolation techniques were investigated, namely the blood protocol (182), RNeasy® (Qiagen), miRNeasy® mini kit (Qiagen), mirVana™ isolation kit (Ambion) and RNAqueous® kit (Ambion) (Table 2.2). Prior to extraction using the RNeasy®, miRNeasy®, mirVana™ and RNAqueous® isolation kits, the following steps were designed within the laboratory and were carried out to permit isolation of miRNA from cell-conditioned media:

- Extractions were performed on 1 mL of cell-conditioned media. 700 µL of Trizol™ reagent was added to the sample and vortexed. The sample was then left to stand for 5 minutes at room temperature.
- 140 µL of chloroform was added and the sample shaken vigorously for 15 seconds.
- Samples were centrifuged at 15,300 x g RCFMax (14,000 RPM Eppendorf 5417R, F45-30-11), at 4°C, for 15 minutes.
- The upper aqueous phase was transferred to a new tube, avoiding the inter-phase.
- The manufacturer's protocol for each kit was then followed from the separation step onwards.

2.3.3.3 Blood RNA extraction

Blood Collection

Ethical approval from the Galway University Hospitals Research Ethics Committee was sought and granted to Biobank blood from consenting informed patients (see appendix 9.1) for use in departmental research studies. Whole blood was collected in Vacuette EDTA K3E blood bottles (Grenier Bioone); one processed for plasma, another unprocessed, and a third sample collected in Vacutainer Serum Separator Tubes II (Becton Dickinson) for serum. Samples for serum collection were left to clot at room temperature for 30 minutes and then all samples destined for serum and plasma collection were centrifuged at 805 x g @ 4°C for 10 minutes. Plasma/serum was removed, aliquoted and stored at -80°C until required. The unprocessed whole blood sample was stored at 4°C until required.

Copurification of total RNA using Trizol

The blood protocol (182) was developed in-house in the Discipline of Surgery, NUI Galway, and is a modified Trizol™ co-purification technique. Previous studies have found this method to provide reliable miRNA yields from whole blood, plasma and serum. Total RNA was extracted from whole blood, serum and plasma samples using a modification of the Tri Reagent® BD (Molecular Research Centre) copurification protocol, as follows: Using 1 mL of whole blood, or its derivatives, phase separation was performed by the addition of 3 ml of Trizol and 200 µL of 1-bromo-4 methoxybenzene to augment the RNA phase separation process. The homogenous lysate was divided between two 2 mL collection tubes and then centrifuged at 12000g for 15 minutes, at 4°C. The clear aqueous phase (approximately 1 mL) from each tube was then removed, transferred to fresh collection tubes respectively and RNA was precipitated by the addition of 1 mL of isopropanol (Merck Millipore®) and centrifugation of the solution at 12000g for 8 minutes at 18°C. Following removal of the supernatant, the RNA pellet was then washed with 1 mL of 75% ethanol. An additional ethanol wash was performed to improve the purity of RNA isolated, which was reflected in an improved 260/280 nm spectrophotometry ratio. Each RNA pellet was briefly air dried and then solubilised using 30 µL of nuclease free water. Each 1 mL of whole

blood yielded 60 µL of total RNA when the two matched RNA pellets were solubilised, combined and finally transferred to storage tubes prior to storage at -80°C.

2.3.3.4 Breast Tissue RNA extraction

Ethical approval from the Galway University Hospitals Research Ethics Committee was sought and granted to Biobank tissues from consenting informed patients (see appendix 9.1) for use in departmental research studies. Patient tissue samples are extremely susceptible to degradation by RNase contamination and therefore precautions were taken to avoid this:

- Samples for RNA extraction were only handled with Gilson pipettes, racks, tubes, etc designated specifically for RNA use.
- Gloves were worn at all times, sprayed with 70% IMS, and changed frequently.
- Extractions were performed on ice, with minimal handling of tubes, in Class II Biological Safety Cabinets.

For the purposes of extracting RNA from breast tissue, both tumour tissue and normal tissue, a separate purification technique was used which firstly involves homogenising the tissue with trizol, with subsequent RNA extraction using the column based RNeasy Isolation kit (Qiagen). Snap frozen tissues were retrieved from the Discipline of Surgery Biobank located at the research laboratory in NUI Galway. Tissues were removed from the tubes and placed on a 10 cm^2 petri dish. Using a scalpel, approximately 100 mg of tissue was cut away. This was then homogenized in 1 mL Trizol™ (Qiagen) using a bench-top homogenizer (Kinematica AG). Homogenized tissue samples were stored at -80°C until required for extraction. Homogenized tissue samples were thawed gently on ice and vortexed. For tissue samples which appeared extra fatty (e.g. normal tissues), the thawed homogenate was centrifuged at 12,000g for 10 minutes at 4°C. This allowed the RNA to migrate to the pink interphase between the fat and the cellular debris. 200 µL of chloroform per ml of Trizol™ was added to the sample and the tube was then shaken vigorously for 15 seconds. The sample was left to stand at room temperature for 5-10 minutes and was then centrifuged at 12,000 g for 15

minutes at 4°C to remove the insoluble material. The fatty material was layered on the surface while the RNA migrated to the upper clear aqueous phase. The upper aqueous phase was transferred to a new collection tube. 3.5 volumes of 100% ethanol was added to the upper aqueous phase and mixed thoroughly by vortexing. 700 µL of the ethanol/aqueous phase mixture was then added to the RNeasy spin column and centrifuged at maximum speed (e.g. 12,000g) for 21 sec at 4°C. The resulting eluate or flow-through was then discarded to waste. 350 µL of buffer RW1 was pipetted onto the RNeasy column and centrifuged at max speed for 21 seconds at 4°C. An optional DNase I treatment was then performed as follows. 80 µL of DNase I mix, made using reagents from an RNase-free DNase set (Qiagen), were applied onto the membrane of the column and left at room temperature for 15 minutes. Following DNase treatment the buffer RW1 wash step was repeated. Two further wash steps, using 500 µL of buffer RPE, were carried out. The second of these steps had an increased centrifugation time of 2 minutes to dry the membrane. The large RNA was eluted from the RNeasy column by applying 50 µL RNase-free water to the membrane and centrifuging at 12,000 g for 1 minute at 4°C. The RNA was stored at -80°C until further use.

2.3.4 RNA Analysis

For miRNA expression analysis it is crucial to first confirm that pure and intact RNA has been isolated as the concentration and integrity of the RNA can be affected by the purification processes used. The Nanodrop Spectrophotomer offers fast and efficient quantitation and estimation of RNA purity, while the Agilent Bioanalyser offers an accurate method to determine the integrity and the concentration of RNA from various sources.

2.3.4.1 Nanodrop Spectrophotometer

Following RNA extraction, all eluates were analysed to determine the RNA concentration in each sample. This was achieved using the Nandrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). Depending on the extraction method used for particular sample types, different impurities can be expected, e.g. Trizol™, carbohydrates, Guanidine

Thiocynate, nucleotides, peptides, EDTA, phenol and proteins. The Nanodrop® system measures 1 µL samples with high accuracy and reproducibility. The spectrophotometer measures the full spectrum (220 nm-750 nm) utilizing a patented sample retention technology that employs surface tension alone to hold the sample in place. A major advantage of the NanoDrop 1000 spectrophotometer is that it is capable of measuring highly concentrated samples without the need for dilution. For measuring large RNA, the sample type 'RNA-40' was selected, while for measuring small RNA, the sample type 'RNA-33' was selected. A 1.1 µL aliquot of the extracted RNA was pipetted onto the apparatus pedestal and the instrument arm was used to compress the sample to form a column held in place by surface tension.

Spectral measurements were made with a tightly controlled pathlength of 0.1 cm. RNA concentration was automatically calculated using the formula:

$$\text{RNA Concentration (ng/µl)} = (A_{260} \times e)/b$$

A_{260} = Absorbance at 260 nm

e = extinction coefficient (ng-cm/µl)

b = pathlength (cm)

The Nanodrop is used for accurate measurement of concentrations (A260), protein contamination (ratio A260/280) and contamination with buffer components or organic compounds (ratio A260/A230). In solution, pure DNA and RNA typically have A260/280 ratios between 1.8 and 2.2, therefore samples producing this result were considered indicative of pure RNA. Samples producing significantly less absorbance ratios were considered to be contaminated with protein or phenol. A ratio at 260 and 230 nm (A260/A230) between 1.8 and 2.2 was considered acceptable. Lower ratios can indicate the presence of contaminants or impurities from extraction chemicals or incompletely removed constituents of cells. The volume of RNA solution required to yield 1 µg of RNA was then calculated.

2.3.4.2 Agilent Bioanalyser

The small-RNA enriched fractions were also analysed using the Small RNA Assay (Agilent Technologies) and the Agilent 2100 Expert software (Version B.02.03). The bioanalyzer provides an electropherogram for each sample giving a good estimate of the RNA integrity. In addition to traditional measurement, the Bioanalyser provides an RNA Integrity Number (RIN) which gives a reliable impression of the quality of the RNA. The small RNA Agilent chip includes 11 sample wells, 4 gel wells and a well for an external standard/ladder. Fabricated in glass, micro-channels create interconnected networks among the wells. The wells were filled with a sieving polymer and a fluorescent dye which form an integrated electrical circuit. To prepare the gel, the Small RNA gel matrix and small RNA dye concentrate were allowed to equilibrate to room temperature for 30 minutes. The gel was filtered by centrifuging at 10,000 x g for 15 minutes. The dye concentrate was vortexed for 10 seconds and briefly centrifuged at 10,000 x g. 2 µL of dye concentrate and 40 µL of the filtered gel were mixed thoroughly by careful pipetting. The gel/dye mix was then spun at 13,000g for 10 minutes. 9 µL of the gel was placed into the relevant wells and the chip was placed on the chip priming station. Using a syringe, the gel was spread through the channels to each of the wells. 1 µL of sample was placed into the sample wells, with 1 µL of the ladder placed into the ladder well. The wells were then aligned with a cartridge containing 16-pin electrodes. The electrodes were cleaned with RNase-free water for 5 minutes prior to use. Charged bio-molecules, e.g. RNA, were electrophoretically driven by a voltage gradient i.e. capillary electrophoresis. As a result of the constant mass-to-charge ratio and the presence of the sieving polymer matrix, the RNA molecules became separated by size. Small RNA fragments migrated faster than the larger RNA fragments, the dye molecules intercalated into RNA strands and these complexes were detected by laser-induced fluorescence. The data was then translated into bands (i.e. gel-like images) and peaks (i.e. electropheropgrams). The resulting data then produced % miRNA content values.

2.3.5 Reverse Transcription for miRNA Expression Analysis

Reverse Transcription (RT reaction) is a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) by using total cellular RNA, a reverse transcriptase enzyme, a primer, dNTPs and an RNase inhibitor. Small RNA (5 ng-100 ng, depending on sample type) was reverse transcribed to cDNA using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). All procedures were carried out in vented hoods, which had been thoroughly cleaned with 70% IMS and had undergone UV cleaning for 15 minutes prior. This created an area free of artificial templates and amplified material. The components of the RT kit were allowed to thaw on ice. Each reaction was primed using a miRNA-specific stem-loop primer. An RT mastermix was created on ice using the components listed below per sample, with p2 and p10 Gilson pipettes used to ensure accurate volumes (Table 2.3).

Component	Master mix Volume
10X Reverse Transcription Buffer	1.5 µL
100Mm dNTPs	0.15 µL
Nuclease Free Water	4.16 µL
miRNA specific Stem-loop Primer (50 nM)	3.0 µL
50 U/µL Multiscribe RT	1.0 µL
20 U/µL RNase Inhibitor	0.19 µL
Master Mix Volume	**10.0 µL**
Small RNA (1 ng/µL)	5.0 µL
Total Volume of Reaction	**15.0 µL**

Table 2.3 Component volumes required for Reverse Transcription Reaction.

All reactions were prepared on a cooling tray. RT master-mix/RNA reactions were centrifuged at 10,000 x g prior to loading onto the Thermal cycler. The reaction was performed using a Gene Amp PCR system 9700 thermal cycler (Applied Biosystems). An RT-negative control was included in each batch of reactions. This consisted of the Reverse Transcription Master Mix without

any RNA, therefore acting as an RT Blank. The following parameter values were programmed into the Thermal Cycler (Table 2.4).

Time	Temperature
30 minutes	16°C
30 minutes	42°C
5 minutes	85°C
Hold/∞	4°C

Table 2.4 Reverse Transcription Thermal Cyler parameters.

Following completion of the RT reaction, all samples were briefly centrifuged at 10,000 x g and transferred to RNA free polypropylene tubes and stored at -20°C until required.

2.3.6 Reverse Transcription for Gene Expression Analysis

cDNA was synthesised using the SuperScript III reverse transcriptase enzyme (Invitrogen). One reaction was completed for each sample using random primers as they hybridise to complementary regions at different points on the mRNA strand. This is beneficial as shorter cDNA fragments are produced, therefore increasing the probability that the 5' ends of the mRNA would be converted to cDNA. RNaseOUT™ and Dithiothreitol (DTT) are also included in the reaction. RNaseOUT™ protects the mRNA template from degradation by ribonucleases, while DTT inhibits RNase activity by reducing disulphide bonds, thereby affecting stability. Reverse Transcription Master Mix blanks were included in each set-up. Mitochondrial Ribosomal Protein L19 (MRPL19) and Peptidyprolyl Isomerase A (PPIA) were employed as endogenous controls (183, 184).

1 µg of mRNA was denatured in the presence of Master Mix 1 (random primers (76 pg/µL) and deoxyribonucleotide triphosphates (dNTPs) (700 µM)) for 4 minutes at 65°C in a total reaction volume of 13 µL. Following this, 7 µL of Master Mix 2 (First Strand Buffer, DTT (14Mm), RNaseOUT™ (6U) and SuperScript® III Reverse Transcriptase (29U)) was added to make a total reaction volume of 20 µL. This complete reaction was incubated for 5

minutes at 25°C, followed by 1 hour at 50°C (the optimal temperature for Superscript® III activity) and a final 15 minute 70°C incubation to denature the enzyme. 30 µL of nuclease free water was added giving a final total volume of 50 µL. Following completion of the RT reaction, all samples were briefly centrifuged and transferred to RNA free polypropylene tubes and stored at -20°C until required.

2.3.7 Real Time Quantitative – Polymerase Chain Reaction (RQ-PCR)

Amplification of Complementary DNA (cDNA) is performed by the Polymerase Chain Reaction. RQ-PCR involves monitoring the progress of the PCR as it is occurring i.e. in Real Time. Reactions are characterised by the point in time during the cycling process when the amplification of a target is first detected. The reaction consists of an exponential phase, in which the amount of amplified product approximately doubles during each cycle of denaturation, primer annealing and template extensions, and a non-exponential or plateau phase where reduced reagents limit the reaction process. The Cycle Threshold (CT) is the point at which enough amplified product has accumulated to produce a detectable fluorescent signal. The greater the amount of starting template, the lower the CT value, therefore if a sample is high in abundance, it will have a low CT value. Where sequences were available, primers were obtained from Applied Biosystems. A No Target Control (NTC) was included in each batch of reactions. This consisted of the PCR Master Mix without any cDNA, therefore acting as a PCR/NTC Blank (Figure 2.4).

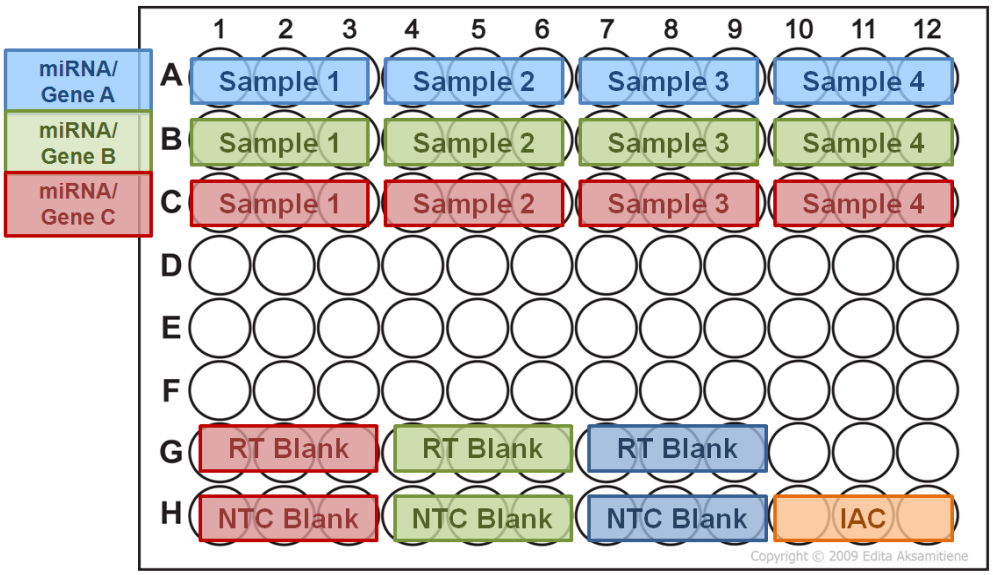


Figure 2.4 Sample plate plan for RQ-PCR. Each sample is in triplicate, with a Reverse Transcription Blank and a PCR/NTC Blank for each target (gene/miRNA), also an Inter Assay Control for each plate.

RQ-PCR reactions were carried out in triplicate using a master-mix comprising of the following components per sample (Table 2.5).

ABI miRNA Kit	Master-mix Volumes
Taqman® Universal Fast Mix	5.0 µL
Nuclease Free Water	3.8 µL
ABI miRNA PDAR	0.5 µL
Total Master-mix Volume	**9.3 µL**
cDNA	0.7 µL
Total Volume of Reaction	**10.00 µL**

Table 2.5 Component volumes required for RQ-PCR Reaction using ABI products.

Primers and Probes obtained from MWG Biotech were carried out using a master-mix comprising of the following components per sample (Table 2.6).

MWG miRNA Kit	Master-mix Volumes
Taqman® Universal Fast Mix	5.0 µL
Nuclease Free Water	1.6 µL
Probe (0.2 uM)	0.5 µL
Forward Primer (1.5 uM)	1.5 µL
Reverse Primer (0.7 uM)	0.7 µL
Total Master-mix Volume	**9.3 µL**
cDNA	0.7 µL
Total Volume of Reaction	**10.00 µL**

Table 2.6 Component volumes required for RQ-PCR Reaction using MWG products.

RQ-PCR reactions were carried out in final volumes of 10 µL, using an AB7900HT (Applied Biosystems). Standard fast thermal cycling conditions were used. First, 40 cycles at 95°C for 15 seconds, which removed the secondary structure from the DNA and split it into single stranded DNA. This was followed by 60°C for 60 seconds to allow the annealing step to take place. On each plate, an inter assay control (IAC) was included to account for any variations between runs.

2.3.8 RQ-PCR Data Statistical Analysis

Relative Quantification was chosen in all studies. This involves quantifying the expression of endogenous controls. Endogenous controls are known as important housekeeping genes as their expression levels remain constant, regardless of the condition of the cell/body fluid/tissue. The miRNA of interest can then be expressed relative to this gene. An average CT value was obtained from the triplicate of each sample. The average CT value for the endogenous controls was subtracted from the target miRNA/gene of interest, and this value was termed the ΔCT value.

$$\Delta CT = CT_{\text{Test Gene}} - CT_{\text{Endogenous Control}}$$

A Reference sample was then chosen. This is the sample with the lowest expression, i.e. the highest ΔCT value. It was then subtracted from all the ΔCT values, this value was termed ΔΔCT.

$$\Delta\Delta CT = \Delta CT_{\text{Sample 1}} - \Delta CT_{\text{Reference Sample}}$$

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

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

The Relative Quantification (RQ) is the fold change compared to the calibrator. All results were log transformed for analysis in order to stabilize the within-group variances and to create a normal distribution for parametric analysis.

Statistical analysis was carried out using the statistical software package Minitab 16.0 (Minitab Ltd). One-way ANOVA and independent two-sample t-tests were used to determine association and comparisons between independent groups and to assess significance. Results with a p-value <0.05 were considered to be statistically significant.

2.3.9 PCR Amplification efficiencies

Theoretically, in well designed PCR assays with optimised primer conditions and reagent concentrations, maximum efficiency should occur during the exponential detection phase with a doubling of amplification product for each cyle. However, amplification efficiencies may fluctuate as a function of non-optimal assay design, enzyme instability or even the presence of co-extracted amplification inhibitors (185, 186).

To determine the amplification efficiency of the probes and primers, serial dilutions (neat to 10^{-6}) of cDNA template were prepared and amplified using standard conditions. A dilution curve was constructed by plotting Cycle Threshold (CT) versus the dilution factor of cDNA.

The amplification efficiency, E, is calculated from the slope of the standard curve using the formula:

$$\text{Amplification efficiencies (E)} = (10^{-1}/\text{slope}^{-1}) \times 100$$

The percent amplification efficiency is calculated using the formula:

$$\%E = (E-1) \times 100\%$$

where 100% efficiency is an indicator of a robust assay.

2.4 miR-504 overexpression *in vitro*

Following ethical approval and registration of Genetically Modified Organisms with the Environmental Protection Agency (see appendix 9.2), lentiviral vectors were used for delivering and maintaining over expression of mature microRNAs, resulting in stable integration into the host genome. In a parallel study within the research group, T47D cells were transduced with a lentiviral mimic over-expressing miR-504, with a separate T47D population transduced with the same lentival backbone, but with a No Target Control (NTC) sequence in place of miR-504. The inital lentiviral transduction was performed by another member of the research group, while all further confirmation and analysis performed by the author. Lentiviral vectors were designed to have strong promoters, the Human cytomegalovirus (hCMV), for miR-504 and a Non-Targeting Control (NTC), individually. The lentiviral vectors also had a Red Fluorescent Protein (RFP) to visually track transduction efficiency. The lentivirus used was Thermo Scientific SMARTchoice shMIMIC Lentiviral microRNAs, see Figure 2.5 for a structural map of the lentiviral construct.

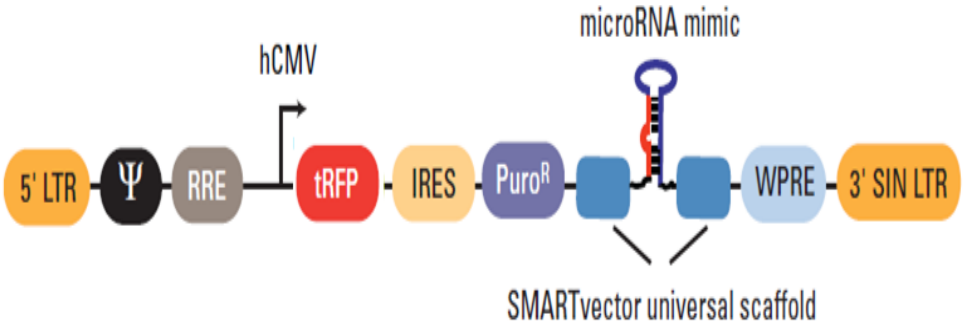


Figure 2.5 Design of the components of the SMARTvector Lentiviral microRNA vector. (Adapted from the SMARTchoice shMIMIC Lentiviral microRNA Technical Manual).

Cultures were supplemented with Polybrene which improves transduction efficiency. Puromycin was used to select out and ablate any non-transduced cells. Prior to transduction, cell density and polybrene/puromycin concentrations were optimised (Figure 2.6).

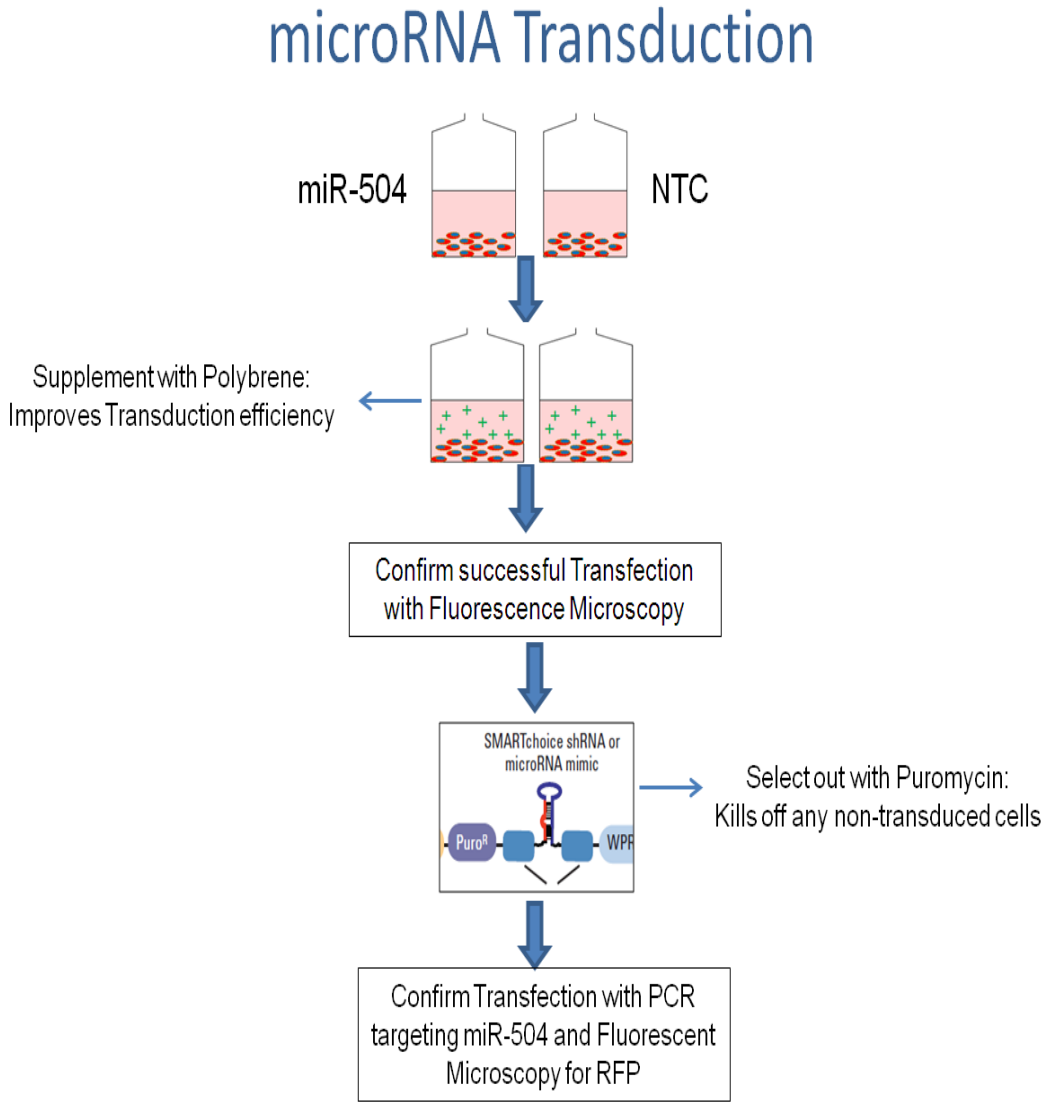


Figure 2.6 Workflow of microRNA transduction protocol.

2.4.1 Transduction Confirmation

Fluorescence Microscopy

Approximately 48-72 hours post-trandsuction, transduced cells begin expressing detectable levels of Red Fluorescent Proteins (RFP) which can be visualised using Fluoescence microscopy. Chamber slides were seeded with 2 x 10^4 cells. One chamber received virus free T47D cells (wildtype), another chamber received the T47D NTC cells, and the other received T47D 504 cells. Following 24 hours incubation, the media was gently pipetted off from the chamber slides. The chambers were washed twice with PBS. 4% Paraformaldehyde was added and left to stand for 20 minutes at room temperature to fix the cells. The walls of the chambers were removed. The slides were washed 3 times for 5 minutes in PBS. A 1:100 dilution of 4',6-diamidino-2-phenylindole (DAPI: 1 µg/mL) stain was added to each slide and left to stand for 5 minutes at room temperature to stain the cell nuclei. This was followed by 3 x 5 minute washes in PBS. A series of alcohol dehydrations was then performed; 75% alcohol – 3 minutes, 95% alcohol – 3 minutes, 100% alcohol – 3 minutes, Xylene – 3 minutes, Xylene – 3 minutes. Grading the alcoholic solutions in a stepwise manner is thought to minimise cellular distortion and reduce cell loss from the glass slide. The protocol was carried out under the protection from light to avoid photo bleaching. Coverslips were mounted using DPX mounting medium. The slides were then viewed by fluorescent microscopy using the appropriate filters, with RFP exciting at 482 nm and emitting at 502 nm. The excitation maximum for DAPI is 358 nm and the emission maximum is 461 nm.

RQ-PCR

Successful overexpression of miR-504 was also confirmed using RQ-PCR. At multiple timepoints following transduction, RNA was extracted from cells and relative expression of miR-504 quantified using RQ-PCR, as previously described.

2.4.2 Cell Proliferation Assay

The effects of miR-504 overexpression on cell proliferation in the T47D 504 cell line were measured using the CellTiter 96® AQueous Non-Radioactive Cell Prolideration Assay (MTS, Promega). This is a colorimetric method for determining the metabolic index/cellular proliferation of cells.

The water soluble MTS tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and the electron coupling reagent (phenazine ethosulfate PES) form a stable solution, which is bio-reduced into a purple formazan product that is soluble in tissue culture media (Figure 2.7).

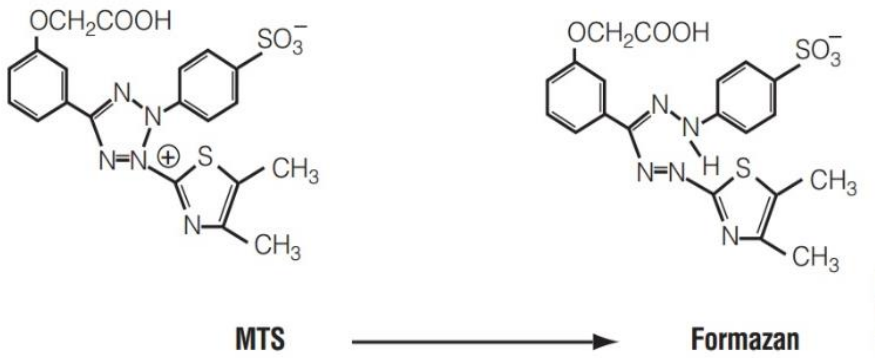


Figure 2.7 Structures of MTS tetrazolium and its formazan product (187).

The absorbance at 490 nm of the formazan product can be measured and is directly proportional to the number of living cells in culture.

T47D virus free (wildtype) cells, T47D NTC cells and T47D 504 cells were seeded into 96-well flat bottom clear walled plates at a density of 6 x 10^4 per well. Each cell line had 8 replicates as outlined in Figure 2.8.

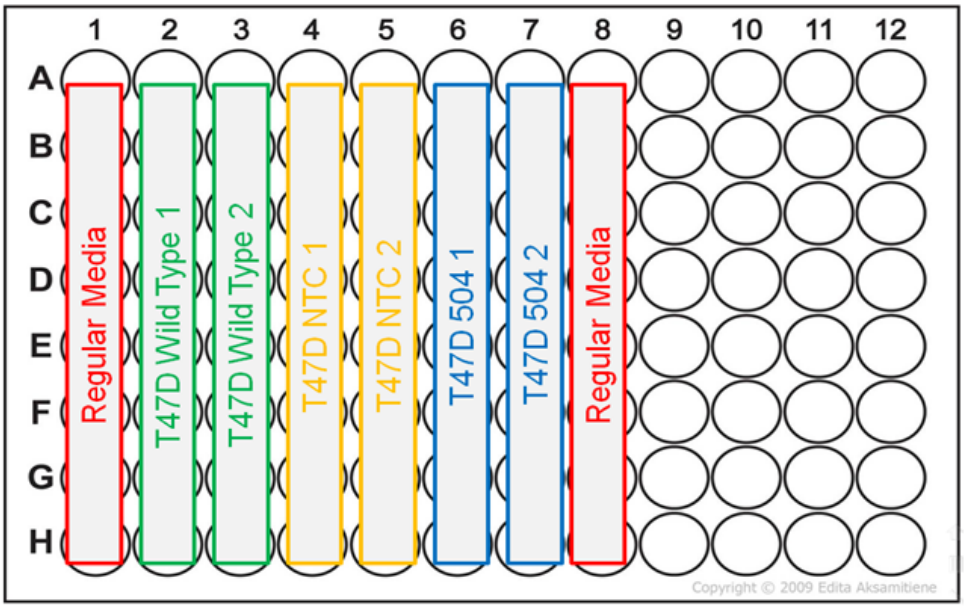


Figure 2.8 Sample plate plan for a 96 well flat bottom cell culture plate.

Cells were allowed to adhere overnight. At the appropriate time point, e.g. 24/48/72 hours, 20 µL of 5 mg/mL MTS solution was added to each well. Wells containing basal media were used as a control. The plates were returned to the incubator at 37°C for 3.5 hours. Following this, the plate was read on the Bio-Rad microplate reader at 490 nm. This assay was repeated at different time points e.g. 24 hours, 48 hours, and 72 hours. Readings from 8 wells were averaged per sample. Each entire assay was repeated in triplicate. The mean of 3 experiments (Mean ± SEM) was plotted against time to generate a growth curve across the different time points.

2.4.3 Cell Migration Assay

Transwell migration assays were performed using Corning Transwell Inserts (Corning Life Sciences) in 24 well plates. The inserts used in this study have a 0.8 µm pore size and a polycarbonate membrane (Figure 2.9).

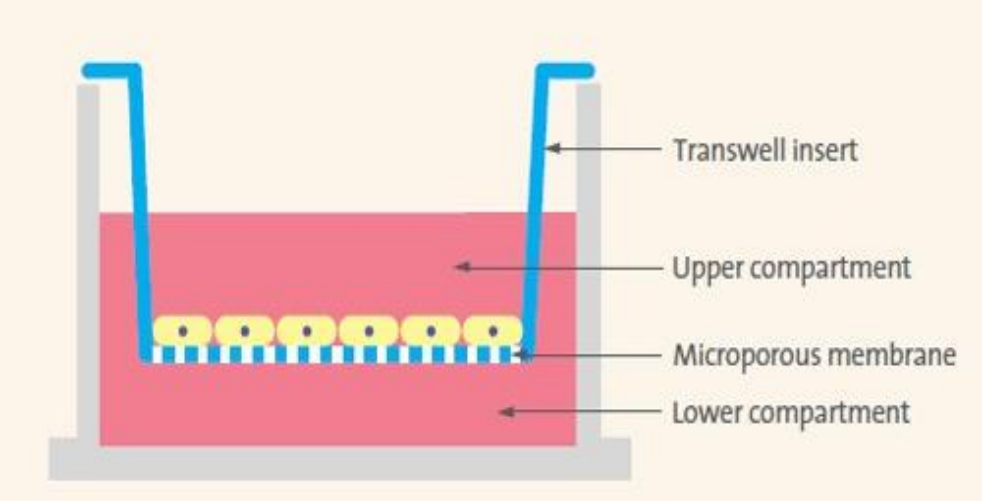


Figure 2.9 Transwell insert membrane set-up (Adapted from the Corning Transwell Membrane Manual (188).

The wells and membranes were rehydrated with basal medium prior to use in order to improve cell attachment. Suspensions of cells (7×10^4 per 100 µL) in serum free media were added to the inserts. Suspensions of cells were prepared in duplicate, with 2% FBS in the lower compartment as the chemoattractant and basal media in the other lower compartment. This was to distinguish between chemotaxis and chemokinesis. Chemotaxis refers to the movement of cells in direct response to a chemical stimulus. Chemokinesis refers to random or non-directional movement. The plates/inserts were returned to the incubator at 37°C for 18 hours. The following day, the remaining cells in the inserts were removed by scrubbing with sterile cotton swabs. The scrubbed inserts were then transferred to ice cold 100% methanol for 15 minutes at room temperature in order to fix the cells on the lower surface of the membrane. Following this, the inserts were transferred to Haemotoxylin (Sigma-Aldrich®) for 3 minutes at room temperature to stain the cells. The inserts were then serially rinsed twice in dH_2O to remove all traces of Haemotoxylin. The inserts were inverted and left to dry at room temperature. The membranes were cut away from the inserts using a scalpel, at an angle, and the membranes were immediately mounted onto a slide using immersion oil and cover slips. The migrated cells on the upper side of the membrane were then counted in five fields of view per membrane using a 40X objective in a Leica Light Microscope.

2.5 miR-504 overexpression *in vivo*

2.5.1 Ethics and Licensing

This in-vivo study was performed following NUI Galway Ethical Committee approval. An animal licence under the Cruelty to Animals Act 1876 by the Department of Health and Children was also obtained. The licence training course provided by Laboratory Animals Science and Teaching (LAST-Ireland) was attended and certification was received upon completion of a written exam and demonstration of animal handling proficiency skills. All handling and procedures were carried out in accordance with published guidelines (189). The guiding principles relating to animal research: Refinement, Reduction and Replacement (3Rs) introduced by Russell and Burch (190) in 1959, were adhered to within this study. In brief, refinement refers to decreasing the incidence or severity of inhumane procedures applied. Reduction refers to reducing the number of animals used to obtain information. Finally, replacement refers to substituting insentient material for conscious living animals where possible.

2.5.2 Animal Facility

All animals were housed and experiments carried out at the animal research facility located at the National Centre for Biomedical Engineering Science (NCBES) at NUI Galway. Prior to entering the animal facility, a strict gowning up procedure must be adhered to in order to prevent the introduction of outside pathogens. Appropriate Personal Protective Equipment, including shoe covers, gown, gloves, hair-net and mask, was worn at all times when inside the facility. On-site, a Named Day-to-Day Care Person was present and responsible for the upkeep and maintenance of animal husbandry, to include changing bedding and feeding the animals. All consumables used at the animal research facility were autoclaved prior to use and bedding was changed twice weekly. 3-4 animals were housed per individually ventilated cage. All animal handling was carried out in Class II Laminar Flow Hoods, to minimise the risk of infection.

2.5.3 Animal Model

Inbred female athymic nude mice (Harlan Sprague-Dawley, Indianapolis) at 6-8 weeks of age were chosen for use in this study. These mice are immune-compromised allowing efficient growth of human-derived tumours. Also, athymic nude mice carry a defect in the Foxn1^{nu} gene causing them to be phenotypically hairless (191, 192). This is beneficial as it allows tumour formation to be more easily monitored. For all surgical procedures, a ketamine/xylazine cocktail (Ketamine (80-100 mg/kg)/ Xylazine (10 mg/mL)) (Sigma-Aldrich®) was used to administer general anaesthesia intramuscularly. A 27 gauge needle and 1 mL syringe was used for injection. 20 µL of the cocktail was administered to each animal. Strict aseptic technique was applied to ensure hygiene was maintained and risk of infection minimized. Post anaesthesia, all mice were monitored until fully awake, capable of moving freely, and able to access food and water. Athymic nude mice express very low endogenous levels of circulating oestrogen, therefore it was necessary to increase oestrogen levels required to support the growth of T47D breast cancer cells, which are Estrogen Receptor positive. Prior to tumour induction, 60-day slow release 17-β-estradiol pellets (0.18 mg) (Innovative Research of America, Sarasota, FL) were implanted subcutaneously into the rear neck of each mouse as depicted in Figure 2.10.

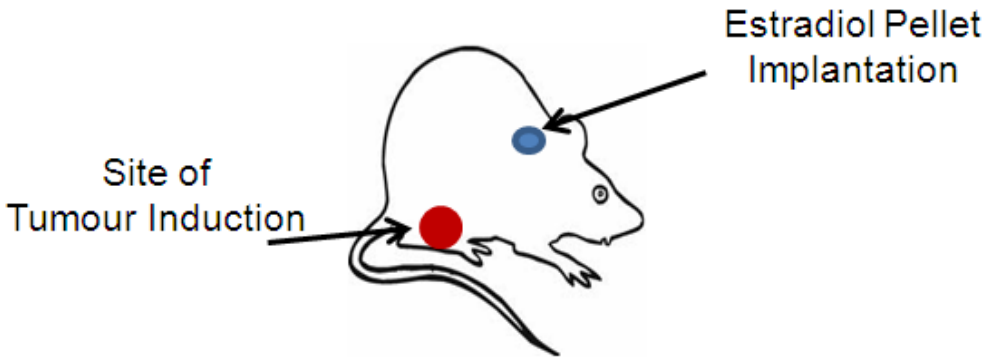


Figure 2.10 Mouse model showing site of tumour induction and estradiol pellet implantation.

Under anaesthetic, as detailed above, a small incision was made in the loose skin of the mouse's neck and a small pocket was bluntly dissected caudolaterally where the pellet was then inserted. Dermal glue was then used to close the incision. All animals were tagged with nickel/copper alloy tag containing a unique 3 digit code.

2.5.4 Tumour Induction

Approximately 14 days following estradiol pellet implantation, mice were divided into two experimental groups:

- Group 1: (n = 5) innoculated with T47D cells transduced with an NTC mimic
- Group 2: (n = 5) innoculated with T47D cells transduced with a miR-504 mimic

Transduced cells (2.5×10^5) were re-suspended in 150 µL RPMI-1640 and administered to the right subcutaneous flank of each animal (Figure 2.10). To confirm, on tumour induction day, that the cells were over-expressing miR-504, RQ-PCR was performed on a sub-aliquot of these cells for the analysis of miR-504 expression. To ensure the cells were stably over-expressing miR-504, an aliquot of the cells were maintained in culture *in vitro*, to quantify the miR-504 expression level, in parallel with tumour growth *in vivo*, at day 3, day 7, day 14 and day 28. Animals were weighed at weekly intervals to monitor the general health of the animal.

2.5.5 Study Endpoint

Following 9 weeks of tumour monitoring, all mice were sacrificed by exposure to increasing concentrations of CO_2 in a gas chamber. Following sacrifice, each mouse was dissected using a Y incision. All organs were examined and removed, followed by harvesting of any tumours and/or enlarged lymph nodes present, which were immediately snap frozen in liquid nitrogen at -80°C until required for processing. Terminal cardiac puncture was performed where possible. The blood was stored at 4°C in 2 mL EDTA tubes until required.

2.6 Isolation and Characterisation of Exosomes

2.6.1 Preparation of Exosome Depleted Medium

It has been established that exosomes are present in Foetal Bovine Serum (FBS), which is used as a growth supplement to DMEM for standard cell culture media. FBS is derived from bovine serum and contains a high abundance of bovine exosome vesicles. These exosomes can interfere or cause significant background issues when performing studies on exosomes isolated from cell cultures (141). To eliminate contamination by these exosomes, the culture medium used to culture cells to be included in exosome studies must be depleted of the contaminating exosomes. There are three options available: (1) do not supplement the culture media with serum, thereby depriving the media of specific metabolic requirements for the culture of cells, (2) purchase commercially available exosome deplete FBS, such as Exo-FBS™, which is very costly, or (3) manually deplete FBS of exosomes in the laboratory by over-night high-speed ultracentrifugation. In order to preserve the optimal conditions for cell growth and also due to cost restrictions, the third option, manual depletion, was utilised in all exosome studies. Optiseal Polyallomer Tubes were filled with FBS and ultracentrifuged at 100,000 x g for 16 hours at 4°C (Sorvall 100SE Ultracentrifuge). The supernatant was removed, taking care not to disturb the exosome pellet, and stored in 50 mL falcons at -20°C until required.

2.6.2 Collection of Cell-Conditioned Medium

Conditioned medium (CM) is spent media harvested from cultured cells. It contains all factors secreted by the cultured cells including metabolites, growth factors and extracellular matrix proteins and, of course, exosomes. The cell-conditioned medium was obtained by incubating the appropriate culture medium in a cell culture flask with an immortalised cell line at a known density for a specified period of time. Following this incubation period, the cell-conditioned media was harvested from the cell culture flask, placed into a falcon and centrifuged at 180 x g for 4 minutes. The resultant supernatant was then either; collected and stored at -20°C until required, or was put through differential ultracentrifugation for exosome isolation.

2.6.3 Collection of Cell Pellet

Cell pellets were routinely collected alongside cell-conditioned medium for comparison purposes. Spent media was removed and the cells were trypsinised as described previously. The cells were then pelleted by centrifugation at 180 x g for 4 minutes. The supernatant was then removed to waste by aspiration and the resulting cell pellet was frozen at -80°C until required.

2.6.4 Exosome Isolation Protocols

In 1996, Raposo *et al.* (193) first described a protocol for exosome purification from cell culture conditioned medium and body fluids. To date, this is the most commonly used and widely reported method. In the interim, there have been other methods developed involving trapping exosomes on beads bearing an antibody specific for exosomal surface molecule (194). In addition, commercially produced products/solutions are available for exosome isolation, for example the SystemBio Exoquick TC solution, which was also employed in this study.

2.6.4.1 Differential Ultracentrifugation

The process of differential ultracentrifugation involves a number of centrifugation, filtration and ultracentrifugation steps (141) as outlined in Figure 2.11.

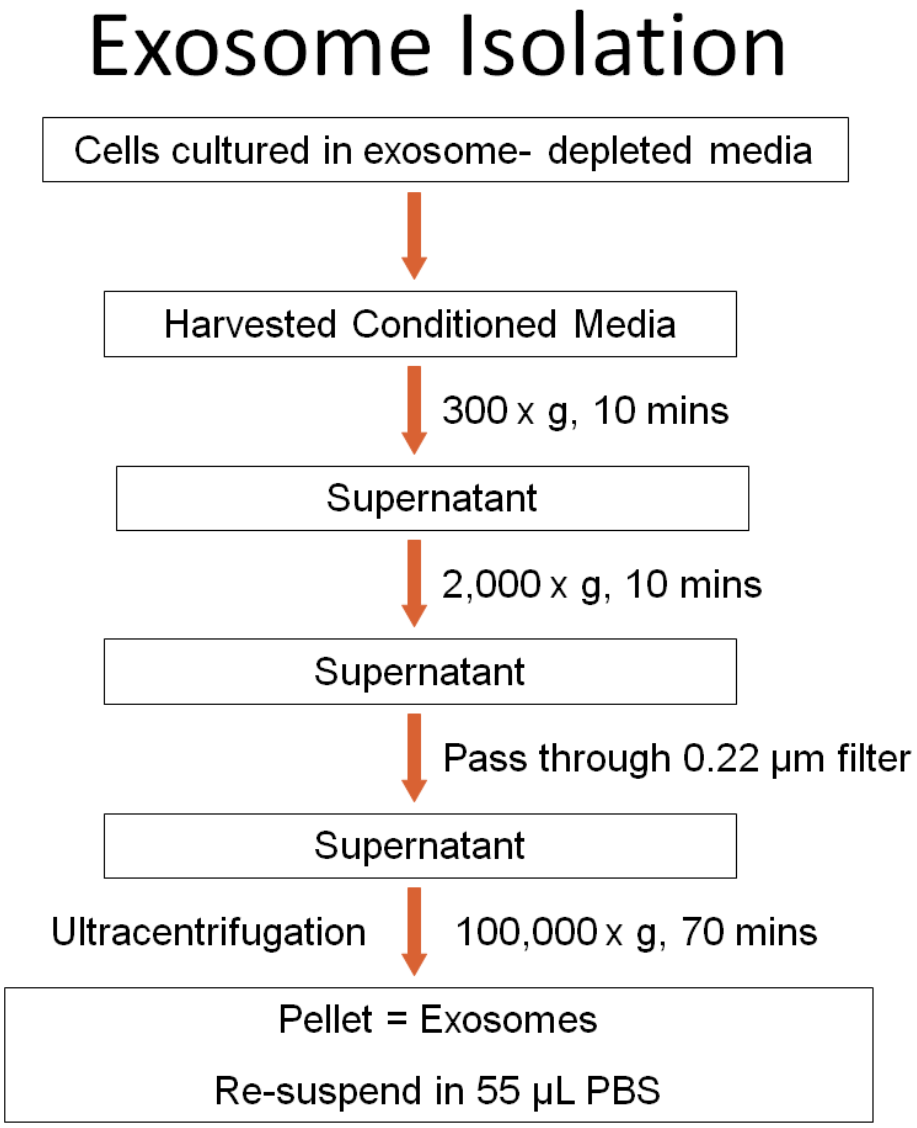


Figure 2.11 Protocol overview for isolation of exosomes from cell-conditioned media using Differential Ultracentrifugation.

Breast cancer cells were grown in the required exosome-depleted media. Cell-conditioned media was harvested following incubation for 48 hours. The cell-conditioned media was then treated as outlined in Figure 2.11. The first steps are designed to eliminate large dead cells and debris by successive centrifugation at increasing speeds. Following each step, the pellet was disposed to waste and the remaining supernatant was carried through to the next step. A 0.2 µm filter (Millipore, Billerica, MA, USA) was used to eliminate all cells greater than 200 nm, while keeping small membranes for further purification by ultracentrifugation. At this stage, if Transmission Electron Microscopy was required, a primary fixative is added, as described

later in section 2.6.6.1. The final supernatant was then placed into Optiseal Pollyallomar tubes (Beckmann Coultour, CA, USA) and ultra-centrifuged to pellet the small vesicles that correspond to exosomes. The resulting supernatant was aspirated and stored at -80°C until required for analysis. The exosome pellet was re-suspended in 55 µL of sterile PBS and stored at -80°C until required. Alternatively, if Western Blot analysis was required, the exosome pellet was resuspended in a protein lysis buffer, as described later in section 2.6.6.2. A Sorvall 100SE Ultra Centrifuge, with a Beckmann Fixed Angle 70Ti rotor was used for all exosome isolations.

2.6.4.2 ExoQuick-TC Solution™

ExoQuick-TC Solution exosome precipitation reagent (SBI, Mountain View, CA, USA) offers quick one-step isolation of exosomes from cell-conditioned media eliminating the need for an ultra-centrifuge. Breast cancer cells were grown in the required exosome-depleted media. Cell-conditioned media was harvested following incubation for 48 hours. The harvested media was then centrifuged at 3,000 x g for 15 minutes. The resulting supernatant was transferred to a fresh 15 mL falcon and 2 mL of the ExoQuick-TC solution was added. The mixture was inverted several times. The sample was then refrigerated overnight (minimum 12 hours) at 4°C. Following this, the sample was centrifuged at 1,500 x g for 30 minutes. A thick white/beige pellet appeared at the bottom of the tube and the supernatant was aspirated. The exosome pellet was re-suspended in 100 µL of sterile PBS and stored at -20°C until required.

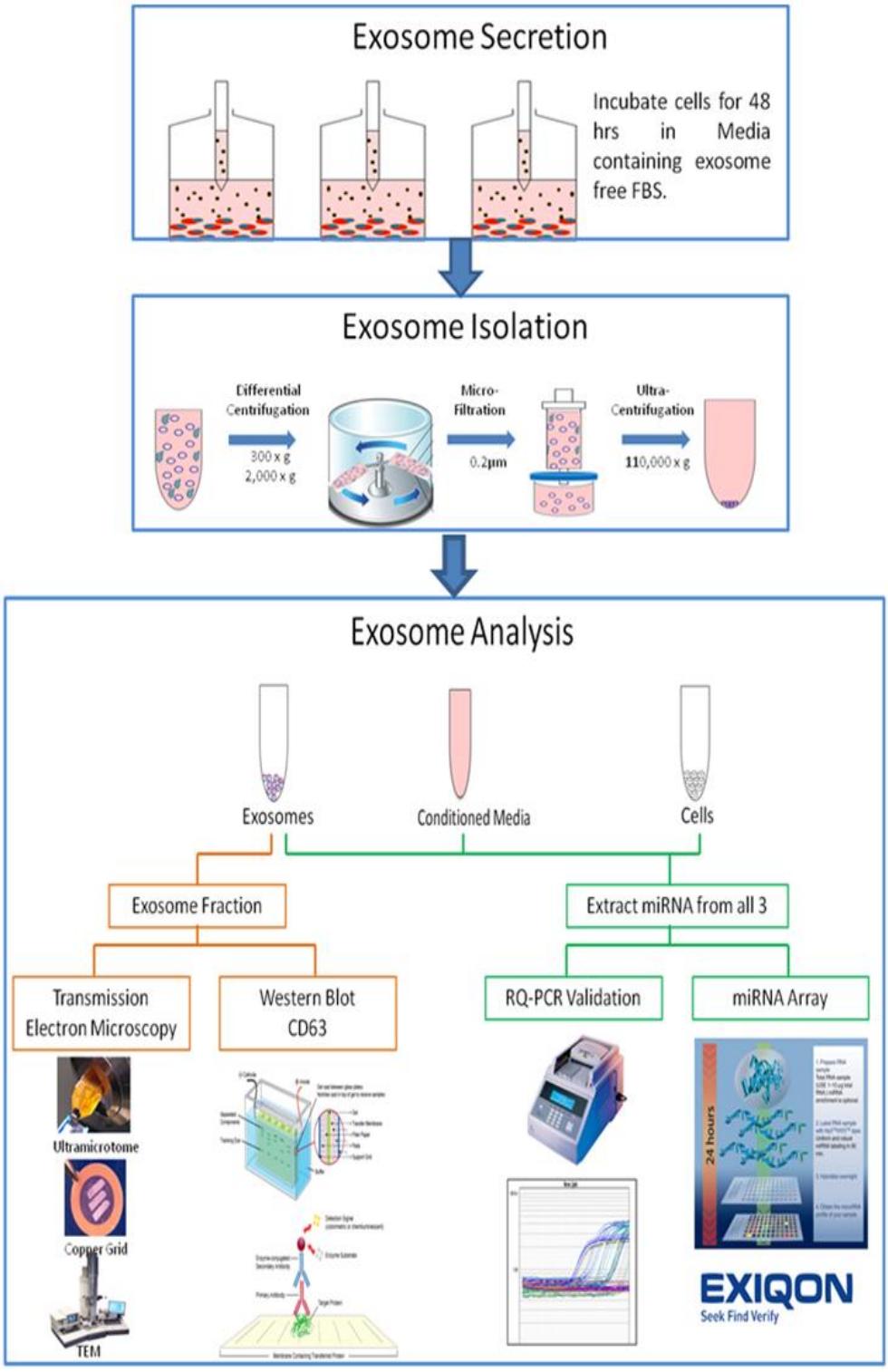


Figure 2.12 Overview of methods showing multiple applications used for downstream analysis of exosome fractions.

2.6.5 Protein content of exosomes

Two methods were employed to analyse the protein content of the isolated exosome fractions. First, purified exosomes were examined for protein content on the Nanodrop Spectrophotometer, ND-1000 (Thermo-Fisher, Waltham, MA, USA) at an absorbance of 280 nm. PBS was used to blank the instrument. This reading was used to give an indication that protein was present in the isolated exosomes. Secondly, the Micro BCA Protein Assay Kit (Pierce/Thermo Scientific, Cramlington, UK) was used for the colorimetric detection and quantitation of total protein in the isolated exosomes. This kit utilizes the bicinchoninic acid (BCA) method, which has been optimized for use with dilute protein samples (0.5-20 µg/mL). 5 µL of the isolated exosomes was added to 145 µL of lysis buffer. 150 µL of each sample and standard were pipette into microplate wells. 150 µL of the Working Reagent (WR) was added to each well the plate was mixed thoroughly on a plate shaker for 30 seconds. The plate was then sealed and incubated at 37°C for 2 hours. The plate was then cooled to room temperature and the absorbance at 562 nm was measured on the plate reader. The average 562 nm absorbance reading of the blank standard replicates was subtracted from the individual samples and standards. The standard curve was then formulated according to the manufacturer's instructions and the protein concentration of each sample was determined.

2.6.6 Characterisation of Exosomes

In order to confirm the successful isolation of exosomes from cell-conditioned media, it is essential to visualise the exosomes and also to detect exosome-associated proteins. Visualisation of isolated intact exosomes, ensuring they are of the correct size and morphology, is achieved using Transmission Electron Microscopy (TEM), while the detection of exosome-associated proteins such as CD63 is achieved using Western Blot analysis.

2.6.6.1 Transmission Electron Microscopy (TEM)

Intact exosomes were desired for visualisation using TEM, therefore a modification to the standard TEM preparation protocol was introduced. Prior to the final ultracentrifugation spin step during the exosome isolation protocol, a primary fixative was added to the cell-conditioned media supernatant in order to protect and maintain the architecture of the exosomes during high speed centrifugation (Figure 2.13).

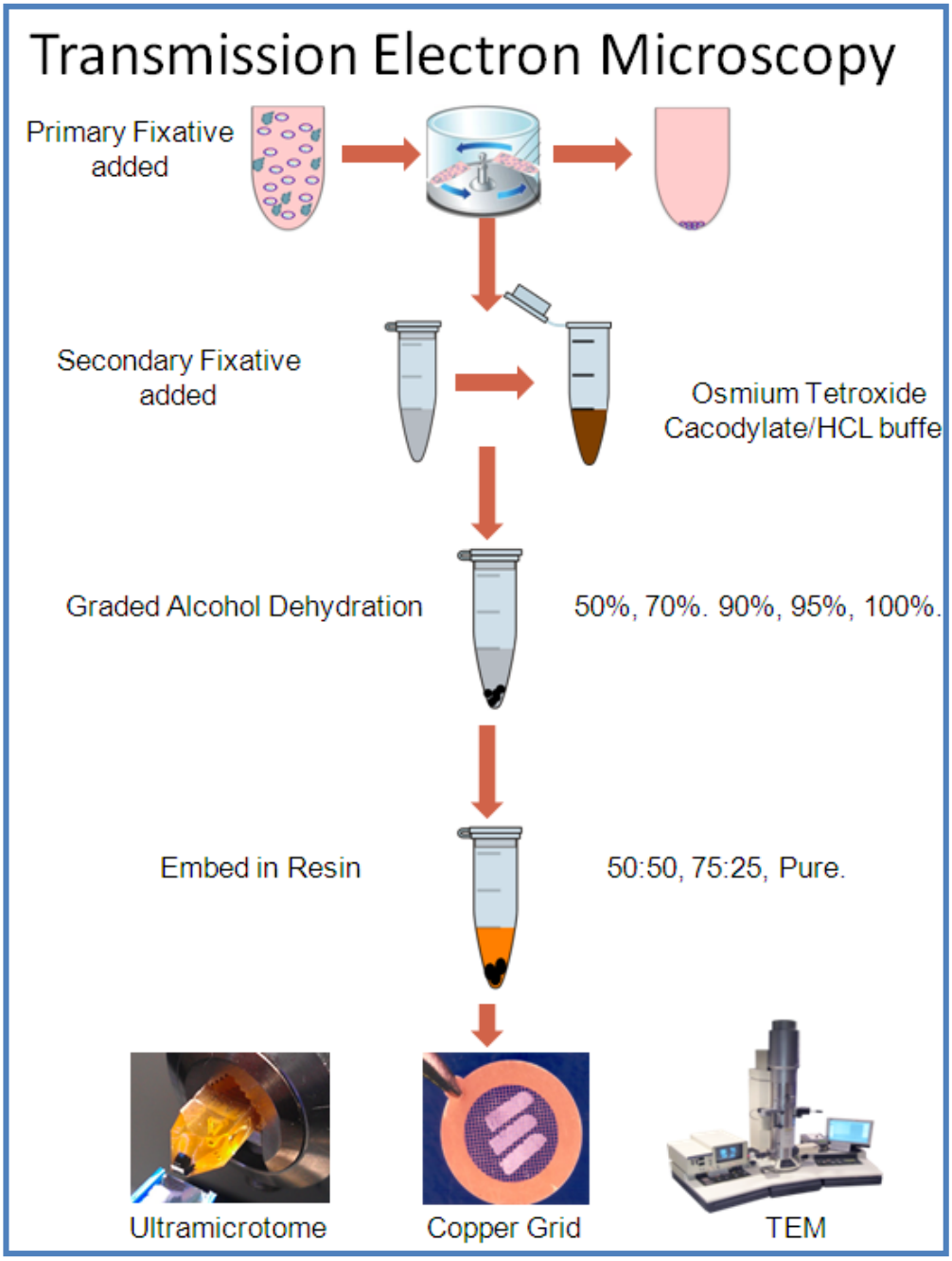


Figure 2.13 Workflow of TEM analysis of exosomes.

This primary fixative was composed of 2% Gluteraldehyde (Polysciences, Warrington, PA), 2% Paraformaldehyde in a 0.1M Sodium Cacodylate /HCL buffer, at a pH of 7.2. Following ultracentrifugation, the supernatant was removed and the exosome pellet was resuspended in 55 µL of sterile PBS. The re-suspended isolated exosomes were then treated with a secondary fixative composed of 1% Osmium Tetroxide in 0.1M Cacodylate/HCL buffer at a pH of 7.2. 2 mL of the secondary fixative was added to 2 mL of sterile PBS and ~200 µL was added to the isolated exosomes. Samples were vortexed for 30 seconds to mix. The samples were allowed to stand at room temperature for approximately 2 hours, causing a density gradient to form resulting in a black/brown pellet. Following this, the osmium supernatant was aspirated to waste into a 2% solution of ascorbic acid, which serves to neutralise the osmium toxicity, taking care not to disturb the black/brown pellet. The pellets were then dehydrated with graded alcohol steps (50%, 70%, 90%, 95%, and pure ethanol). Each dehydration step was performed for 15 minutes twice. The pure ethanol was aspirated to waste and Propylene Oxide (Sigma-Aldrich®) was added for 20 minutes twice to act as a transition solvent between the final alcohol stage and the following impregnation with an Epon based resin (Ladd Industries, Burlington, VT). The resin was prepared using a commercial kit. The exosome pellets were placed into a 50:50 mixture of resin and Propylene Oxide for 2 hours. The resin was then replaced with a 75:25 mixture of resin and Propylene Oxide for 4 hours. The resin was then replaced again with pure resin overnight. The following morning the pure resin was replaced at least twice and the sample was placed into a 65°C oven to incubate for at least 48 hours to allow the sample to polymerise. After polymerisation, the resin blocks were trimmed to expose the embedded exosomes. Sections of 1 micron thickness were cut onto glass slides with 1% Toluidine Blue and were viewed using a light microscope. These sections were referred to as ‘Scout Sections’ as they allowed preliminary analysis of the sample structure and components. Regions of interest were selected for subsequent trimming using an ultramicrotome (Reichert-Jung Ultracut E) and ultrathin sections of thickness 80 nm-100 nm were cut and lifted onto copper grids. The grids were then stained in 1.5% aqueous Uranyl Acetate for 30 minutes and Lead Citrate for 10 minutes. The

sections were allowed to dry and were then viewed using a Hitachi H7000 Transmission Electron Microscope.

2.6.6.2 Western Blot Analysis

Western Blot Analysis, targeting the exosome-associated protein CD63, was used to confirm that exosomes had successfully been isolated. An important step in this experiment was to use a suitable protein isolation method prior to loading on to the Western Blot. It was also crucial to measure the amount of total proteins present in the purified exosome fractions, as this allowed the correct amount of exosome protein lysate to be loaded onto the western blot. This also gave an estimate of the amount of exosomes secreted by the cells. Following determination of the protein content of the isolated exosome fractions using the Micro BCA Protein Assay, the appropriate concentration of the fractions was calculated for use in the western blot analysis. BioRad Mini-Protean TGX Precast Gels (4-15%), 10-well comb, 30 µL/well, were used in this study. Each sample was mixed with 4X sample buffer, 10X reducing agent, at a final volume of 10 µL, and were placed in the PCR sprint machine to denature at 70°C for 10 minutes. The gels were positioned in the clamp assemblies and the chamber was filled with 10x Tris-Glycine-SDS Running Buffer (2.5 mM Tris/19.2 mM Glycine/0.1% SDS/H_2O). Each well was loaded with the appropriate sample with the Molecular Weight (MW) marker added to the first and last well. The gel was run at 100 V for 1 hour. The gels were placed on nitrocellulose membranes to transfer the proteins. The gel sandwich was prepared in the following order: fibre pad, filter paper, equilibrated gel, nitrocellulose membrane, filter paper, fibre pad as outlined in Figure 2.14.

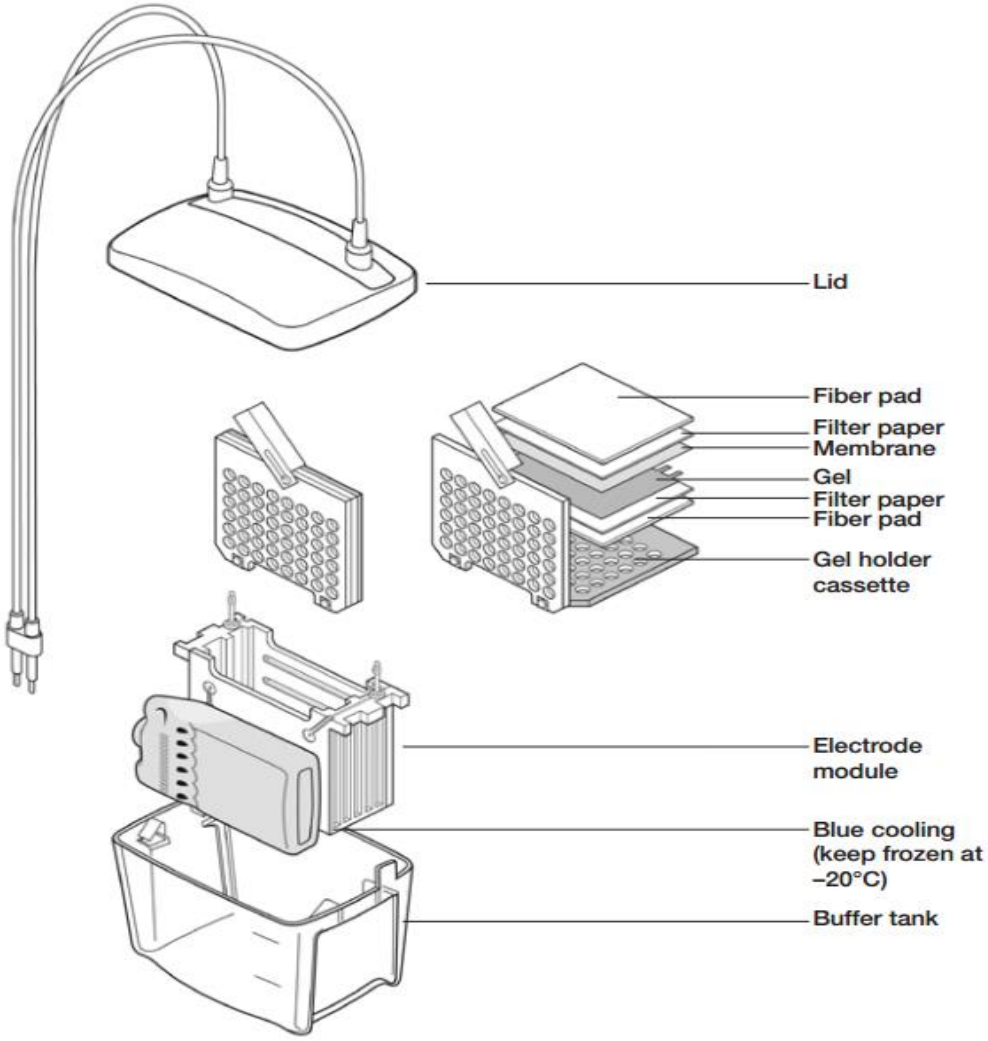


Figure 2.14 Mini Trans-Blot Cell set-up and assembly of parts for transfer of protein from gel to membrane (Adapted from Mini Trans-Blot® Electrophoretic Transfer Cell Instruction Manual, BIO-RAD).

The filter papers, nitrocellulose membranes and fibre pads were pre-soaked in 1x Transfer Buffer (2.5 mM Tris/19.2 mM Glycine/20% Methanol/H_2O). The transfer was run at 100 V for 30 minutes. The nitrocellulose membrane was then removed from the sandwich, placed into 5% milk solution, and mixed on a shaking tray at 120 RPM for 1 hour. The membrane was removed from the milk solution and placed into a washing buffer: TBS-Tween (0.5 M Tris/1.5M NaCl/0.05% Tween/H_2O). The membrane was washed at 120 RPM for 15 minutes, followed by two further 5 minute washes in the washing buffer. A 1:1,000 dilution of the primary antibody (abcam rabbit polyclonal to CD63: 100 µg at 1 mg/mL), in 0.1% milk solution (Blocking Buffer) was

added to the membrane and mixed at 120 RPM overnight at 4°C and covered to minimise evaporation. The following day, the membrane was washed in the washing buffer for 15 minutes, followed by two 5 minute washes. A 1:3,000 dilution of the secondary antibody (abcam goat anti-rabbit IgG HRP: 1 mg at 2 mg/mL), in 0.1% milk solution (Blocking Buffer) was added to the membrane and mixed at 120 RPM for 90 minutes. The membrane was washed in the washing buffer for 15 minutes, followed by four 5 minute washes. The Supersignal West Pico Chemiluminescent Substrate was then prepared according to the manufacturers instructions, immediately prior to use. The membrane was placed on clingfilm and the substrate solution was added to the membrane and allowed to incubate for 5 minutes at room temperature. The membrane was then removed from the substrate solution, blotted on filter paper, placed into clean fresh cling film and covered over. Using the Syngene G-Box and GenSnap software the images were captured every five minutes for 30 minutes.

2.6.7 Exosome microRNA extraction

Exosomes were isolated using the methods described previously (differential ultracentrifugation and ExoQuick-TC Solution™) and were resuspended in 55 µL of sterile PBS. microRNA was isolated using the miRVana miRNA isolation kit as per the manufacturer's instructions. 300 µL of lysis binding solution was added to the re-suspended exosome pellet and vortexed. 1/10 volume of miRNA homogenate additive was added to the lysate, vortexed and left on ice for 10 minutes. An equal volume of acid-phenol:chloroform was added and the sample was centrifuged for 15 minutes at maximum speed at 4°C. The resultant clear upper aqueous phase was removed to a fresh tube. 1/3 volume of 100% ethanol was added to the aqueous phase and vortexed. 700 µL of the lysate was placed into the spin cartridge and centrifuged for 15 seconds at 10,621 x g at 4°C. The filtrate was collected to a fresh collection tube. This was repeated until all the lysate had passed through the spin cartridge which was then discarded. 2/3 volume of 100% ethanol was added to the filtrate and vortexed. A new spin cartridge was added to the collection tube and 700 µL of the lysate was placed into the spin cartridge and centrifuged for 15 seconds at 10, 621 x g at 4°C. The filtrate was discarded to

waste and the step was repeated until all the lysate was passed through the spin cartridge. 700 µL of Wash Solution 1 was added to the spin cartridge and centrifuged for 15 seconds at 10,621 x g at 4°C. The flow through was discarded to waste. 500 µL of Wash Solution 2/3 was added to the spin cartridge and centrifuged for 15 seconds at 10,621 x g at 4°C. The flow through was discarded to waste and the step was repeated again but was centrifuged for 1 minute to dry the membrane. The spin cartridge was transferred to a fresh collection tube and 100 µL of pre-heated (95°C) elution solution was added to the membrane. This was left to stand for 5 minutes and was then centrifuged for 1 minute at 10,621 x g at 4°C. The eluate was collected and transferred to an RNA free tapered collection tube and stored at -80°C until required.

2.6.8 microRNA profiling of Exosomes

2.6.8.1 Exiqon miRCURY™ LNA Array

Exosomes were harvested from 4 breast cancer cell lines: T47D, SK-BR-3, MDA-MB-231 and BT-20. The RNA content was extracted from each exosome sample using the miRVana miRNA isolation kit. Trizol was not used in these extractions as it was reported by Kim *et al.* (195), that structured small RNAs with low GC content are not recovered efficiently when isolating RNA with Trizol. Following extraction the RNA quantity and quality was determined using the Nanodrop Spectrophotometer and Agilent Bioanalyser, as detailed previously. The RNA samples (2 µg per sample) were then sent to Exiqon Services in Denmark for analysis. Three biological replicates per sample were examined to allow statistical tests of data comparisons. Exiqon re-assessed the quality of the total RNA by an Agilent 2100 Bioanalyzer profile. The RNA samples were labelled with Hy3™ and Hy5™ fluorophores using the miRCURY™ LNA microRNA Hi-Power Labelling Kit. The Hy3™ labelled samples and a Hy5™ labelled reference RNA sample were mixed pair-wise and were then hybridized to the miRCURY™ LNA microRNA Array 7th Gen (Exiqon, Denmark) according to our specified experimental set-up. The array contained captured probes targeting all miRNAs for human, mouse, or rat registered in the miRBASE 18.0 (2083). 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. The miRCURY LNA™ microRNA Array slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and the image analysis was carried out using the ImaGene 9.0 software (BioDiscovery, Inc., USA). After scanning, a technical quality assessment of the data was performed based on results from spike in controls, flagging of spots, background intensity levels and signal intensity distribution. The quantified signals were background corrected (Normexp with offset value 10 (196)) and normalized using quantile normalization method. After normalization, the data was assessed by Principal Component Analysis (PCA) and two way hierarchical clustering heat-maps. PCA is a method used to reduce the dimensions of large data sets to lower dimensions for analysis, thereby determining the key features of high-dimensional datasets. In this array data, PCA clustered arrays by groups of the most significantly dysregulated miRNAs, first by the most significant group and then by progressively less significant groups. PCA 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 is obtained. Two-way hierarchial clustering was performed using the complete-linkage method together with the Euclidean distance to normalised values of 2083 targets across all 4 breast cancer cell lines. The top ranked miRNAs for each sample were then validated by RQ-PCR, as described previously.

2.6.9 Exosome Transfer

2.6.9.1 Visualisation - Confocal Microscopy

Confocal microscopy was performed to visualise the transfer of exosomes to recipient cells. The recipient cells were grown on UV sterilised glass coverslips in a 6 well cell culture plate. Cells were seeded at approximately 2 x 10^5 per well and were allowed to adhere overnight at 37°C. The previously transduced T47D-504 cell line (protocol discussed in section 2.4) was used to secrete exosomes as the donor cell population, as the transduced cells already contained a Red Fluorescent Protein (RFP) in the lentiviral construct used to transduce the cells. The RFP tagged transduced cells were used as it eliminated the need to fluoresecently tag an alternative batch of cells (Figure 2.15).

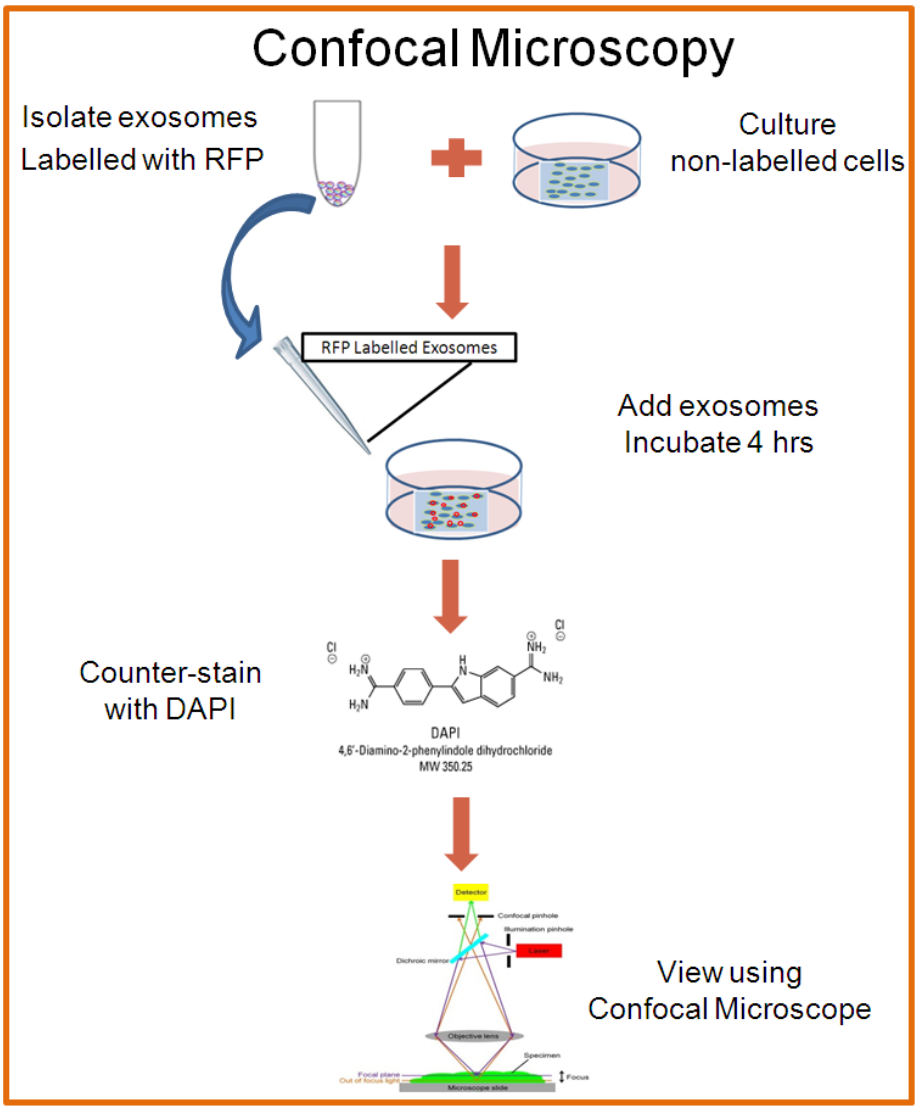


Figure 2.15 Schematic of exosome transfer set-up for confocal microscopy.

Exosomes were harvested as described previously in section 2.6.4, however in this setup the resultant pellets were re-suspended in a smaller volume of sterile PBS, ~30 µL. A humidification box was prepared with moist tissue paper lining the bottom. A strip of parafilm was placed into the box. The coverslips with the adhered recipient cells were removed from the 6-well plate and placed cell side up, on to the parafilm in the humidification box. 20 µL of fresh exosome-depleted media and 20 µL of sterile PBS were placed onto the control coverslip. 20 µL of fresh exosome depleted media, and 20 µL of exosomes harvested from RFP labelled cells, were placed onto the test coverslips. A small piece of parafilm was placed directly on top of each coverslip, causing the liquid to cover the entire surface of the coverslip. The humidification box was then allowed to incubate at 37°C for 4 hours. The coverslips/cells were removed from the humidifcation box and immersed in sterile PBS (in a 6 well plate). The cells were then fixed in 4% paraformaldehyde/PBS for 10 minutes. This was followed by another rinse in sterile PBS. 1 mL of a 0.1% Triton X-100/PBS solution was added to each coverslip for 10 minutes to permeabilise the cells. 1 mL of a 5% Foetal Calf Serum/0.1% Triton X solution was then added to each coverslip and covered for 1 hour. This step was to block any non-specific binding of antibodies. Each coverslip was then washed three times in 0.1% Tritron X-100/PBS. Alexaflour 488 Phalloidin was diluted 1:100 and 30 µL was placed on small squares of parafilm in the humidifcation box. The coverslips were placed cell side down on each square of parafilm and allowed to incubate at 37°C for 1 hour. Each coverslip was then washed three times in 0.1% Triton X-100/PBS. A 1:1000 dilution of DAPI (stock solution: 1 mg/mL) in 0.1% Triton X-100/PBS was added to each cover slip in a 6 well plate, placed in the dark, and left to stand for 2 minutes. Each coverslip was rinsed three times in PBS. 20 µL of slowfade gold anti-fade reagent (Invitrogen) was added to a glass microscope slide. 9 µL of mounting media was also added and the coverslip was placed cell side down onto the slide. The edges were gently blotted and the corners were sealed with nail varnish. The slides were labelled and stored in the dark until required for visualisation on the confocal microscope. 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. All Z stack images were processed using Andor IQ 2.3 software and all images are presented as maximum intensity projections (MIP).

2.6.9.2 Functional effects of exosome transfer

Detection of miRNA enrichment into exosomes using RQ-PCR

Exosomes were harvested from T47D-Wildtype cells, T47D-NTC cells and T47D-504 cells, as described previously. miRNA was extracted from the exosomes, conditioned media and cells, and RQ-PCR was performed targeting miR-504.

Impact of exosome transfer on miRNA expression in recipient cells

Two 12-well cell culture plates were seeded with T47D-Wildtype cells and two 12-well cell culture plates were seeded with MDA-MB-231 cells, each at a density of 1.6 x 10^5 per well. The cells were allowed to adhere overnight. Two plates per cell type were seeded so as to allow 4 hour and 24 hour transfer incubations. Exosomes were harvested from T47D-NTC cells and T47D-504 cells, as described previously, however the exosome pellets were re-suspended in 900 µL of fresh exosome-free media. The wells were rinsed twice with sterile PBS. 100 µL of exosomes and 1.1 mL of fresh exosome free media were added to each well (Figure 2.16).

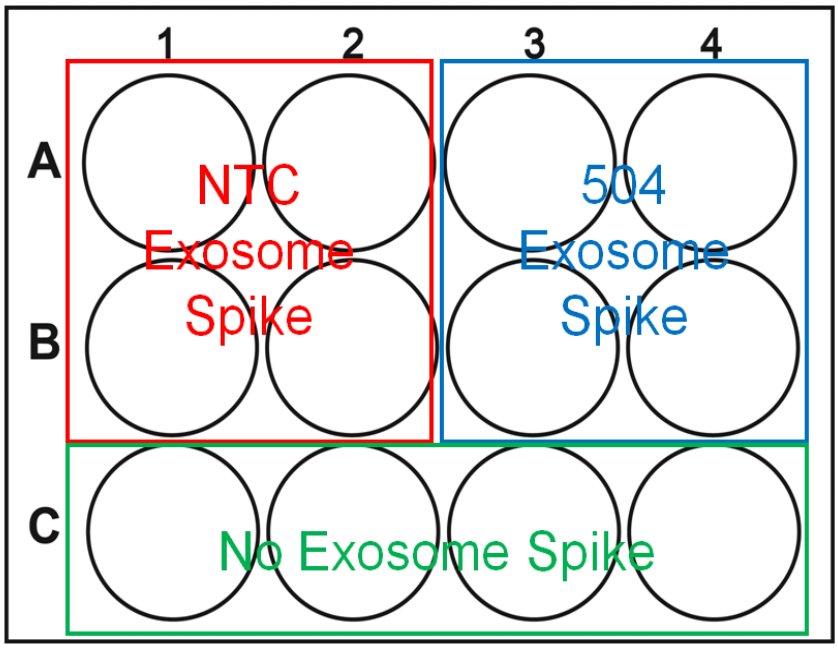


Figure 2.16 Twelve-well plate plan for exosome transfer experiments.

Fresh exosome free media alone was added to the control wells. Following the required transfer incubation time (4 hrs/24 hrs), the spent media was removed and the wells were rinsed twice with sterile PBS. The RNeasy® RNA isolation method was used to extract RNA from the recipient cells, where 500 µL of trizol was added directly to each well, combining two wells as one sample, resulting in 6 RNA samples per plate. The manufacturers' protocol was followed, as described previously. MiRNA expression analysis was performed by RQ-PCR on the extracted RNA as described previously.

Impact of exosome transfer on cell proliferation in recipient cells

Exosomes were harvested from T47D-NTC and T47D-504 cells and were transferred to T47D-Wildtype cells seeded at 4 x 10^4 in a 96-well plate. Control cells included T47D cells growing in regular media and T47D cells growing in exosome free media (Figure 2.17).

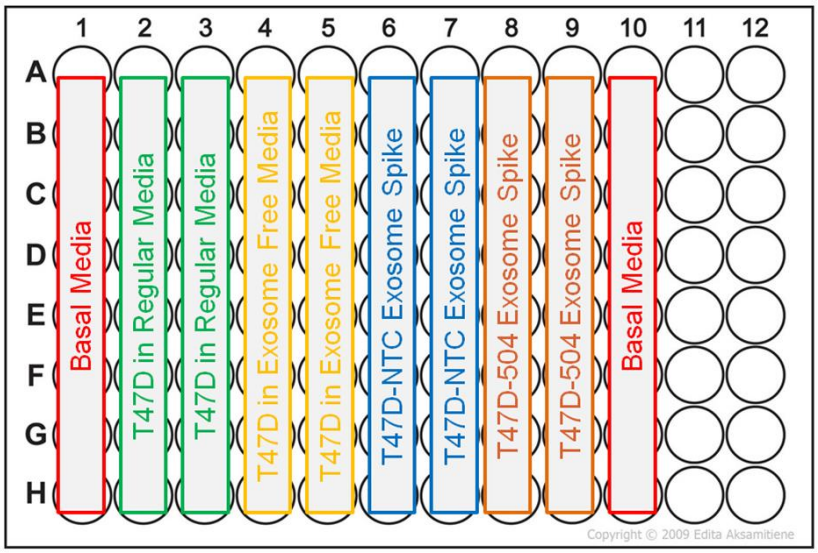


Figure 2.17 Cell proliferation measured using the CellTiter 96® AQueous Non-Radioactive Cell Prolideration Assay.

At the appropriate time point, e.g. 48/72 hours, 20 µL of 5 mg/mL MTT solution was added to each well. The plates were returned to the incubator at 37°C for 3.5 hours. Following this, the plate was read on the Bio-Rad microplate reader at 490 nm.

Impact of exosome transfer on cellular migration in recipient cells

Exosomes were harvested from T47D-NTC cells and T47D-504 cells, and were transferred into MDA-MB-231 and T47D cells growing in a 12-well plate (Figure 2.16). Following exosome uptake, the cells were trypsinised, counted and seeded into Corning Transwell Inserts at a density of 7.0 x 10^4. Each suspension of cells were prepared in duplicate, with 2% FBS in the lower compartment as the chemoattractant. The plates/inserts were returned to the incubator at 37°C for 18 hours. The porous membranes were treated, excised and viewed as previously described in section 2.4.3.

Chapter 3

microRNA dysregulation in breast cancer and the influence of menstrual cycle hormones

3 Chapter 3: microRNA dysregulation in breast cancer and the influence of menstrual cycle hormones

3.1 Introduction

miRNAs are now known to play a central role in a wide range of biological and pathological processes, most notably cancer development and progression. Previous reports, with a focus on breast cancer specifically, have shown that the expression levels of certain miRNAs differ between malignant and normal breast tissues. Indeed, these differences are capable of classifying tumours according to clinicopathological variables, such as proliferation index, nodal status, and tumour stage (74, 80). Moreover, differential expression has been shown across the four clinically utilised molecular subtypes of breast cancer i.e. Luminal A, Luminal B, Her2/neu and Basal-like (83). Using a murine model of breast cancer, our laboratory recently reported significant dysregulation of circulating miR-106a, miR-202, miR-138, and miR-191, during disease progression (197). Furthermore, dysregulation of these miRNAs was investigated in the circulation of breast cancer patients. Therefore, the first part of this study was to investigate if these changes in the circulation were reflective of dysregulation in the primary tumour microenvironent. This was achieved by examining the presence of these miRNAs in patient breast tumour tissue samples and by identifying any dysregulation when compared to patient normal healthy controls. This is a very exciting area of research as it highlights the overwhelming potential of identifying miRNA signatures, or miRNA fingerprints, to be used as diagnostic and prognostic indicators of disease, providing a more individualised therapeutic regime.

To further add to this overwhelming potential, one of the most exciting findings in the last decade was by Mitchell *et al.* (88), who discovered miRNAs to be present in the circulation, and moreover to be remarkably stable. As a result of this finding, there has been widespread interest in miRNAs as potential non-invasive circulating biomarkers of disease. In particular, in the breast cancer setting, miRNAs have been discovered to be dysregulated in the circulation of patients with breast cancer (198, 199). It is

crucial, however, to first establish that these findings are not being affected by normal physiological processes. Although the measurement of circulating miRNAs is indeed very promising as a non-invasive biomarker, it is a 'point in time' measurement. Therefore, the relationship between circulating hormones and miRNAs must be investigated. In hormone responsive cancers, such as breast cancer, it is essential to determine whether levels of circulating miRNAs fluctuate with variations in menstrual hormones (Figure 3.1).

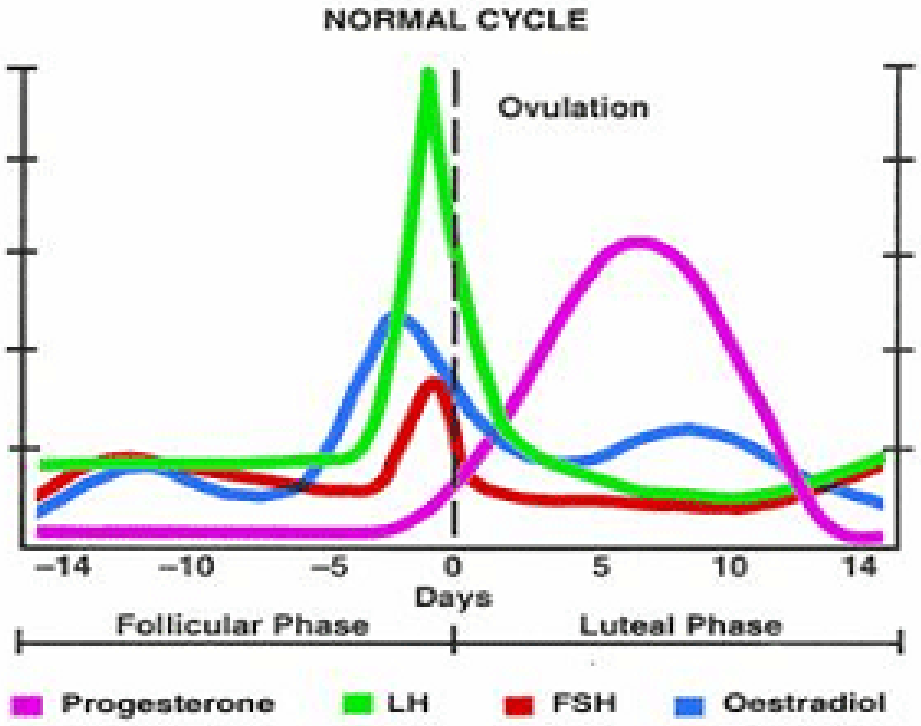


Figure 3.1 Hormone fluctuations expected during a typical menstrual cycle (200).

The onset of menstruation is associated with the expression of a complex network of highly active substances with inflammatory and immune related activities. The effect of a breast cancer patients menstrual status has been widely studied and is considered when determining therapeutic treatments (201-204). For example, Vascular Endothelial Growth Factor (VEGF) is a very potent angiogenic cytokine and it has been established that the reduced serum levels of VEGF in the luteal phase of the menstrual cycle provides evidence of a more suitable time point for surgical intervention of pre-menopausal breast cancer patients (205, 206). In 2010, Kuokkanen *et al.*

(207), suggested in a study focusing on the human endometrial tissue, that certain miRNAs are differentially expressed during the phases of the menstrual cycle, suggesting that these miRNAs are hormonally regulated. Currently, however, there are no published reports on the relationship, if any, between steroid hormone fluctuations associated with the menstrual cycle and miRNA expression levels in the circulation. Given the emerging role of miRNAs as potential biomarkers in the clinical setting, it is essential to evaluate the relationship between menstrual cycle hormones and miRNA expression.

3.2 Aims

The aims of this study were as follows:

- To investigate the expression levels of miR-106a, miR-202, miR-138 and miR-191 in patient breast tumour tissues and compare them to normal healthy tissues and to further investigate the relationship with clinicopathological characteristics.
- To investigate the potential fluctuation of circulating miRNA levels throughout the menstrual cycle and to determine any relationship with circulating hormones.

3.3 Materials and Methods

Patient Tissue Samples

Following informed patient consent, breast tissues were harvested in theatre during surgical resection including malignant (n = 55), benign (n = 15) and normal healthy controls (n = 40) harvested during breast reduction mammoplasty, with the patient clinicopathological details listed in Table 3.1.

Number of Patients	*Total n= 110*		
Tissue Type	**Malignant**	**Benign**	**Normal**
***n* =**	**55**	**15**	**40**
Epithelial Subtype	***n***		
Luminal A	18		
Luminal B	7		
HER2	14		
Basal	16		
Tumour Grade	***n***		
Grade 0	2		
Grade 1	2		
Grade 2	12		
Grade 3	38		
Tumour Stage	***n***		
Stage 1	13		
Stage 2	24		
Stage 3	9		
Stage 4	3		
Menopausal Status	***n***		
Pre	15		
Peri	2		
Post	33		

Table 3.1 Patient clinicopathological characteristics.

Each sample underwent Trizol® homogenisation, followed by extraction using the RNeasy™ miRNA isolation kit (Qiagen), as detailed in section 2.3.3.4. Samples were then amplified by RQ-PCR targeting miR-106a, miR-

202, miR-138, miR-191 and the endogenous controls miR-16 and let-7a. Any relationship between miRNA expression and patient clinicopathological characteristics was also investigated.

Healthy Volunteer Blood Samples

Plasma samples with pre-established levels of circulating hormones and chemokines were obtained from the Discipline of Surgery Biobank. The samples had previously been collected from 11 healthy premenopausal female volunteers every week for four consecutive weeks (total n = 44). miRNA was extracted from the plasma samples using the mirVana miRNA isolation kit (Ambion), as described previously. RQ-PCR was then performed targeting miRNAs which have previously been associated with carcinogenesis, including miR-16, miR-195, miR-497, miR-379, and miR-106a, with the endogenous control miR-122.

3.4 Results

3.4.1 miRNA expression in patient breast tissue samples

Each miRNA of interest was detectable in all malignant, benign and normal breast tissue samples (n = 110). miR-106a was found to be significantly elevated in breast tumours compared to healthy breast tissue ($p<0.001$, Figure 3.2).

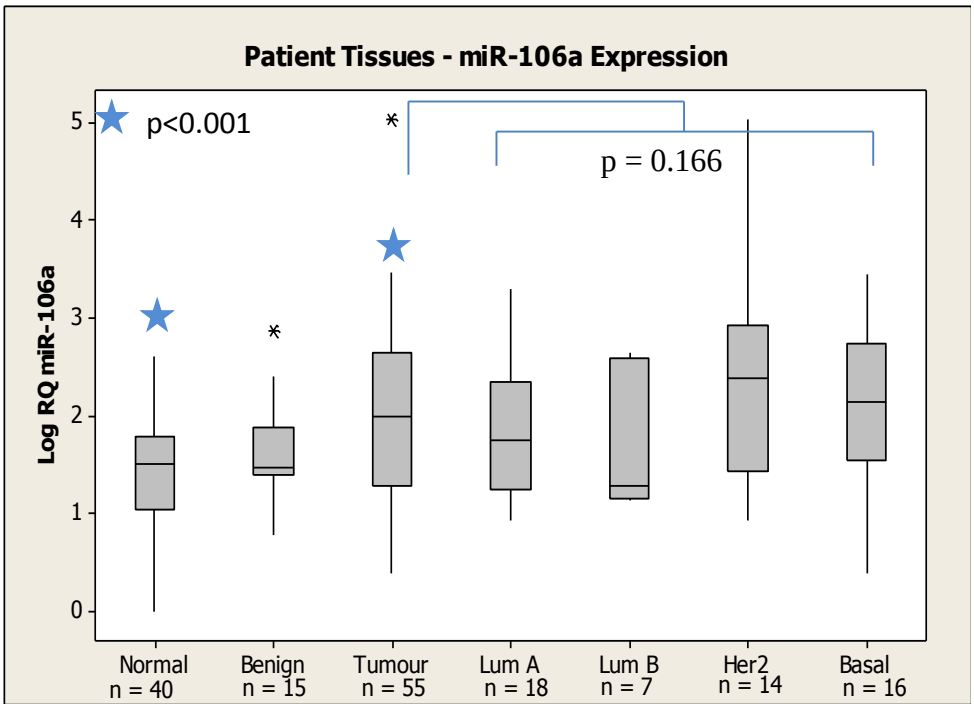


Figure 3.2 miR-106 levels in tissue of breast cancer patients and healthy controls. ★ represents a comparison between Tumour and Normal samples, while '*' respresents outliers within the group. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

When subdividing the tumours based upon their epithelial subtypes, the upregulation of miR-106a observed in the tumours, as a collective group held true (Figure 3.2). No significant dysregulation was observed across the epithelial subtypes. In addition, no significant dysregulation was observed across patient clinicopathological characteristics such as tumour stage, grade, or menopausal status (see appendix 9.3).

No significant dysregulation was observed with miR-138 expression when comparing tumour to normal tissue (Figure 3.3).

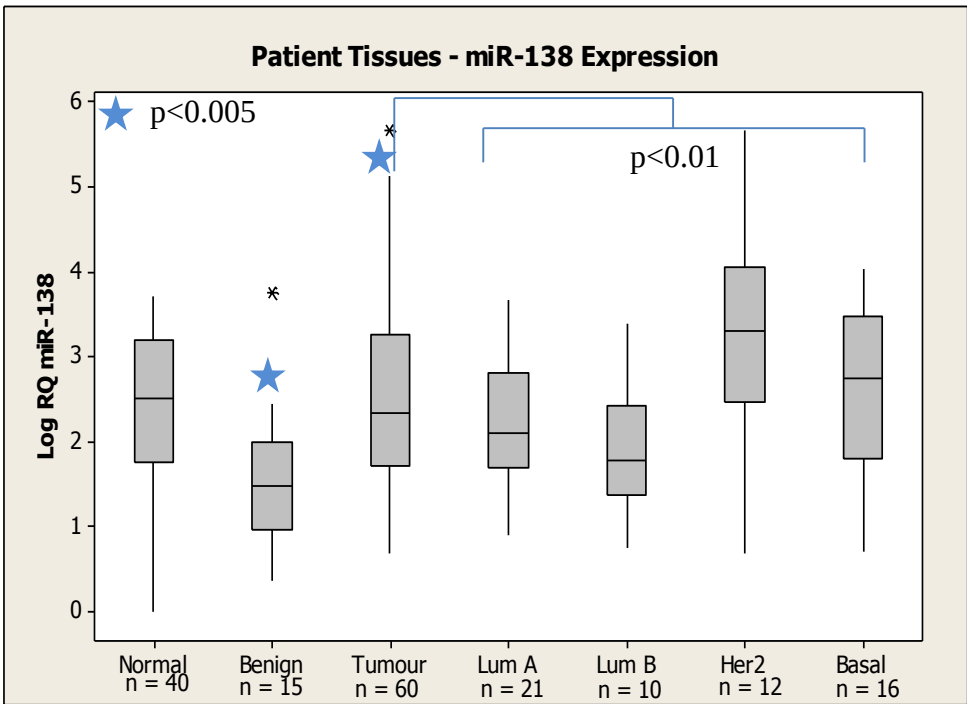


Figure 3.3 miR-138 levels in tissue of breast cancer patients and healthy controls. ★ represents a comparison between Tumour and Benign samples, while '*' respresents outliers within the group. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

However, miR-138 was found to be significantly downregulated in benign compared to tumour tissue ($p<0.005$, Figure 3.3). Interestingly, a significant dysregulation across epithelial subtypes was observed with miR-138 (ANOVA $p<0.01$), with the highest levels detected in the HER2 subtype tissues (Figure 3.3). In addition, no significant dysregulation was observed across patient clinicopathological characteristics such as tumour stage, grade, or menopausal status (see appendix 9.3).

No significant dysregulation of miR-202 expression was observed across all of the breast tissue samples (p = 0.902, Figure 3.4).

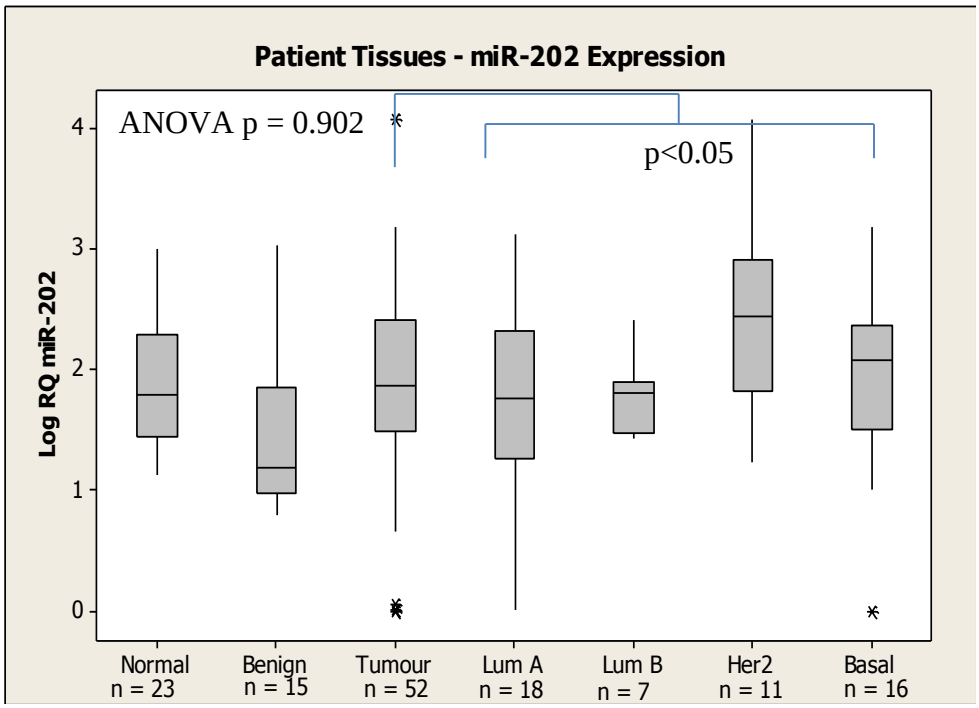


Figure 3.4 miR-202 levels in tissue of breast cancer patients and healthy controls. '*' respresents outliers within the group. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

Significant dysregulation, however, was observed across epithelial subtypes (Figure 3.4). Similar to findings with miR-138, the highest expression level was observed in the HER2 subtype tissues. In addition, no significant dysregulation was observed across patient clinicopathological characteristics such as tumour stage, grade, or menopausal status (see appendix 9.3).

A significant upregulation of miR-191 ($p<0.001$, Figure 3.5) was found in tumour when compared to normal breast tissue.

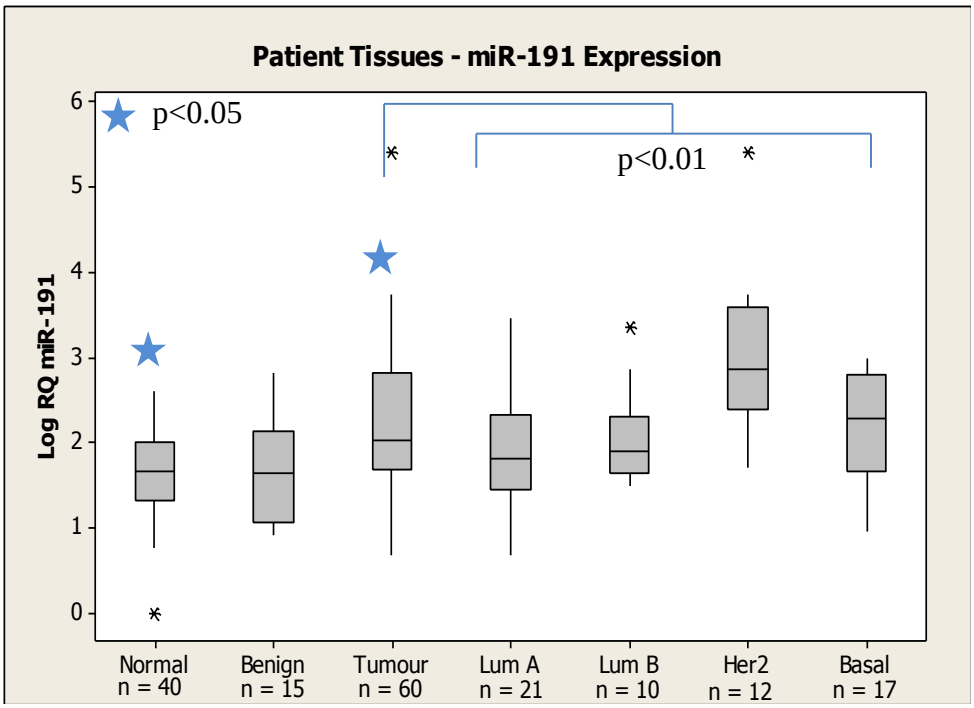


Figure 3.5 miR-191 levels in tissue of breast cancer patients and healthy controls. ★represents a comparison between Tumour and Normal samples, while '*' respresents outliers within the group. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

Interestingly, a significant dysregulation across epithelial subtypes was also observed with miR-191 (ANOVA $p<0.001$), with the highest levels detected in the HER2 subtype tissues (Figure 3.5). In addition, no significant dysregulation was observed across patient clinicopathological characteristics such as tumour stage, grade, or menopausal status (see appendix 9.3).

miR-106a and miR-191 were both observed to be significantly upregulated in tumour tissues when compared to normal healthy tissues. Therefore, it was hypothesized that the combination of these two miRNAs, rather than assessing them individually, would provide superior sensitivity and specificity values for the purpose of differentiating between malignant and normal tissue. In terms of a clinical test, sensitivity refers to its ability to detect the presence of a disease, while specificity refers to its ability to detect the absence of a disease. Following statistical analysis using binary logistic regression, the combination of miR-106a and miR-191 produced sensitivity and specificity values of 70% and 60%, respectively (Figure 3.6).

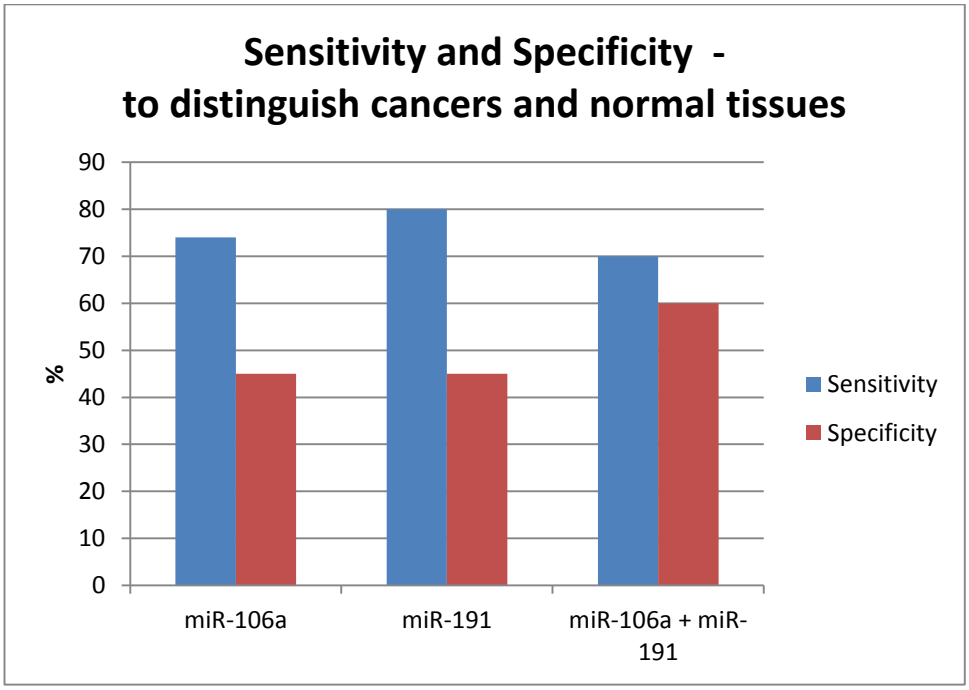


Figure 3.6 Sensitivity and Specificity for miR-106a and miR-191 to differentiate between Cancer and Control.

This produced a slight decrease in sensitivity when comparing the miRNAs individually, even though the specificity was significantly increased.

miR-138 and miR-191 were both observed to display significant dysregulation across the epithelial subtypes of breast cancer, with the highest levels observed in the HER2 subgroup for both miRNAs. Therefore, it was hypothesised that the combination of these two miRNAS would provide

superior sensitivity and specificity values, for epithelial subtype specificity. Following statistical analysis using binary logistic regression, the combination of miR-138 and miR-191, produced sensitivity and specificity values which were in most instances higher than the miRNAs examined individually (Table 3.2).

	HER2 vs Lum A	HER2 vs Lum B	HER2 vs Basal
miR-138	Sensitivity = 60% Specificity = 90%	Sensitivity = 79% Specificity = 60%	Sensitivity = 40% Specificity = 65%
miR-191	Sensitivity = 67% Specificity = 86%	Sensitivity = 79% Specificity = 70%	Sensitivity = 67% Specificity = 55%
miR-138 + miR-191	Sensitivity = 67% Specificity = 86%	Sensitivity = 86% Specificity = 70%	Sensitivity = 67% Specificity = 75%

Table 3.2 Sensitivity and specificity for miR-138 and miR-191 to display epithelial subtype specificity.

3.4.2 Relationship between menstrual cycle hormones and circulating miRNAs

Each volunteer had a normal ovulatory cycle. The Leutenizing Hormone (LH) peak was taken to denote mid-cycle, while the mid-luteal phase was marked by a peak in systemic Progesterone. Other phases of the cycle were extrapolated from these two time points. In general, miRNA concentrations ranging between 30-300 ng/µL per of miRNA were obtained per sample. Each miRNA of interest was detected in all samples (n = 44), with the exception of miR-497, which was only detectable in half the samples (n = 22/44). Five samples were excluded from the study as the endogenous control miRNA levels were outside the acceptable range for inclusion. Circulating levels of the miRNAs were not found to significantly change across the phases of the menstrual cycle (Figures 3.7 and 3.8), with the exception of miR-202 (Figure 3.8 A).

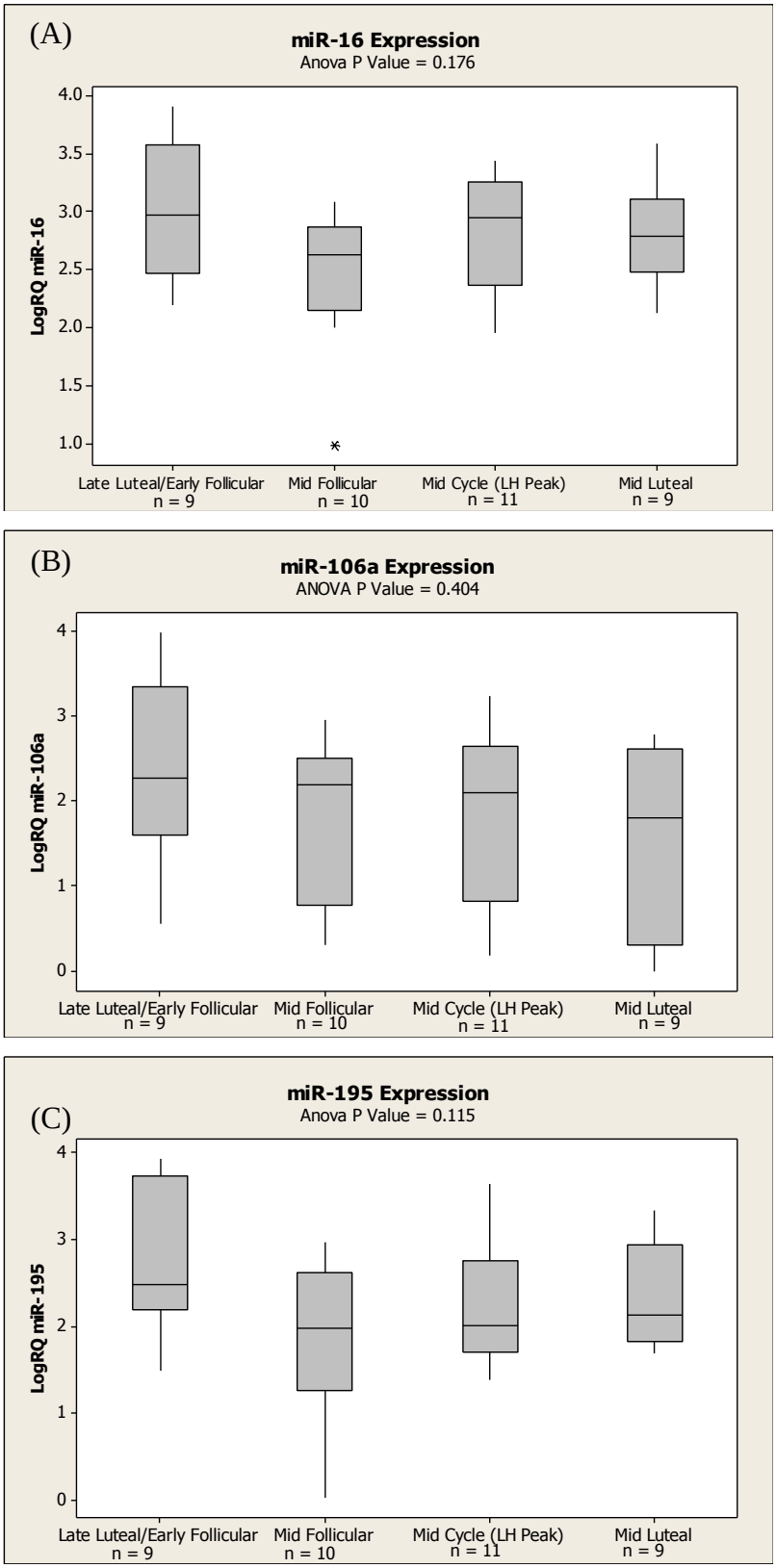


Figure 3.7 Variation of (A) miR-16, (B) miR-106a, and (C) miR-195 across the phases of the menstrual cycle. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data, while '*' respresents outliers within the group.

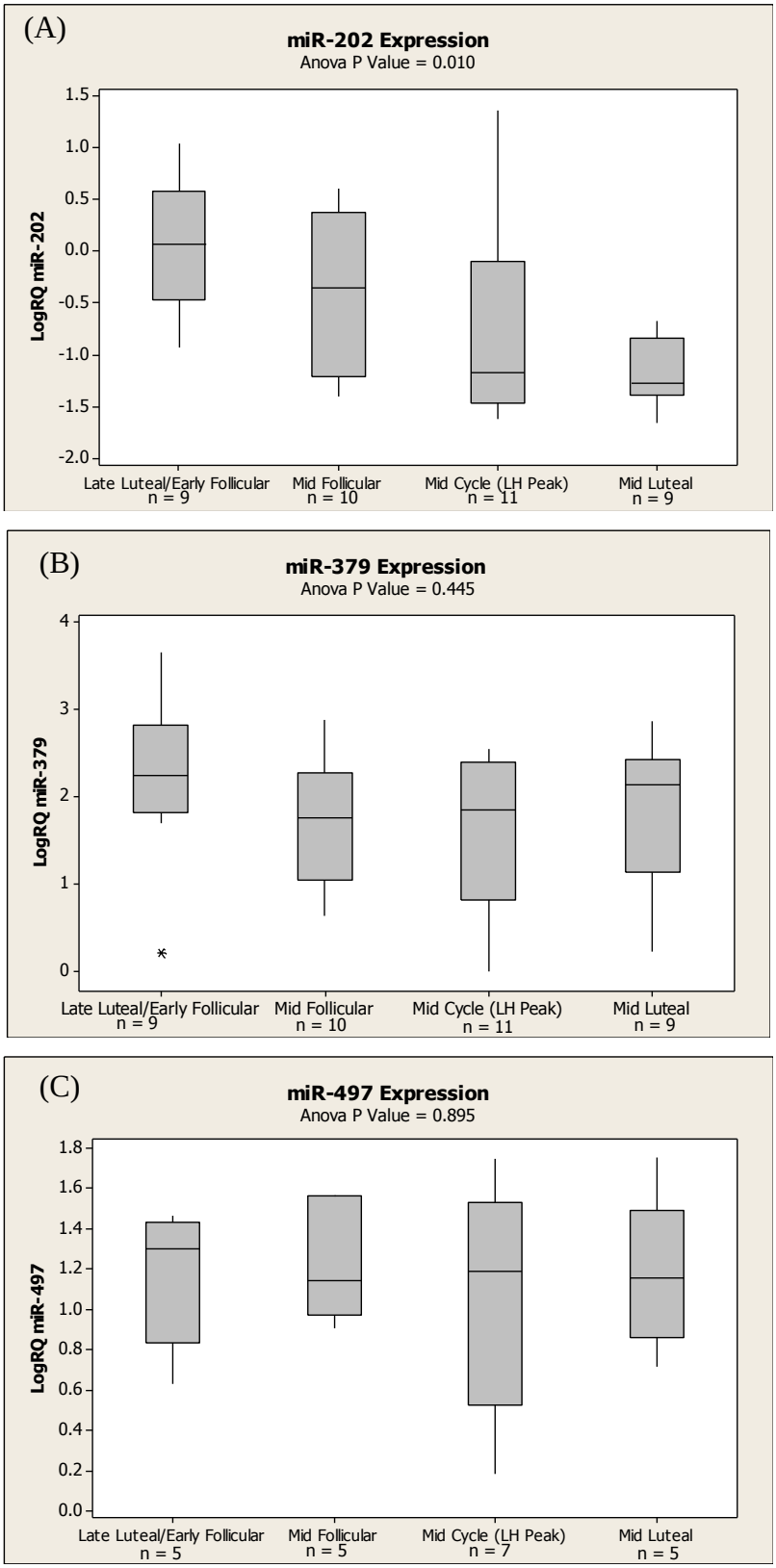


Figure 3.8 Variation of (A) miR-202, (B) miR-379, and (C) miR-497 across the phases of the menstrual cycle. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data, while '*' respresents outliers within the group.

The levels of expression for each target were expressed relative to the endogenous control miR-122, which was stably expressed. The only significant dysregulation observed was with miR-202, where a steady decrease across the phases of the menstrual cycle was evident (Figure 3.8A, ANOVA p value<0.01).

As these samples had previously been analysed for levels of some circulating hormones and chemokines, any relationship between these and the miRNAs of interest was also investigated. The miRNA expression values at each timepoint were pooled for each miRNA and pearson correlations were used to determine any relationships with hormones/chemokines. Significant correlations were observed between the circulating hormones/chemokines, LH and CCL5, and miR-497. Significance was also observed between LH and miR-379 (Table 3.3). Relationships between miRNAs and hormone/chemokines are shown using p values associated with pearson correlation, with p values greater than 0.05 indicating a significant relationship.

Target	miR-16	miR-195	miR-497	miR-379	miR-106a
SDF-1	0.459	0.219	0.881	0.153	0.181
MCP-1	0.968	0.231	0.287	0.24	0.15
FSH	0.56	0.475	0.318	0.126	0.842
LH	0.459	0.502	0.026	0.003	0.682
Progesterone	0.128	0.162	0.923	0.25	0.369
Oestradiol	0.845	0.821	0.648	0.181	0.393
CCL5 (pg/mL)	0.405	0.269	0.038	0.076	0.429
TGFβ (pg/mL)	0.339	0.63	0.936	0.259	0.209
					Significant

Table 3.3 Relationships between miRNAs and hormones/chemokines with p values associated with pearson correlation. Green areas highlight significant relationships.

This study included samples taken over various time points i.e. longitudinal data. Therefore, linear mixed models with a patient specific random effect were used to estimate and test the effect on each of the miRNAs with the phase of the menstrual cycle and specific hormones as covariates for each. To examine any potential relationship between the miRNAs of interest and circulating hormones, a linear mixed model was fitted to account for the longitudinal data. A significant relationship was observed between miRNA-379 and both FSH and LH (Table 3.4).

microRNA	Co-variables	Co-efficient	Standard Error	P- value
miR-379	Cycle Phase	-0.25	0.14	0.09
	FSH	-0.12	0.03	<0.005
miR-379	Cycle Phase	-0.12	0.12	0.35
	LH	-0.03	0.01	<0.01

Table 3.4 Linear mixed model showing relationship between miR-379 and both FSH and LH.

3.5 Discussion

miRNA expression in patient breast tissue samples

Although microRNAs represent ideal circulating biomarkers of disease, their implementation as biomarkers in clinical practice still faces some challenges. Breast cancer is a heterogenous disease and the impact of tumour characteristics on circulating miRNAs, and indeed the relationship between the two, requires further elucidation in order to reveal their full potential.

In 2012, the Discipline of Surgery research laboratory published a study which investigated circulating miRNAs, previously associated with breast cancer, in a murine model of breast cancer (86). MicroArrays were used to investigate alterations in circulating miRNA expression during tumour progression in an animal model. The levels of 77 miRNAs were found to be changed during disease progression and the top 4 miRNAs with changes >2-fold were chosen for further investigation in the circulation of breast cancer patients. It was crucial, therefore to investigate the identified targets in tissue

samples from breast cancer patients. The first part of this study investigates the expression of the identified targets: miR-106a, miR-138, miR-202 and miR-191, in patient breast tissue samples.

miR-106a belongs to the miR-17-96 family of miRNAs, which has attracted much interest in recent years due to its oncogenic activity (208). Indeed, the miR-17-92 family is perhaps the most widely studied family due to its members being implicated in carcinogenesis. Interestingly, however, miR-106a appears to only exist in mammals, suggesting a specific role for this miRNA (209). It has been suggested that miR-106a, in particular, plays an important role in the development and progression of breast cancer, with evidence supporting its role as an oncomiR (83, 210). In addition to this, RUNX3 has been confirmed as a target gene of miR-106a which results in multidrug resistance in gastric cancer (211). In 2010, Wang *et al.* (212) reported miR-106a to be consistently and significantly upregulated in both breast tumour tissues and matching serum samples, when compared to healthy normal tissue. Interestingly, they also found miR-106a levels to be higher in estrogen and progesterone receptor negative cancers, when compared to estrogen and progesterone receptor positive cancers. In a previous study published by our group, miR-106a was shown to significantly decrease in the circulation of a murine model as the disease progressed (86). Following this, miR-106a expression in the circulation of breast cancer patients (n = 83) was not significantly altered when compared to healthy controls (n = 83) (197). Therefore, we were interested to see if miR-106a would be altered in the tumour tissue of breast cancer patients. In this study, miR-106a was found to be significantly upregulated in breast tumours tissues (n = 60), when compared to healthy normal tissues (n = 40). No significant correlation to patient clinicopathological characteristics, such as tumour stage, grade, epithelial subtype and menopausal status, was observed. The data presented suggests miR-106a is a potential oncomiR and supports its important role in the tumour microenvironment but not as a circulating biomarker.

miR-138 has been shown to be a multifunctional molecular regulator (213) with numerous studies supporting its roles in cell migration (214), EMT (215), potential involvement in DNA damage response and repair (216) and

differentiation (217). In addition, on a cellular level, miR-138 has been implicated as a tumour suppressor miRNA (218-220), potentially through targeting Neutrophil gelatinase-associated lipocalcin (NGAL) (218) or Htert (220). However, until now, miR-138 has not been investigated in patient breast tissue samples. In a previous study published from our group, miR-138 was shown to significantly increase in the circulation of a murine model as the disease progressed (86). miR-138 was subsequently found to be significantly upregulated ($p<0.005$) in the circulation of breast cancer patients (197). Therefore, we were interested to see if this increase in the circulation would be reflected in the tumour tissue. In this study, miR-138 was not shown to be significantly altered in breast tumour samples when compared to normal healthy tissues. Interestingly, however, significant dysregulation was observed across epithelial subtype, with the highest levels detected in HER2 amplified samples. However, within the cohort examined, the HER2 subtype included only 12 tumour samples out of a total of 60 tumour tissues, therefore the results may change if a larger number of HER2 samples were to be included in the study.The data presented here raises the potential for miR-138 as a potential subtype specific biomarker for breast cancer.

miR-202 is a member of the Let-7 family of miRNAs and functional data has been published regarding the role of this miRNA and other members of the Let-7 family in carcinogenesis (221, 222). In 2010, miR-202 was shown to have the highest number of targets in significant biological pathways and Gene Ontology categories of approximately 900 miRNAs analysed (223). miR-202 has previously been reported to be dysregulated in colorectal cancer (224), follicular lymphoma (225) and chronic gastritis (226). In addition, the therapeutic potential of miR-202 has recently been suggested by Zhao *et al.* (227), where miR-202 was found to be decreased in human gastric cancer, and indeed restoration of the miRNA caused inhibition of proliferation, suggesting a potential tumour suppressor role for this miRNA. With regards to breast cancer, miR-202 has previously been shown to be significantly upregulated in the circulation of breast cancer patients when compared to controls (86, 228). In a previous study published from our group, miR-202 was shown to significantly increase in the circulation of a murine model as the disease progressed (86). However, in a subsequent study, unpublished

data found miR-202 was not significantly altered in the circulation of breast cancer patients (n = 50) when compared to healthy controls (n = 50). Therefore, we were interested to see if miR-202 would be altered in the tumour tissue of breast cancer patients. In this study, miR-202 was not shown to be significantly altered in breast tumour samples when compared to normal healthy tissues. Therefore the increase observed in the circulation in other breast cancer studies, is not reflected in the tumour tissue. Similarly to miR-138, however, significant dysregulation was observed across epithelial subtype.

miR-191 has been extensively reported to be dysregulated in a whole range of cancers including colon, liver, thyroid, prostate, pancreas, gastric, and ovarian cancers (80, 229-234). In the breast cancer setting, miR-191 has been shown to be elevated in the tumour tissues of patients with estrogen receptor positive breast cancers, when compared to patients with estrogen receptor negative breast cancers (80, 235). In a more recent study by Nagpal *et al.* (236), miR-191 was shown *in vitro* to be an estrogen-inducible miRNA in an ER-dependent manner in breast cancer. As a consequence of the extensive evidence displaying a correlation between miR-191 and cancer, further investigation of this miRNA is warranted, particularly in the breast cancer setting. In a previous study published from our group, miR-191 was shown to significantly decrease in the circulation of a murine model as the disease progressed (86). Following this, miR-191 was found not to be significantly altered in the circulation of breast cancer patients when compared to healthy controls (197). Therefore, we were interested to see if this decrease in the circulation would be reflected in the tumour tissue. In this study, miR-191 was found to be significantly increased in breast cancer tissues when compared to normal healthy controls, supporting the suggested oncogenic role of this miRNA. Furthermore, miR-191 expression was found to be significantly dysregulated across epithelial subtypes, with the highest levels detected in the HER2 and Basal subtypes. Although the upregulation of miR-191 in breast cancer tissues correlates with previous studies, the higher levels in the HER2 and Basal subtypes observed in this study does not correlate with previous studies suggesting miR-191 is elevated in ER+ breast cancers (236). This discrepancy requires further investigation. However, this adds yet

another dimension to the role of this miRNA not only as a potential oncomiR, but also as a potential subtype specific biomarker for breast cancer. Further statistical analysis was performed to investigate the impact on the sensitivity and specificity of miRNAs by combining certain ones, rather than using them isolation. miR-106a and miR-191 were both observed to be upregulated in the tumour tissues, suggesting a potential oncogenic role for these miRNAs. By combining these two miRNAs, the sensitivity of distinguishing between tumour and normal tissue slightly decreased, although the specificity was increased by 15%. It is most likely that in the future it will not be one individual miRNA that will used as a biomarker, but rather a panel of miRNAs, which will have increased sensitivity and specificity. In addition, miR-138 and miR-191 were observed to be significantly dysregulated across the epithelial subtypes, with the highest expression observed in the HER2 subtype tissues. For distinguishing between epithelial subtypes of tumours, by combining these two miRNAs, the sensitivity and specificity was increased in a number of assessments. For example, the sensitivity and specificity was increased when combining the two miRNAs for distinguishing between HER2 and Basal subtype tissues. Therefore, this raises the question if we were to examine a cohort composed of just one subtype of tumour, compared to normal tissue, would this then increase the dysregulation observed. This highlights the importance of including all epithelial subtypes when looking at the relationship of tumour characteristics as it could potentially bias results otherwise.

Impact of menstrual cycle hormones on circulating miRNAs

It is important to reveal if the dysregulation of miRNAs observed in the circulation of breast cancer patients is as a result of the disease, or as a result of normal physiological processes, such as the menstrual cycle. It is important to also consider the relationship other factors in the circulation are having on the circulating miRNA expression levels. These factors can be wide ranging from factors associated with gender (237), age (238), and ethnic origin (239), to normal physiological processes such as circulating steroid hormones and chemokines. As breast cancer is indeed a hormone responsive cancer, it is important to investigate circulating hormones associated with the menstrual

cycle and their relationship with circulating miRNA expression levels. The relationship between circulating miRNA expression levels in breast cancer and the menstrual cycle has never previously been published. Therefore, the second part of this study investigates this potential relationship in the circulation of healthy premenopausal women.

Throughout the course of the menstrual cycle, there are many physiological and hormonal changes in the body, with major alterations occurring in the endometrium. Endometrial miRNA profiles have been shown to fluctuate over the course of the menstrual cycle (207, 240-242) and it has been shown that miRNAs regulate many cellular processes that occur as a result of the cyclic changes in the endometrium and other tissues.

In the current study, based on 11 healthy premenopausal subjects and 44 samples in total taken weekly over 4 weeks, a stable miRNA expression profile of several miRNAs, with the exception of miR-202, was observed across the phases of the menstrual cycle. All subjects included in this study had normal ovulatory cycles and were not at the time on the oral contraceptive pill. Pre-determined hormone levels of the samples were used to establish the phases of the subjects menstrual cycle, with the LH peak denoting the mid-cycle, and the progesterone peak denoting the mid-luteal phase. The miRNAs chosen for investigation in this study all have previous associations with carcinogenesis and indeed with breast cancer.

miR-16 is probably one of the most widely studied miRNAs. miR-16 is ubiquitously expressed and highly conserved, indeed it was one of the first miRNAs to be linked with human cancers (67). Evidence has shown miR-16 to have many functions including modulation of the cell cycle, inhibition of cell proliferation, promotion of apoptosis and suppression of tumourigenicity, both *in vitro* and *in vivo* (243). It is also well established that miR-16 is frequently deleted and/or downregulated in a range of cancers, including chronic lymphocytic leukaemia (79), prostate cancer (244) and lung cancer (245). Conversely, miR-16 has been reported to remain unchanged in breast cancer, and indeed represent a suitable endogenous control (184, 246). Therefore, given the importance of miR-16 during tumourigenesis, this miRNA was included in this investigation. The miRNA expression levels of miR-16 were found to remain stable across the phases of the menstrual cycle.

As mentioned previously, miR-16 is ubiquitously expressed and it was found in abundance in all samples included in this study. In addition, miR-16 was found to be stably expressed within sample groups, with little inter-sample variation observed.

As previously discussed, miR-106a has been suggested to play an important role in the development and progression of breast cancer at both a circulating and a tissue level, with evidence supporting its role as an oncomiR (83, 210). In this study, miR-106a expression levels were found to remain stable across the phases of the menstrual cycle.

As mentioned earlier, miR-191 has been shown to be an estrogen-inducible miRNA in an ER-dependent manner in breast cancer. As a result, this miRNA was of particular interest in this study. miR-191 expression levels were found to remain stable across the phases of the menstrual cycle, thereby suggesting the circulating hormones associated with the menstrual cycle do not have an influence on, nor are associated with, changes in miR-191 expression levels.

miR-497 has been shown to be significantly down regulated in breast cancer patients and in addition as the disease progresses, the levels of miR-497 decrease (247-249). In this study, miR-497 expression was found to remain stable across menstrual cycle phases. Interestingly, miR-497 and miR-195 are part of the same miRNA cluster located at Chromosome 17p12.1 (85, 249). However, studies have shown miR-195 to be significantly increased in the circulation of breast cancer patients before receiving surgery and the levels are then significantly decreased after surgery (182). In this study, miR-195 expression levels were observed to remain stable across the menstrual cycle phases of this cohort of samples. This result perhaps adds more weight to the significant dysregulation previously observed in the circulation of breast cancer patients (89) as it means the alterations in expression levels are not as a result of influences from menstrual cycle hormones.

miR-379 is of particular interest to this research group as we have previously reported miR-379 as a novel regulator of Cyclin B1 expression with significant loss of miR-379 in breast tumour tissue (250). However, very little is known about the role of miR-379 in normal physiology and indeed in the circulation. In this current study, miR-379 expression was observed to remain stable across the menstrual cycle phases.

miR-202 is known to have a large number of biologically relevant pathways and was therefore chosen for investigation in this study. miR-202 has previously been shown to be significantly upregulated in the circulation of breast cancer patients when compared to age matched healthy controls(n = 48) (228). In this study, miR-202 showed significant dysregulation across the phases of the menstrual cycle suggesting that a potential relationship exists between miR-202 and circulating hormones associated with the menstrual cycle. This finding must therefore be taken into account when evaluating dysregulation of miR-202 observed in the circulation of breast cancer patients when compared to healthy controls (228). This was the only miRNA included in this study which displayed dysregulation across the phases. Therefore, in order to further validate this finding, the sample numbers would need to be increased significantly and a wider range of miRNAs should also be investigated.

Based on the overall findings, it is suggested that miRNA levels in the plasma of this cohort of healthy young women are not influenced by the circulating hormones and chemokines associated with the menstrual cycle, with the exception of miR-202. There are no previously published studies investigating the relationship between circulating menstrual cycle hormones and miRNA dysregulation in the circulation of breast cancer patients. Therefore, this finding could prove valuable in the future should circulating miRNAs be introduced into clinical practice as biomarkers for disease. In 1997, a pioneering study by Holdaway *et al.* (251), reported the circulating hormone profile of the menstrual cycle to be maintained in patients with breast cancer. Thus it is reasonable to extrapolate these findings in normal premenopausal women to those with breast cancer. It has long been established that the timing of surgical intervention in premenopausal breast cancer patients must be considered, with numerous major studies all supporting the second half of the menstrual cycle as optimal for surgical intervention (202, 252-255). Therefore, as circulating miRNA expression profiling is a point in time measurement, it is essential to investigate if a relationship exists between menstrual cycle hormones and all miRNA expression levels found to be dysregulated in the circulation of breast cancer patients. Ultimately this information will ensure that changes in miRNA

levels observed are related to the disease, and not simply a product of the phase of the menstrual cycle.

3.6 Conclusion

The first part of this study emphasizes the divergent roles of miRNAs, with two miRNAs demonstrating an association with breast cancer in patient tissues. The data also supports potential subtype specificity of miRNAs which has important implications for both diagnosis and therapy of the disease and therefore warrants further investigation.

The second part of this study revealed expression of a selection of miRNAs to remain stable throughout the menstrual cycle. Circulating levels of these miRNAs have previously been reported to be dysregulated in breast cancer patients. This is an important finding as it reveals that variations in these miRNAs are not a reflection of changes in circulating hormones.

miR-202 was highlighted as potentially dysregulated across the phases of the menstrual cycle and could therefore be an important consideration for studies going forward, investigating the potential role of this miRNA as a circulating biomarker of disease.

Chapter 4

Active secretion of microRNAs by breast cancer cells

4 Chapter 4: Active Secretion of microRNAs by Breast Cancer Cells

4.1 Introduction

In recent years, there has been a rapid growth of interest in microRNAs (miRNAs) as potential biomarkers of disease, particularly with regards to their presence in the circulation (256-258). Novel circulating biomarkers could improve the early detection, diagnosis, and ultimately the clinical management of cancer (259). The use of circulating miRNAs as potential biomarkers of disease is a rapidly evolving field of study. This hot topic has initiated widespread study of the source, abundance, half-life, and stability of miRNAs in the circulation, which require careful consideration if they are to be implemented in the clinical setting (260). Numerous studies have been performed assessing the effect of specimen type, the presence of cellular contaminants, and stability (256, 261-263). Mitchell *et al.* (88), first reported the presence of miRNAs in the circulation in a remarkably stable form, while subsequent studies have also found circulating miRNAs to be significantly more stable than their protein counterparts during long term storage (263, 264). It has been suggested that dysregulation of some miRNAs may be disease specific, for example, in the context of breast cancer, Heneghan *et al.* (89) reported that circulating miR-195 was significantly elevated only in breast cancer patients and not those with other selected tumour types.

However, the true source of miRNAs in the circulation and their exact secretory mechanism is relatively unknown (35). A number of hypotheses have been put forward. Chin and Slack (95), suggested two possible pathways: (1) tumour miRNAs are present as a result of cell death and lysis and (2) miRNAs are present as a result of being actively secreted by cells (95). In terms of active secretion, more recent studies have suggested that this may involve encapsulation of miRNAs into microvesicles, including exosomes and shedding vesicles (126, 265, 266). Alternatively, active secretion could be achieved using a microvesicle-free, RNA binding, protein-dependent pathway (105). Microvesicles, and indeed exosomes, have been identified as novel regulators of cell to cell communication and thus could also provide a mechanism of miRNA transfer between cells (267-269). An accumulating body of evidence suggests that extracellular vesicles such as

exosomes have the ability to be secreted and taken up by acceptor cells (101, 126, 270). To establish a signature specifically associated with breast cancer, there is a need to identify miRNAs secreted by breast cancer cells, with breast cancer cell lines and their associated cell-conditioned media representing a good starting point.

There have been relatively few published reports on the isolation of miRNAs from cell-conditioned media to examine their secretion by cells (271, 272). In addition, there is currently no commercially available extraction kit/technique designed specifically for miRNA isolation from cell-conditioned media. Two research groups have reported successful isolation of miRNAs from the media of four cell lines (93, 103). Kosaka *et al.* (93), reported miRNA isolation from the cell-conditioned media of HEK293T cells and COS-7 cells using the mirVana™ miRNA isolation kit (Ambion). Turchinovich *et al.* (103), reported use of the miRNeasy® micro Kit (Qiagen) with cell-conditioned media from HEK293T cells and two breast cancer lines, MCF7 and MDA-MB-231. However, it is worth noting the latter group drew attention to the very low yields which they were obtaining (103). There are no published reports describing analysis of miRNA secretion from tumour tissue explants. Successful RNA analysis relies heavily on the yield, purity, and integrity of the extracted RNA. Therefore, an optimal extraction protocol is essential.

More recently, it has been discovered that miRNAs are packaged into protective vesicles, called exosomes, for delivery to a new target location (126). Exosomal transport of miRNAs allows delivery of genetic information and cell to cell communication through this emerging and novel pathway with unprecedented potential. This exchange of miRNAs is an exciting and novel dimension to the regulation of a cell's phenotype and is particularly important in cancer. Precisely which miRNAs are being selectively packaged into the exosomes and what effect they are having in their new location is poorly understood and requires clarification. Further identification and characterisation of these exosomal miRNAs is central to the generation of novel biomarkers of disease and identification of their role in intercellular communication.

4.2 Aims

The aims of this study were as follows:

- To characterise miRNA secretion from cell-conditioned media and to identify the most reliable and reproducible method for miRNA isolation.
- To isolate the exosome fraction from within cell-conditioned media investigating both traditional and commercial methods, to characterise the exosomes by Transmission Electron Microscopy and Western Blot, and to detect a miRNA signal from within the exosomes investigating any selective packaging of miRNAs.

4.3 Materials and Methods

Cell-conditioned media was harvested from T47D, SK-BR-3, MDA-MB-231 and BT-20 breast cancer cell lines and also primary tissue explants, as described previously in section 2.6.2. miRNA was isolated from cell-conditioned media samples using five different miRNA isolation techniques: the blood protocol (89), RNeasy® (Qiagen), miRNeasy® mini kit (Qiagen), mirVana™ isolation kit (Ambion) and RNAqueous® kit (Ambion) (Section 2.3.3). The concentration and purity of miRNA was assessed using a NanoDrop™ 1000 spectrophotometer (Nanodrop Technologies, Willmington, DE, USA). A panel of 4 cancer associated miRNAs were targeted using RQ-PCR: miR-16, miR-195, miR-497 and miR-10b.

Exosomes secreted by breast cancer cells were isolated from the cell-conditioned media using either: Differential Ultracentrifugation or the ExoQuick TC Solution™. For morphological analysis, exosomes were characterised using Transmission Electron Microscopy (TEM), while further characterisation was performed by Western Blot, targeting the exosome-associated protein CD63. miRNA was isolated from the purified exosomes using the miRVana™ miRNA isolation kit. RQ-PCR was performed targeting a number of miRNAs of interest in order to detect a miRNA signal.

4.4 Results

4.4.1 Optimal method for isolation of secreted miRNAs

Conditioned media from a wide variety of sample types were extracted using the different extraction techniques. All cell line and stromal cultures were treated in a standardised manner, with approximately 2 x 10^6 cells in each flask (pooling 3) and incubated for 48 hours. All tissue explants were incubated in fresh media for 24 hours. Tissue explants were of varying sizes/mass and therefore the results presented below display a 'snapshot' of the typical yields available from such cultures. This study included breast cancer cell lines (T47D, SK-BR-3, MDA-MB-231), tissue explants (tumour, tumour associated normal (TAN) and diseased lymph node) and primary cell populations, e.g. stromal cells. A total of n = 90 samples were extracted using the different techniques. A yield of >20 ng/µL was required for RQ-PCR amplification and therefore anything less than this was not considered sufficient. Only 20 out of the 90 samples of cell-conditioned media yielded sufficient miRNA for progression. Examples of yields obtained from individual samples (i.e. single estimates) can be seen in Table 4.1.

Sample Identifier	Yield ng/µL
Lymph node Explant A	232.4
SK-BR-3	65.7
Tumour Explant A	64.9
T47D	63.2
Primary stromal cells	42.9
Tumour Explant B	37.1
Lymph node Explant A	32.7
MDA-MB-231	31.5
Tumour Explant	27.9
TAN Explant	24.2
SK-BR-3	21.9

Table 4.1 miRNA yield from conditioned media harvested from breast cancer cell lines and tissue explants (Adapted from Glynn *et al.* (25)).

The results were widely variable with typically the highest yields obtained from tissue explants, which is to be expected due to cellularity, with the highest yield obtained from diseased tumour lymph node tissue. It is worth noting, however, that miRNAs were also detected in the cell-conditioned media of breast cancer cell lines and primary stromal cells. Of the top 10 yields obtained from all samples, 8 of these were obtained using the mirVana™ miRNA isolation kit. The blood protocol and RNeasy® miRNA isolation techniques were found to be the least successful for the isolation of miRNA from cell-conditioned media.

This suggested that the mirVana™ isolation kit may be optimal for miRNA extraction from cell-conditioned media. A direct comparison of methods was then performed using the mirVana™, miRNeasy® and RNAqueous® isolation techniques on aliquots of the same samples. Here, 3 aliquots of the same samples were extracted using each of the 3 extraction techniques. There was significant variability of results obtained using the different extraction techniques, even from within the same sample, which can be seen in Table 4.2.

Sample Identifier	Method of Extraction	Yield ng/µl
T47D	mirVana™	63.2
	miRNeasy®	4.6
	RNAqueous®	12.9
MDA-MB-231	mirVana™	31.5
	miRNeasy®	4.2
	RNAqueous®	18.7
SK-BR-3	mirVana™	65.7
	miRNeasy®	4.6
	RNAqueous®	12.7

Table 4.2 miRNA yields obtained from aliquots of the same samples using the mirVana™, miRNeasy® and RNAqueous® techniques.

The results showed that consistent reproducible results were not obtained using the miRNeasy® or RNAqueous® techniques. In all cases, the mirVana™ miRNA isolation kit provided relatively high and reproducible yields for the successful isolation of miRNAs from cell-conditioned media.

4.4.2 Detection of miRNAs in cell-conditioned media

A range of miRNAs, including miR-16, miR-195, miR-497 and miR-10b, were successfully amplified from each of these samples using RQ-PCR (Table 4.3), confirming the presence of intact miRNAs. The average CT values for each sample are listed below. The lower the CT value, the higher the expression. Samples were considered 'undetected' if they fell beyond the 40 CT Limit of Detection for the assays i.e. the expression was too low to be detected.

		miR-16	miR-195	miR-497	miR-10b
Breast Cell Lines	T47D	33.74	31.04		×
	SK-BR-3	32.69	38.30	×	×
	MDA-MB-231	32.81	36.47	×	×
	BT-20	28.62	32.80	×	×
	MCF10-2a	29.75	30.05	×	×
	MCF12a	31.05	30.65	×	×
Primary Culture	Tumour Exp	25.78	28.94	32.36	28.29
	TAN Exp	23.80	27.79	33.39	27.72
	TL Exp	23.25	29.36	30.78	27.69
Stromal Cells	Cap Stro	30.17	35.47	×	×
	Tum Stro	31.26	33.75	×	×
	Normal Stro	31.35	34.85	×	×

Table 4.3 Amplification of miR-16, miR-195, miR-497 and miR-10b in cell-conditioned media samples from breast cancer cell lines, primary culture and stromal cells ('×' denotes not detected).

TAN = Tumour Associated Normal, TL = Tumour Lymh node, Cap Stro = Capsulectomy Stromal cells.

While miR-16 and miR-195 were detectable in all cell-conditioned media samples collected from both fresh tissue explants and breast cancer cell lines, miR-497 and miR-10b were only detectable in media exposed to the fresh tissue explants. Samples were considered 'undetected' if they fell beyond the 40 CT Limit of Detection for the assays i.e. the expression was too low to be detected.

4.4.3 Exosome Isolation

To further investigate the source/location of miRNAs in cell-conditioned media, the exosome fraction of the conditioned media was investigated. Exosomes were isolated from the conditioned media of a range of breast cancer cell lines, including T47D, SKBR3, BT20 and a non-tumourigenic breast cell line MCF12a and the miRNA content was extracted. Differential Ultracentrifugation (traditional method) and the ExoQuick TC™ Solution (commercial method) were investigated. No significant difference was observed between the miRNA yield obtained using either exosome isolation (Figure 4.1).

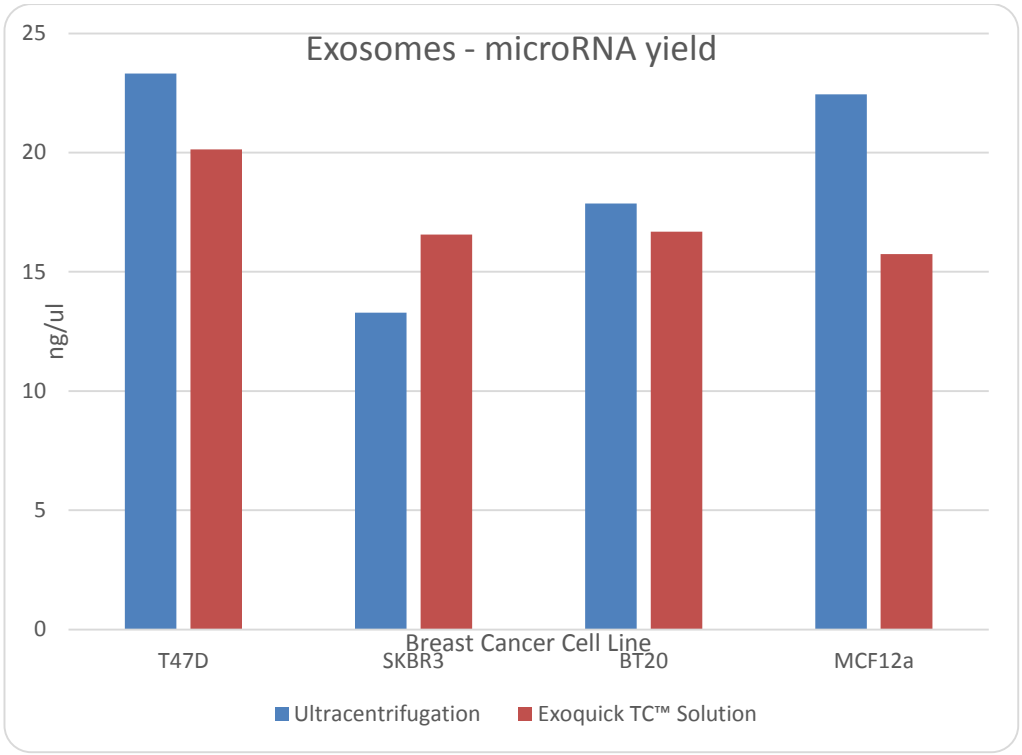


Figure 4.1 miRNA yields obtained from exosomes isolated from a range of breast cancer cell lines using either Differential Ultracentrifugation or ExoQuick TC™ Solution.

Due to the high cost of the ExoQuick TC Solution (approximately €320 for 4 samples) the differential ultracentrifugation method was deemed the most appropriate method to use in all subsequent experiments.

4.4.4 Exosome Characterisation

4.4.4.1 Transmission Electron Microscopy (TEM)

Exosomes were isolated, and were fixed, dehydrated, and embedded in resin for visualisation, which results in the grainy appearance in the images. TEM allows confirmation that the exosomes are of the correct size (~40-100 nm in diameter) and shape (oval/rounded) which was confirmed (Figure 4.2).

A majority of the vesicles visualized were in the 80-100 nm size range, which are indicative of exosomes. Additional vesicles were also present in size ranges both larger (>100 nm) and smaller (<40 nm) than exosomes, suggesting the presence of microvesicles in addition to exosomes. The majority of the exosomes visualized were intact, with clear images of the

membranes. However, a small number of images revealed broken membranes indicating degradation of the exosomes, possibly as a result of the ultracentrifugation process (Figure 4.2 (D)).

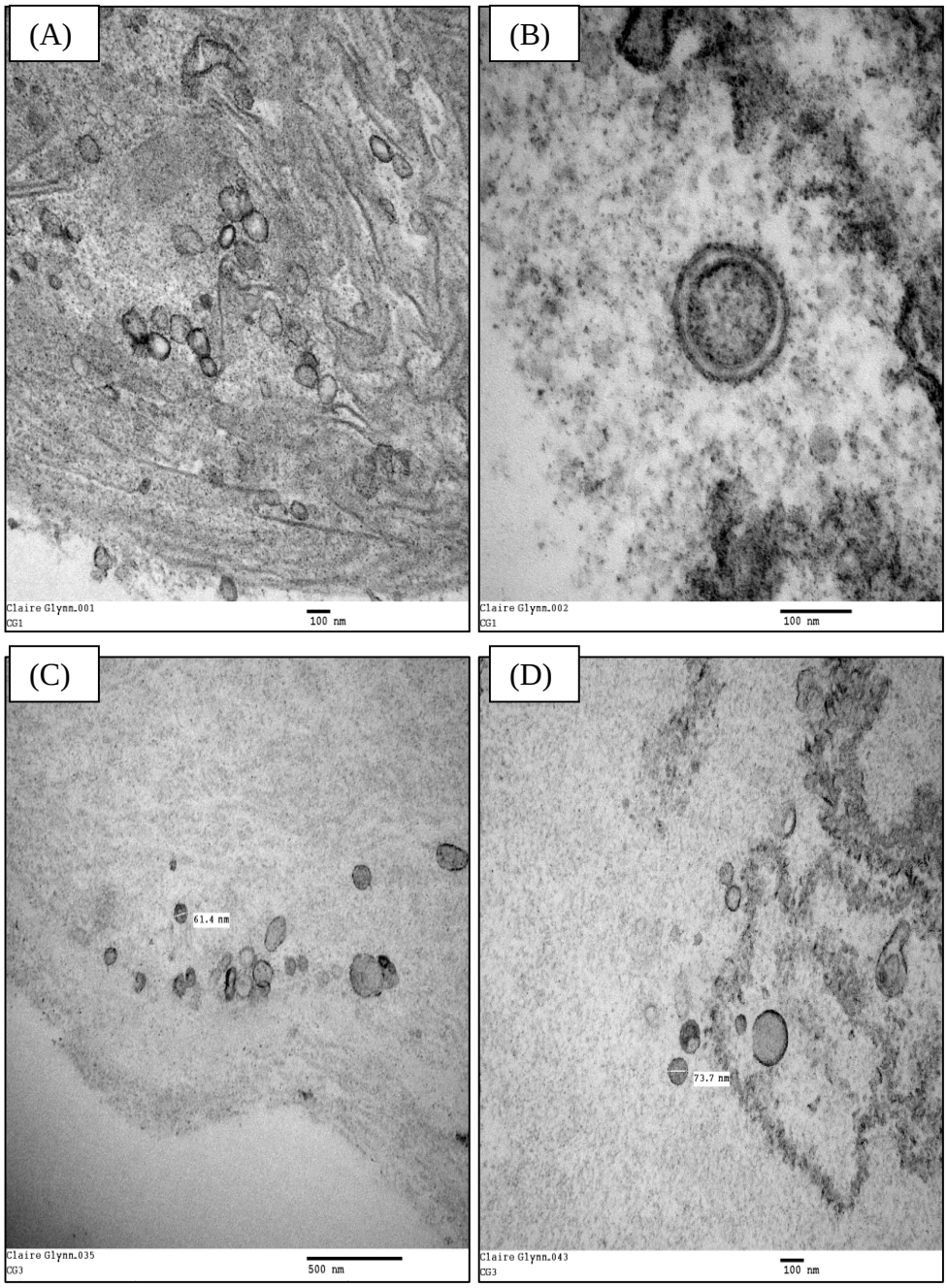


Figure 4.2 TEM images of exosomes isolated from the conditioned media of breast cancer cell lines. (A) T47D cell-secreted exosomes at magnification 50,000x, (B) T47D cell-secreted single exosomes at magnification 150,000x, (C & D) SKBR3 cell-secreted exosomes at magnification 50,000x.

4.4.4.2 Western Blot

Western Blot analysis revealed that the exosomes collected from both T47D and SK-BR-3 breast cancer cells expressed the tetraspanin molecule CD63, a widely reported exosome-associated protein (Figure 4.3). The Western Blot revealed bands of the expected size (50-60 kDa).

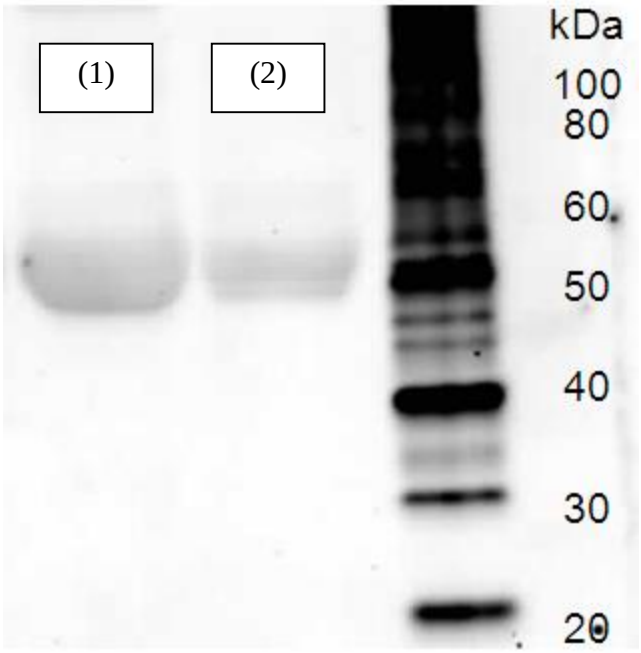


Figure 4.3 Western blot analysis of exosomes isolated from (1) T47D cells and, (2) SKBR3 cells targeting the exosome-associated protein CD63.

4.4.5 Detection of miRNAs in exosomes

As the miRVana miRNA isolation kit had previously been deemed the most appropriate method for miRNA isolation from conditioned media, the method was further employed for the extraction of miRNA from exosomes. Initial experiments were performed to determine if there was a detectable miRNA signature in the exosomes. The same amount of total RNA (100ng) was analysed in each sample. A range of miRNAs, including miR-16, miR-138, miR-379 and miR-504, were successfully amplified from exosomes isolated from a range of breast cancer cell lines using RQ-PCR (Table 4.4), confirming the presence of intact miRNAs. The matching cell source and conditioned media for each sample were also investigated (Table 4.4). The average CT values for each sample are listed below. The lower the CT value, the higher the expression. Samples were considered ‘undetected’ if they fell beyond the

40 CT Limit of Detection for the assays i.e. the expression was too low to be detected.

Cell Line	Sample	miR-16	miR-138	miR-379	miR-504
MCF12A	**Exosomes**	28.69	×	36.4	×
	Cells	21.72	24.72	30.80	32.80
	CM	31.05	×	37.2	×
T47D	**Exosomes**	30.2	×	35.23	×
	Cells	17.8	33.03	31.89	34.75
	CM	32.38	×	35.93	×
SKBR3	**Exosomes**	28.75	×	34.11	×
	Cells	19.53	29.58	32.69	36.85
	CM	32.8	×	36.49	×
BT20	**Exosomes**	26.53	×	33.92	×
	Cells	21.85	24.85	31.88	33.53
	CM	28.62	×	35.27	×

Table 4.4 Amplification of miR-16, miR-138, miR-379 and miR-504 in miRNA extracted from matched exosome, cell conditioned media, and cells, isolated from T47D, SKBR3, BT20 and MCF12a breast cancer cell lines. ('×' denotes not detectable).

Interestingly, selective packaging of particular miRNAs was observed. All miRNAs were detectable in the cells from each cell line, while miR-16 and miR-379 were the only two miRNAs detectable in the exosomes and conditioned media. miR-138 and miR-504 were not found to be detectable in the exosomes or the conditioned media. This result was observed across all 4 breast cancer cell lines.

4.5 Discussion

microRNAs (miRNAs) have been found to be relatively stable in the circulation of cancer patients, raising their potential as circulating biomarkers of disease. The specific source and role, however, of miRNAs in the circulation is unknown and requires elucidation to determine their true potential. The reproducibility and efficiency of 5 methods/kits for miRNA extraction were tested and compared on conditioned media samples from a range of breast cancer cell lines. A prerequisite for successful amplification using RQ-PCR is an optimal miRNA yield. Therefore an efficient method for RNA extraction is critical. A panel of four candidate miRNAs (miR-16, miR-195, miR-497 and miR-10b) were chosen for amplification by RQ-PCR in cell-conditioned media samples. These miRNAs have previously been implicated in the circulation of breast cancer patients (89, 273). In this study, along with primary tissue explants and primary stromal cells, a range of breast cancer cell lines were employed, including T47D, MDA-MB-231 and SK-BR-3. miRNA was successfully isolated from all media samples collected from cell lines, primary cells and fresh tissue explants, using the 5 miRNA isolation protocols previously described. However, there was remarkable variation in yield depending on the extraction method used. Aliquots of the same samples were extracted, revealing the two column extraction protocol of the mirVana® miRNA isolation kit to be the most suitable approach. A range of miRNAs, including miR-16, miR-195, miR-497 and miR-10b, were successfully amplified. While miR-16 and miR-195 were detected in media from both cell lines and tissue explants, miR-497 and miR-10b were only detected in secretions from whole tissue explants. This suggests that other cellular components of the tumour may be the source of the miRNAs and not just the epithelial cells alone. This is a very interesting area and does require further investigation. The stability, and indeed the persistence of action, of miRNAs is a biophysical parameter which warrants careful consideration. Generally, miRNAs are considered to be relatively stable with long half lives of approximately 2 weeks (88). However, some studies have shown selected miRNAs to have relatively short half lives (~1-3.5hrs), with several brain abundant miRNAs shown to be surprisingly restricted (274). In the current

study, all of the cell-conditioned media samples were collected in the same way and were frozen at -20°C within 20 minutes of harvest. Early investigations found there to be no loss in miRNA content in cell-conditioned media samples frozen at -20°C and -80°C, which is in agreement with previous studies reporting the stability of miRNAs (93, 103).

Recent exciting developments have highlighted miRNA transport in exosomes. This novel mode of transport has the potential to support miRNA-mediated intercellular communication. Cell culture media, and indeed biological fluids, contain a wide range of secreted vesicles and shed membrane fragments. Therefore, it is essential to first employ a high quality isolation method to obtain a purified exosome fraction, and secondly to characterize the isolations to confirm the presence of exosomes. In this study, a robust and widely used differential ultracentrifugation method and a commercial kit, ExoQuick TC™ Solution, designed for exosome isolation from fluids, were compared. The results obtained showed no difference between the two methods, by examining the miRNA content extracted from each exosome isolation. The differential ultracentrifugation method is based on repeated centrifugation, microfiltration, and ultracentrifugation. The method can be time consuming and access to a high speed ultracentrifuge is essential. However, up to 8 samples can be isolated in one run and the method has widely been reported to be robust and reproducible (141). The ExoQuick TC Solution only facilitates a small number of samples to be isolated from each kit and is quite costly. Therefore, as there was no significant benefit offered by the ExoQuick TC Solution, the cost could not be justified, and the differential ultracentrifugation method was deemed the most suitable method to employ in all future experiments.

Characterization of the purified exosome fraction is essential to distinguish the exosomes from other vesicles and particles. The exosomal morphology was examined by Transmission Electron Microscopy. Standard electron microscopic methods employ fixation, dehydration, resin embedding and ultrathin sectioning. In addition, to isolate the exosomes, high speed ultracentrifugation was employed. All of these methods combined could have

potentially detrimental effects on the membranes of the exosomes. Therefore, after careful consideration, a key step in this experiment was to add a primary fixative to the conditioned media prior to ultracentrifugation. This ensured the architecture of the exosomes was maintained during the spinning process, whereby the centrifugal force could potentially disrupt or break down the membranes of the exosomes. A secondary fixative employed immediately following ultracentrifugation ensured the exosomes were protected during dehydration and embedding protocols. The images obtained from exosomes isolated from the conditioned media of both T47D and SKBR3 breast cancer cells showed the typical morphology and size expected of exosomes. Electron microscopy can also be performed using immunostaining to show the presence of surface bound membrane proteins. However, the electron microscopy employed in this study identified the presence of exosomes based solely on morphology and size. Conclusive validation of purified exosome fractions was obtained using the well established Western Blot method, targeting the exosome-associated protein CD63. Currently, as there are no exosome specific markers, proteins that are enriched in exosomes arising from all different cellular origins are commonly used to confirm the presence of exosomes. In this study, the exosome-associated protein CD63 was employed for confirmatory characterization. This was carried out on multiple occasions using a variety of exosomes isolated from several different cell types. The expected bands (50-60 kDa) were observed at the correct size (~53 kDa), thereby confirming the presence of exosomes. Little is known about the actual function of the CD63 tetraspanin, although it is thought to play a role in the regulation of the sorting process for particular proteins into exosomes (275).

Determination of precisely which miRNAs are being selectively packaged into exosomes, particularly in breast cancer, and their functional effects in target locations remains poorly understood, and clearly requires further study. In this study, miRNA was isolated from exosomes secreted from a range of breast cancer cell lines. An initial validation of the presence of a miRNA signal was firstly performed. This provided evidence that the exosomes did indeed contain miRNAs. The next step was to target a range of miRNAs in

the exosomes in order to investigate selective packaging. Matching conditioned media and cells were also examined for comparison. Selective packaging of particular candidate miRNAs into exosomes secreted from breast cancer cells was observed as some were present in the exosomes and some were not, while robust expression was observed in the donor cells. It was particularly interesting as this result was repeated across 4 cell lines. miR-138 and miR-504 were not found to be present in the exosomes or the cell-conditioned media, suggesting neither miRNA is destined for release to a target location. This data also highlights the selective nature of the mechanism of packaging miRNAs into exosomes. The results obtained in this study correlate with the findings observed in separate studies and support the hypothesis of the selective packaging of miRNAs into exosomes for transport. These particular miRNAs are well defined mediators of carcinogenesis, although they simply represent a small panel of miRNAs of interest. The entire miRNA secretion profile must be considered in order to fully appreciate the selective nature of the packaging. This is further discussed in chapter 5.

4.6 Conclusion

The successful isolation of intact miRNAs from cell conditioned media in an *in vitro* setting is a prerequisite for expression analyses of secreted miRNAs. This study found that the mirVana™ miRNA isolation kit provided the most reproducible and consistently high yields, when compared to other methods available. The molecular mechanisms which drive this secretion have yet to be understood. The use of an optimal method for isolating these secreted miRNAs will support elucidation of the mechanism of secretion, the cellular source, and role of these molecules in the circulation. Further identification and functional characterisation of how miRNAs enter the circulation is essential to determine their potential as biomarkers and indeed to understanding their role in intercellular communication.

Chapter 5

Analysis of exosome-encapsulated microRNAs secreted by breast cancer cells

5 Chapter 5: Analysis of exosome-encapsulated microRNAs secreted by breast cancer cells

5.1 Introduction

microRNA (miRNA) dysregulation has widely been reported to play a critical role in carcinogenesis (276). The multi-cellular tumour microenvironment and the reciprocal interactions between different cell types play a fundamental role in cancer initiation and progression (277). As outlined in the previous chapter, miRNAs can be transported in protective vesicles called exosomes. This mode of transport allows delivery of genetic information and cell-to-cell communication through this recently identified pathway and has potentially important implications in the cancer setting (126). The release of miRNAs secreted in exosomes from donor cells and their uptake by recipient cells, where an array of biological processes can be impacted, must be investigated. However, precisely which miRNAs are being selectively packaged into exosomes, and their true role in the tumour microenvironment, is poorly understood and requires investigation.

In leading research conducted by Valadi *et al.* (126), it was shown that exosomes contain a combination of essential proteins and in addition express cell recognition molecules on their surface, thereby facilitating their selective targeting of, and uptake by, recipient cells (126). In the previous chapter, it was shown that miRNAs are indeed detectable within exosomes secreted from a range of breast cancer cell lines. Additionally it was shown, with a small panel of miRNAs of interest, that only some miRNAs are selectively packaged into exosomes for secretion. In order to fully unravel this selective miRNA content, it is therefore important to investigate specifically which miRNAs are packaged into exosomes by performing array analysis for the detection of all known miRNAs. Exosomes represent a unique and exquisite mechanism for both local and systemic transfer of genetic information (266, 278). This groundbreaking research has ignited interest in the role of exosomes in cell-to-cell communication and also their implications as biomarkers of disease. The genetic information which these exosomes contain represent a molecular fingerprint of the releasing cell type and its status, which opens a new avenue of investigation in terms of biomarker discovery.

It is only within the last decade that the importance of exosomes has come to light. Previously, exosomes were considered to serve little function and existed only to act as garbage disposal mechanisms for unwanted cellular material. However, the role of exosomes in both normal and pathological processes has now been acknowledged and has provoked widespread research in this area. Traditionally, intercellular communication has been considered to be limited to gap junctions or secreted signals, including hormones, neurotransmitters and chemokines which function in an autocrine/paracrine manner. However, the release, and ultimately the transfer, of exosome-mediated miRNAs, offers a new dimension in intercellular communication, similar to that of cell-cell contact, only this mechanism is a 'long-distance relationship'. The discovery of miRNAs within exosomes has opened a new avenue of investigation in cell-to-cell communication and offers much potential in terms of biomarker discovery.

5.2 Aims

The aims of this study were as follows:

- To identify a full exosome-encapsulated miRNA secretion profile from a range of breast cancer cell lines *in vitro* and to validate a selection of identified targets using RQ-PCR.
- To visualise the transfer and uptake of exosomes into a new cell population, and to determine any effects once transferred in terms of miRNA expression, cell proliferation and migration.

5.3 Materials and Methods

microRNA Array and RQ-PCR Validation

Four breast cancer cell lines were employed in this study: T47D (Luminal A), SK-BR-3 (HER2), BT-20 and MDA-MB-231 (both basal). Secreted exosomes were isolated from the conditioned media of individual cell lines, and miRNA extraction was performed using the miRVana™ miRNA isolation kit. The extracted miRNA was subjected to global miRNA expression analysis using the Exiqon miRCURY™ LNA Array (7th Generation) by Exiqon, Denmark. Following statistical analysis, the top ranked miRNAs were validated by RQ-PCR in miRNA extracted from exosome fractions harvested from the conditioned media of the same breast cancer cell lines.

Exosome Transfer

In a separate experiment, isolated exosomes tagged with a red fluorescent protein were transferred to recipient unlabelled T47D cells, and their uptake was viewed using a confocal microscope, see section 2.6.9 for detail. Isolated exosomes were also transferred to MDA-MB-231 and T47D cells growing in a 12-well culture plate. The exosomes were incubated for 4 hours and 24 hours to allow uptake and the cells were then harvested and miRNA extracted. RQ-PCR was performed on the extracted miRNA samples targeting miRNAs previously identified to be present in exosomes. Proliferation assays were performed to investigate the effect of exosomes on cellular proliferation. In addition, migration assays were performed to investigate the effect of exosomes on the migratory power of cells.

5.4 Results

5.4.1 Exosome-encapsulated miRNA secretion profile

Exosomes were successfully isolated from conditioned media of each cell population: T47D, SKBR3, BT-20 and MDA-MB-231. miRNA was extracted from the exosomes and was sent to Exiqon, Denmark, where the miRNA content of the exosomes were profiled. Data normalisation was performed using Quantile normalization. Quantile normalisation is based on the assumption that two sets of closely related data should sort themselves in a linear fashion forming a diagonal when plotted against each other. It enforces an equal distribution on the data. Exiqon have reported that quantile normalization, in their experience, has produced the best between-slide normalization to minimize the intensity-dependent differences between the samples. The signal distribution plot, seen below in Figure 5.1, for this data showed that the distributions of the samples were similar and the assumption of the Quantile normalisation was fulfilled.

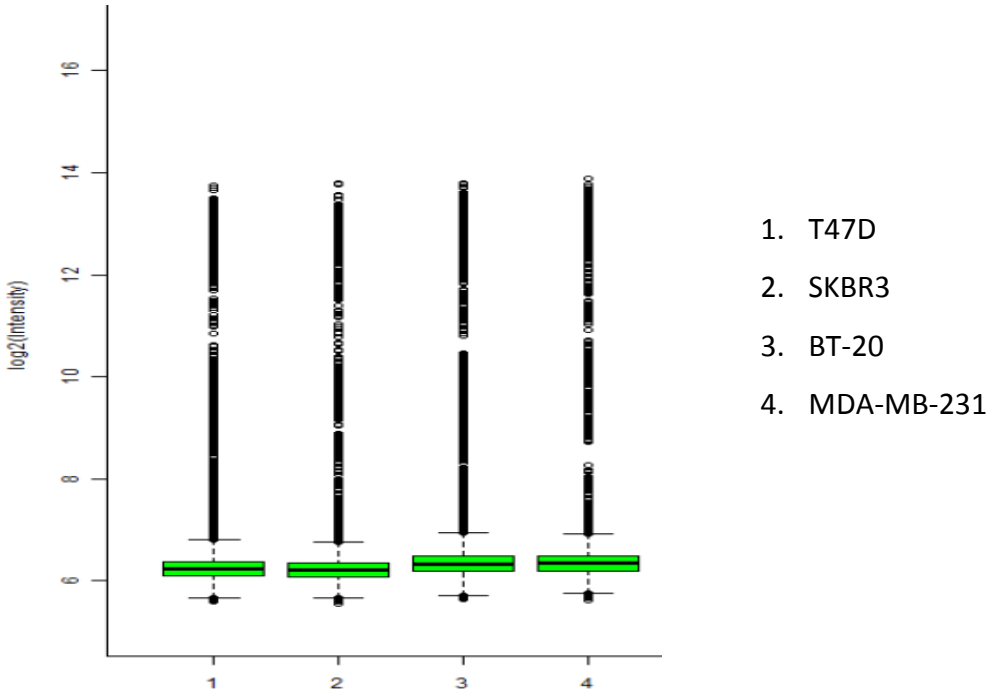


Figure 5.1 Signal distribution plot showing the distributions of the samples are similar.

Array data revealed each of the breast cancer cell lines secreted from 381 to 394 miRNAs in exosomes from a total of 2083 targets analysed (see appendix 9.4). The 2083 miRNAs targeted were the miRNAs annotated on miRBase at the time of profiling (Table 5.1). Of these, 287 were common to all four breast cancer cell lines.

	T47D	SKBR3	BT-20	MDA-MB-231
# miRNAs detected	390	381	381	394

Table 5.1 Number of miRNAs detected in all samples out of a total of 2083.

Interestingly, a subset of these miRNAs are known to target genes associated with cell cycle regulation and apoptosis (e.g. miR-15a-5p, miR-204-5p, miR-221-3p, and miR-223-3p). Two-way hierarchial clustering of miRNAs and samples was performed using the complete-linkage method together with the Euclidean distance measure. Two-way clustering discovers the homogenous subsets of genes and samples i.e. biclusters, by iteratively performing one-way clustering on the genes and samples. While hierarchical clustering finds similarities of genes over all conditions or vice versa, two-way hierarchical clustering (biclustering) searches for local patterns that may feature important biological implications. The miRNA clustering dendogram tree was cut at height 5, resulting in 5 clusters of interest (Figure 5.2).

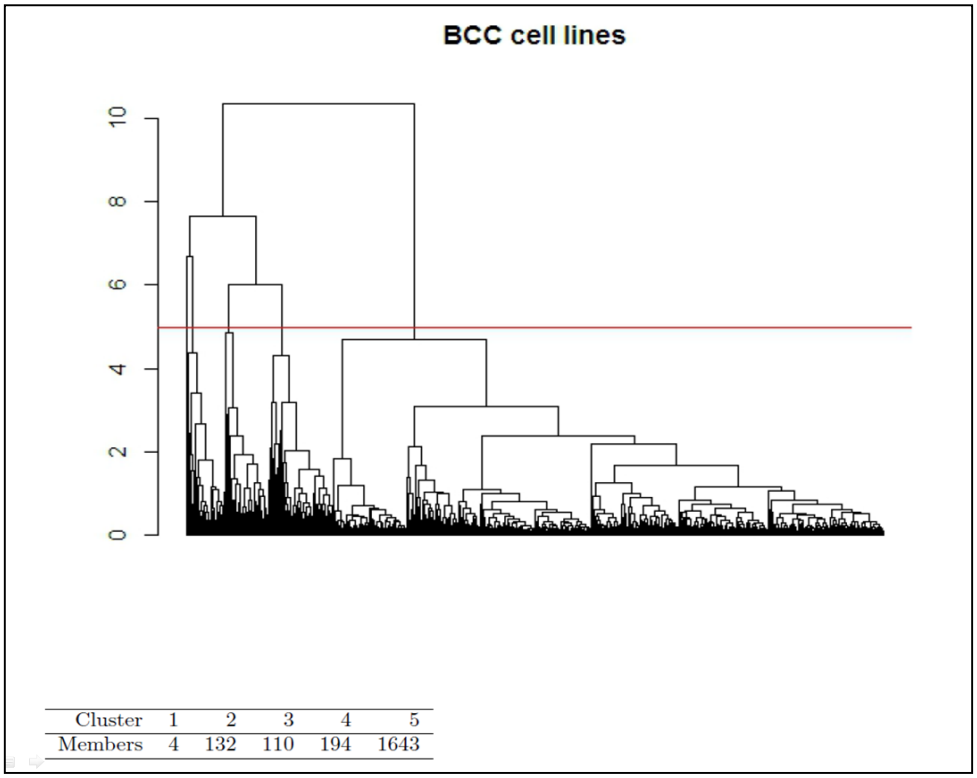


Figure 5.2 Dendogram trees forming clusters, cut at height 5, identifying 5 clusters of interest. Table inset displays the number of miRNAs in each cluster.

The unsupervised analysis of the samples separates the miRNA profiles of the individual cell lines (Figure 5.3 A). Furthermore, 3 clusters were extrapolated (Figure 5.3 B-D), highlighting the differential profiles. Yellow/green signifies high expression, while blue signifies low expression.

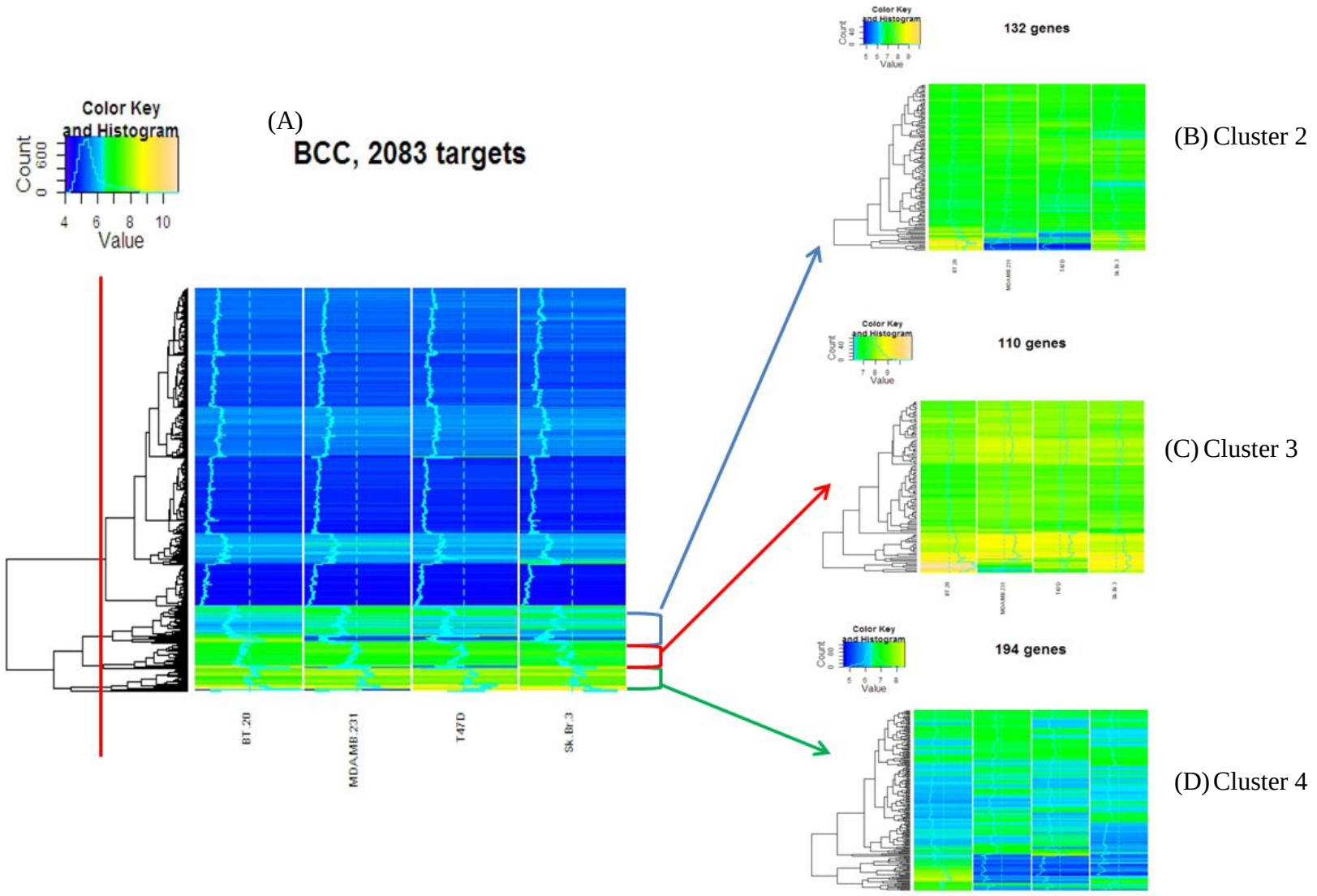


Figure 5.3 Extrapolation of clusters from original heat map.

Small panels of miRNAs in each cluster appear to have differential expression with reference to epithelial subtype. While there were similarities in the profiles across the 4 cell lines, there were also significant differences. Cluster 2 contained 132 miRNAs, with clear similarities observed throughout the heat map, however differential expression was observed across the 4 cell lines in the lower part (Figure 5.3 B). Cluster 3 contained 110 miRNAs with considerable differential expression observed in the lower part of the heatmap (Figure 5.3 C). Cluster 4 contained 194 miRNAs, with some similarities observed throughout, although there are areas of distinct differential expression (Figure 5.3 D). Interestingly, considerable differences were observed in certain portions of the cluster heat maps, with the BT-20 and MDA-MB-231 showing differential expression, despite them both belonging to the Basal epithelial subtype. The differential expression, however, may not be related to epithelial subtype, indeed it could be related to invasive versus non-invsasive or other characteristics of the cell lines.

Following normalisation of the data, the miRNAs were ranked based on their expression. Initial studies focused on well characterised miRNAs (i.e. <hsa-miR-1000). Following this, the miRNAs showing greatest variation between sample types were selected, in addition to miRNAs showing high expression in all sample types. From the top ranked, miRNAs which were previously or currently of interest in cancer were also selected. Four miRNAs of interest were chosen, including miR-10b, miR-145 and miR-498 coming from cluster 3, and miR-492 coming from cluster 4. The relative levels of expression of each miRNA are shown in the table below (Table 5.2).

miRNAs of Interest – Levels of Expression					
		BT20	MDA-MB-231	T47D	SkBr3
1	hsa-miR-10b-3p	8.41	8.89	8.66	8.58
2	hsa-miR-498	8.15	7.59	9.66	7.44
3	hsa-miR-145-5p	8.04	8.57	8.18	8.26
4	hsa-miR-492	7.84	5.56	5.64	5.79

Table 5.2 Relative levels of expression across epithelial subtypes of top 4 miRNAs of interest.

From the array data, miR-10b and miR-145 were found to be highly expressed (8.038 – 8.891) with no significant variation across the sample types. miR-492 was found to be higher in the BT20 samples (7.84) and lower in the other 3 sample types (~5.5). miR-498 was found to be highest in the T47D (9.66), lower in the BT20 (8.146), and lower again in the MDA-MB-231 (7.587) and SKBR3 (7.445).

5.4.2 RQ-PCR validation of exosome-encapsulated miRNAs

miR-10b, miR-145, miR-492 and miR-498 were selected for further RQ-PCR validation. Three individual preparations of exosomes were isolated from the conditioned media of each T47D, SKBR3, BT20 and MDA-MB-231 cells. The miRNA was extracted and RQ-PCR was performed, targeting the miRNAs of interest required for validation. In the RQ-PCR validation data, miR-10b, miR-145, and miR-492 were found to be detectable in 3 preparations of exosomes collected from all 4 breast cancer cell lines. miR-498, however, was undetectable in all samples (Table 5.3).

	T47D (n=3)	SKBR3 (n=3)	MDA-MB-231 (n=3)	BT20 (n=3)
miR-10b	✓	✓	✓	✓
miR-145	✓	✓	✓	✓
miR-492	✓	✓	✓	✓
miR-498	×	×	×	×

Table 5.3 RQ-PCR validation of miR-10b, miR-145, miR-492 and miR-498 in 3 preparations of exosomes isolated from the conditioned media of T47D, SKBR3, MDA-MB-231 and BT20 cells.

5.4.3 Visualisation of exosome transfer and uptake

Exosomes were isolated from T47D breast cancer cells which had previously been tagged with a red fluorescent protein. The isolated exosomes were transferred to a recipient cell population and were incubated to allow transfer and uptake. Using confocal microscopy, the red exosomes were visualised in the unlabelled recipient cell population with the DAPI stained cell nuclei (Figure 5.4). A higher magnification image of the lower left region of the image, shows the exosomes more clearly associating with the cell surface (Figure 5.4). The images were obtained by z-stacked confocal imaging, whereby a series of slices of images are compressed into one. The images are then presented as Maximum Intensity Projections (MIP) where the brightest part of each image is taken and projected as one image. Essentially they are representing 3-D images.

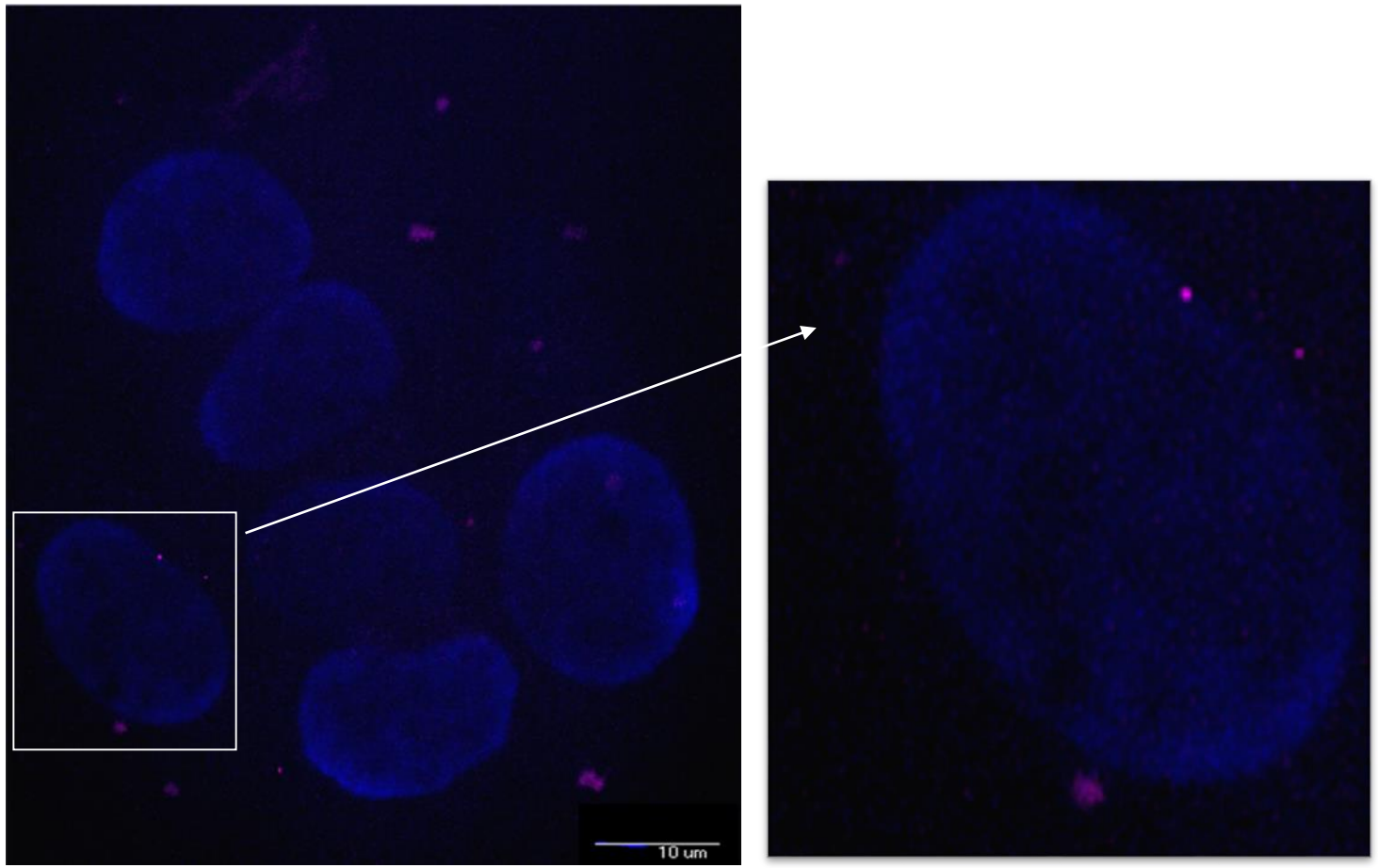


Figure 5.4 Fluorescently labelled exosomes (red) transferred to DAPI stained nuclei (blue) of recipient cell population (63x mag).

In a separate set-up, the actin filaments were visualised by staining of the cytoskeleton using Alexaflour 488 Phalloidin stain (Figure 5.5).

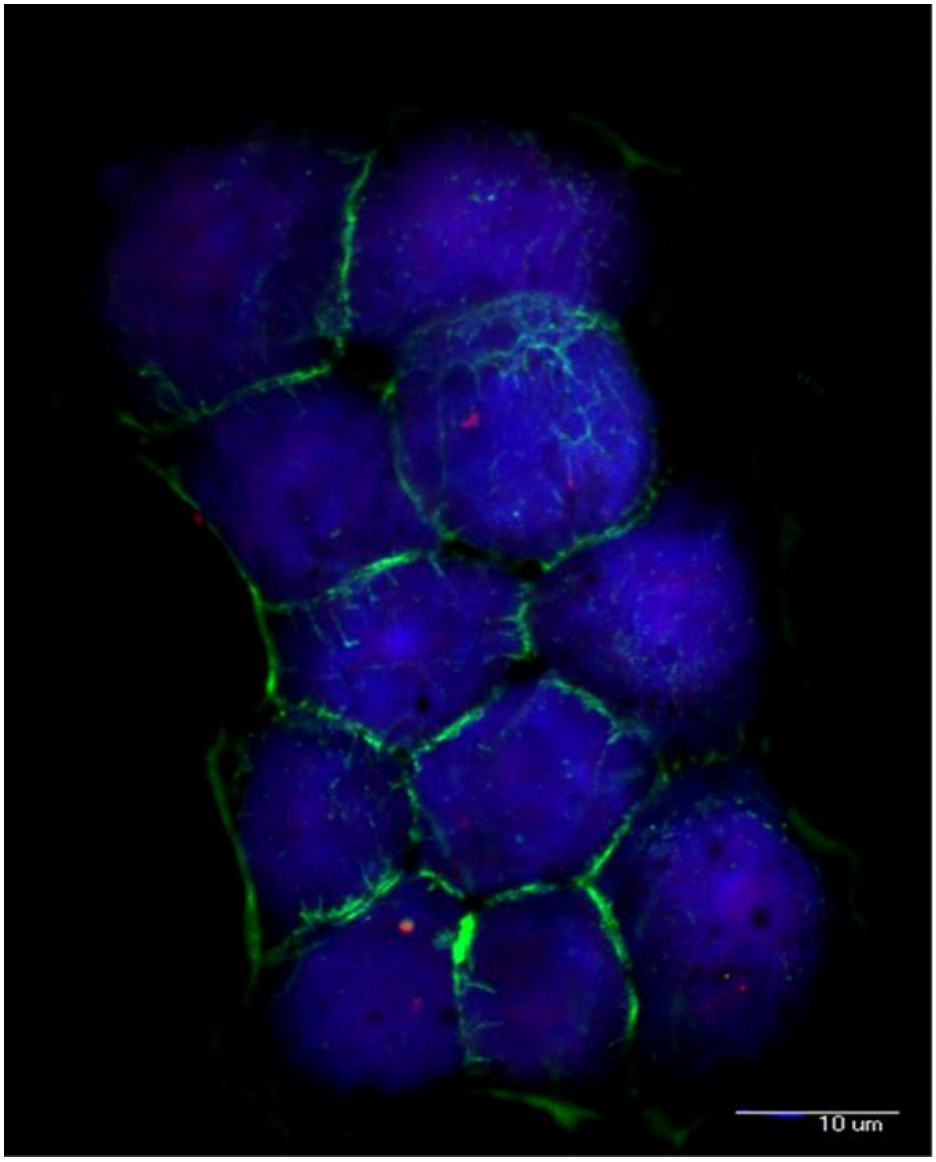


Figure 5.5 Fluorescently labelled exosomes (red) transferred to DAPI stained nuclei of (blue) of recipient cell population, stained with Alexflour 499-phalloidin (green) to view the cytoskeleton.

5.4.4 Impact of exosome transfer on miRNA expression

Exosomes from T47D cells were isolated and transferred to two different recipient cell populations, T47D and MDA-MB-231 breast cancer cells. Two incubation periods were investigated, 4 hours and 24 hours, for each recipient cell population. Recipient cells which did not receive exosomes acted as a control. Following the required incubation period, the cells were harvested and miRNA was extracted. RQ-PCR was performed targeting 3 of the miRNAs identified to be present in exosomes, namely miR-10b, miR-145, and miR-492. miR-16 expression was also investigated and used as an endogenous control. The levels of expression for each miRNA were investigated in each recipient cell line. As depicted in the boxplots (Figure 5.6), there were no significant changes observed with any of the miRNAs investigated, between the cells which received exosomes and those which did not (Figure 5.6 (A-C), t-test: $p>0.0$). In addition, a longer incubation period of 24 hours did not appear to have an effect on expression levels of the miRNAs investigated.

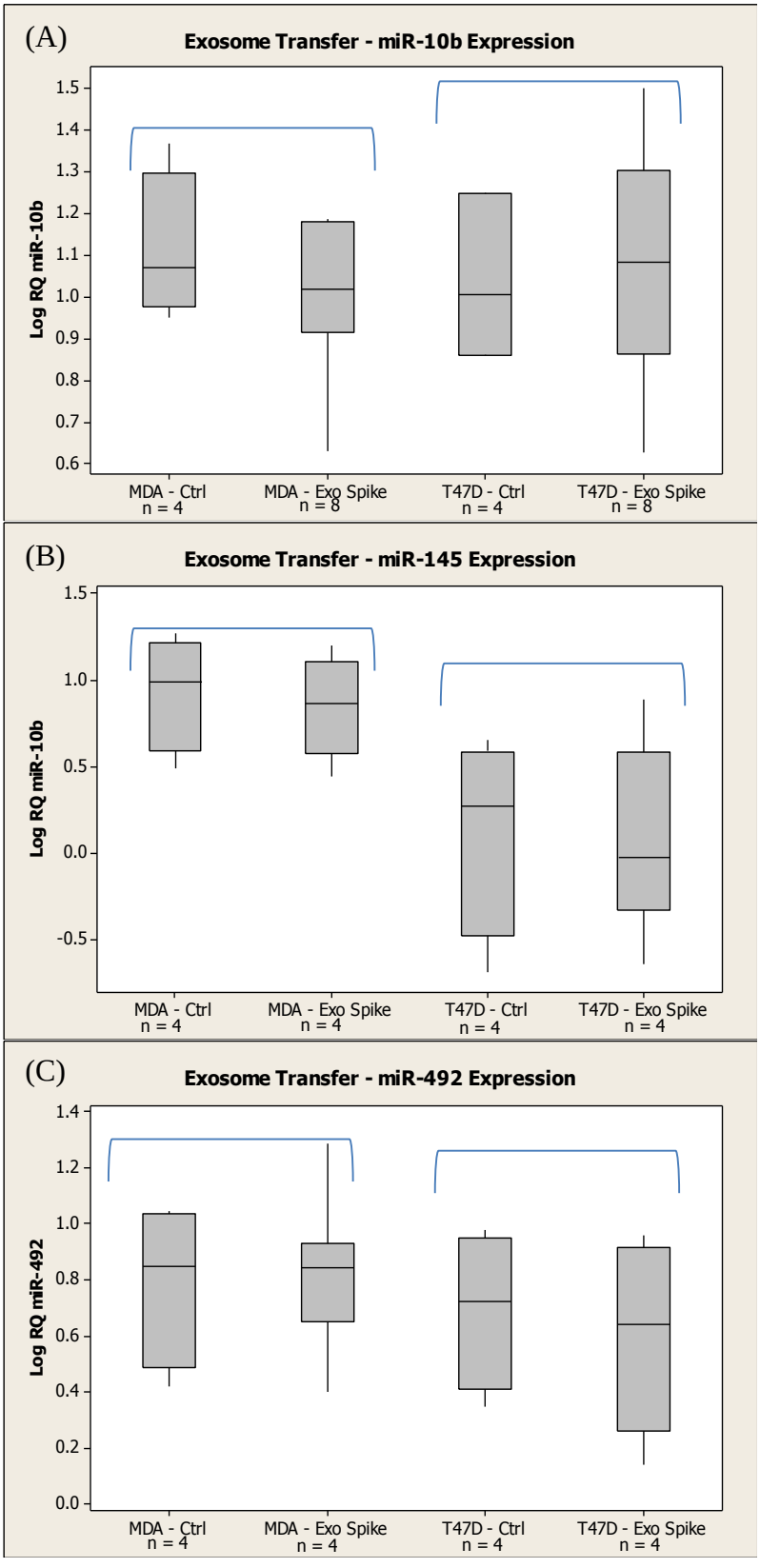


Figure 5.6 miRNA expression levels in cells which received exosome transfer/spike compared to cells which did not receive exosomes. (A) miR-10b, (B) miR-145, (C) miR-492, showing no significant difference between each group (2-tail T-test: $p > 0.05$).

5.4.5 Impact of exosome transfer on cell proliferation

Exosomes were isolated from T47D cells and the purified exosome pellet was resuspended in fresh exosome-depleted media. The exosomes were 'spiked' into a 96-well culture plate containing T47D cells in exosome-depleted media. Control wells contained T47D cells growing in either exosome-depleted media, or T47D cells growing in regular complete media. The CellTiter 96® Aqueous Non-Radioactive Cell proliferation assay (Promega) was used to compare the cell proliferation of the cells at 48 hours and 72 hours after spiking the test wells with exosomes. The results of 8 wells were averaged per sample, and three replicates of the assay were completed. Statistical analysis using ANOVA show a significant increase ($p<0.05$) in cell proliferation at both 48 hours and 72 hours in the cells which received the spike of exosomes (Figure 5.7, $p<0.05$).

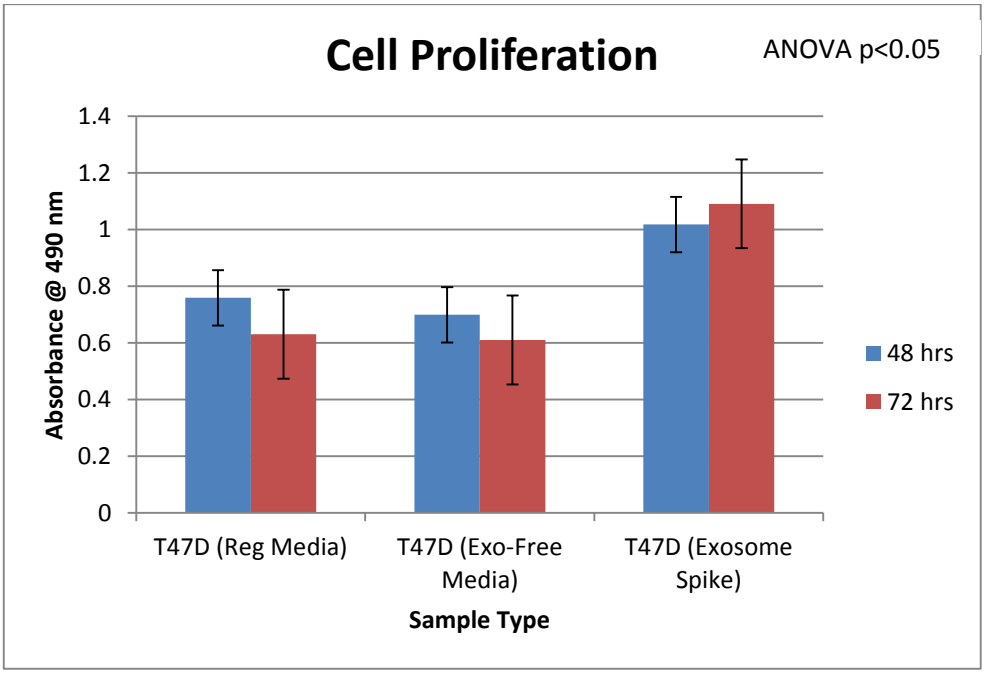


Figure 5.7 Absorbance values at 490 nm for T47D cells growing in regular media, exosome depleted media, and cells which received a spike of exosomes.

5.4.6 Impact of exosome transfer on cell migration

Exosomes were isolated from the cell-conditioned media of T47D cells and were transferred to MDA-MB-231 and T47D cells, where the impact of exosomes on the ability of the cells to migrate was assessed. Control samples included cells which only received fresh exosome-depleted media. This text was carried out in duplicate, with means displayed in the graph below. While the MDA-MB-231 cells did migrate, there did not appear to be any significant difference in the numbers of cells that migrated between the controls and cells which received exosomes (Figure 5.8, $p > 0.05$). The T47D cells did not migrate at all, showing that the addition of exosomes did not stimulate migration in these cells.

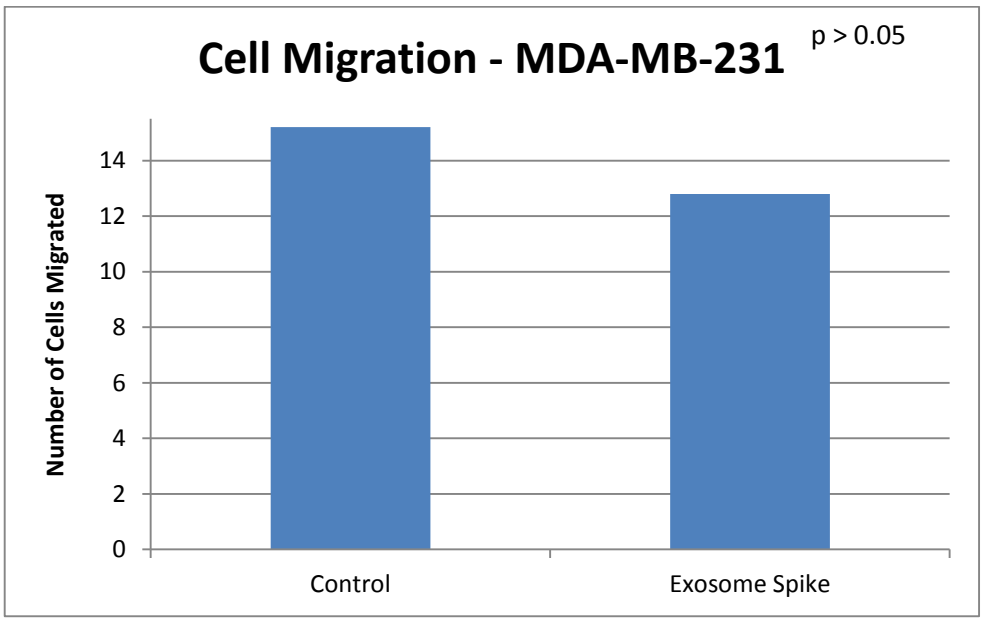


Figure 5.8 Impact of exosomes on cell migration.

5.5 Discussion

Extracellular RNAs have been proposed to exist in 4 forms: freely circulating RNAs, Argonaute 2 bound RNA, high density lipoprotein bound RNA, and vesicle associated RNA. This study focused on the vesicle-associated miRNA, more precisely, the exosome-encapsulated miRNA. Exosomes are released by most but not all cell types and have been found to be secreted in larger amounts from cancer cells (282). The transfer of genetic information, including miRNAs, via exosome-mediated delivery, may facilitate tumour initiation and progression by delivery of oncogenic proteins. Exosomes operate very efficiently as mediators of cell-to-cell communication and play crucial roles within the tumour microenvironment. Their utility, however, does not end there. Exosomes represent an attractive source of biomarkers as they confer the following favourable characteristics: (i) they are actively released from living tumour cells, (ii) they reflect the characteristics of the donor cell, (iii) they can be obtained non-invasively from biological fluids, and (iv) they are remarkably stable. The mechanism of secretion of exosomes containing miRNAs is a hot topic and the current undefined process suggests miRNAs are sorted into multivesicular bodies, which fuse with the plasma membrane via inward budding and subsequently releases the exosomes into the extracellular space (120). The ability of the exosomes/multivesicular bodies to translocate across the cell membrane, is not only important in the release of exosomes but also in the uptake and entry into recipient cells whereby they can mediate cell-to-cell communication. In a recent paper by Kobayashi *et al.* (283), it was shown that many of the miRNAs enriched in extracellular vesicles may not be abundant, or even detectable, in the parent cell, suggesting active selection during compartmentalisation into exosomes. This confirms the hypothesis of selective packaging of miRNAs into vesicles/exosomes for release into the extracellular environment. Indeed, data presented here revealed some miRNAs to be present in exosomes secreted from breast cancer cell lines, while some miRNAs were not. The strength of exosomes as biomarkers of disease relies on the enrichment of highly selected markers during the exosomal sorting process, which represents less than 0.01% of the total proteome of body fluids. In this setting ‘less is more’, as

although the cargo of the exosomes is small in size, the information they convey regarding the pathological condition of the cell source is potentially great.

To our knowledge, this is the first report investigating the full range of miRNAs contained within exosomes isolated from the conditioned media of breast cancer cells. This study investigated the isolation of exosomes from a range of breast cancer cell lines and subsequent miRNA profiling of their content. An array was performed by Exiqon (Denmark), which targeted 2083 currently known miRNAs. Data normalisation was performed using Quantile normalization. Quantile normalisation is based on the assumption that two sets of closely related data should sort themselves in a linear fashion forming a diagonal when plotted against each other. In essence, it enforces an equal distribution on the data. The signal distribution plot for this data showed that the distributions of the samples were similar and the assumption of the Quantile normalisation was fulfilled. Between 381 and 394 miRNAs were detectable in exosomes from each breast cancer cell line, with 287 miRNAs common to all 4 cell lines. It is worth noting that many of the detected miRNAs have high numbering, i.e. >miR-2000, which is not entirely desirable. This is due to the fact that many of these newly discovered miRNAs have not been as well characterised as the lower numbered miRNAs, although all miRNAs included in the array have been annotated on miRBase. Following sorting and ranking, miRNAs of interest were selected for further RQ-PCR validation based on differential and similar expression profiles across the four breast cnacer cell lines. It was hypothesised that the miRNAs which may be of interest to this investigation are not only highly expressed in the samples but also show greater variance between samples, i.e. relative expression of a miRNA in one cell line, with low expression in another, and also have previous associations with cancer. Therefore, four miRNAs were chosen for further validation. Specifically miR-10b, miR-145, miR-492 and miR-498.

miR-10b has well established associations with many cancers and, in particular, with breast cancer (284-287). One of the most recent studies showed that miR-10b was significantly upregulated in breast cancer tissues and cells and indeed the expression of miR-10b was shown to be closely

correlated with aggressiveness in breast cancer (288). Furthermore, the same publication reported inhibition of miR-10b could also partially suppress the EMT, invasion and proliferation induced by TGF-β1 in breast cancer cells. With this evidence supporting miR-10b as a potential target for breast cancer prevention and therapy, this miRNA surely warrants further investigation in terms of exosome-mediated delivery. This potential for exosome-mediated transfer adds a novel dimension to this compelling story. Three individual preparations of exosomes were isolated from 4 breast cancer cell lines of varying epithelial subtype. miR-10b expression was investigated in these exosomes and was found to be present in all exosome samples. This suggests miR-10b is packaged into exosomes by breast cancer cells to mediate cell-to-cell communication. Based on the known role of miR-10b, it is reasonable to speculate that the packaging of miR-10b into exosomes may support oncogenic transformation of adjacent cells.

miR-145 expression has been reported to be decreased in many cancers (289) and it has been suggested that miR-145 plays an important role in suppressing tumour growth and promoting apoptosis (290-292). However, the function of miR-145 appears to be cell type specific (293-295) and therefore it can be hypothesised that miR-145 may play different roles in normal cells and tumour cells. In this current study, miR-145 was found to be expressed in exosomes collected from the conditioned media of the 4 breast cancer cell lines demonstrating active packaging of this miRNA.

miR-492, and its family members, have previously been shown to be highly expressed in Basal breast cancer cells (72). This finding holds true to its expression in exosomes isolated from the basal breast cancer cell lines BT-20 and MDA-MB-231, where miR-492 was found to be more highly expressed, although not significantly, when compared to the other epithelial subtype cell lines.

miR-498 has recently been identified as a novel regulator of the HER2 3'UTR (296). In the array data, miR-498 expression was lowest in the exosomes isolated from the HER2 overexpressing SKBR3 cell line. Interestingly, upon further validation by RQ-PCR, miR-498 was not found to be detectable in any exosome preparations isolated from all 4 breast cancer subtypes. The array used probes produced by Exiqon, while the probes used for RQ-PCR

validation were producd by Applied Biosystems. It was confirmed that the ABI Taqman probes and the Exiqon probes target the same sequence (UUUCAAGCCAGGGGGCGUUUUUC). Therefore, the issue can not be that different sequences were targeted as different companies supplied the probes. This highlights the importance of validation in multiple samples following array profiling.

It has been shown that exosomes have the ability to bind to, and be taken-up by, target cells (297). Furthermore, it has been shown that exosome uptake can alter target cells which may differentiate, dedifferentiate, or become activated, depending upon the content delivered (298). Therefore, in this study, the transfer of exosomes from one cell population to another was investigated. Exosomes were isolated from the conditioned media of T47D breast cancer cells which had previously been tagged during lentiviral transduction with a red fluorescent protein (see Chapter 6). Exosomes were allowed to bind and adhere to the recipient cell line for both 4 hours and 24 hours. In addition, a separate preparation was included to stain with Alexaflour-488 phalloidin in order to view the cytoskeleton of the cells. Numerous images were obtained from all preparations showing the RFP-labelled exosomes adhering to the DAPI stained cell surfaces. The images obtained are Z-stack images and presented as Maximum Intensity Projections (MIP). Using these techniques, the shallow depth of field due to the high numerical aperture required, can be corrected by focusing up and down through the sample capturing a series of slices, which are then compressed into one image whereby the brightest part of each image is taken and projected. This results in the microscopy data of a 3-D structure to be presented in 2-D focus stacking. Therefore, there is no depth in these images when they are presented, and as a result it appears as if the exosomes are in the nucleus, when they are actually adhering to the cell surface. The images obtained correlate to what has been observed in other reports in terms of number of fluorescently labelled exosomes transferred and in terms of positioning (162, 279-281).

It was also clear that the exosomes were targeting the cell surfaces as there were few or no exosomes visible in the gaps between cell colonies.

It was hypothesised that the miRNAs previously identified to be highly expressed in exosomes, would then, once transferred, increase the level of expression in the recipient cells. Previous studies have shown increased levels of particular miRNAs in recipient cells treated with exosomes containing enriched levels of those particular miRNAs (162, 270, 299). This was investigated in this study by transferring preparations of isolated exosomes, onto recipient cells, allowing them to bind and adhere, and then directly extracting the miRNA content from the recipient cells. The three miRNAs of interest, previously identified to be present in exosomes, were investigated by RQ-PCR. No significant difference or increase in expression was observed in the samples which received the exosomes. However, it must be considered that quantification of the number of exosomes transferred was not been conducted prior to transfer. Indeed the number of exosomes transferred, or length of time allowed for uptake, may have an important impact on this experiment. Known target genes or protein products could also potentially reveal changes in expression following exosome transfer.

In order to view the impact of exosomes on cell proliferation, MTS assays were prepared. T47D cells were grown in regular media, in exosome free media, and exosomes were spiked into cells in media. Increased cell proliferation was observed in the cells which were spiked with exosomes. This is perhaps as a result of numerous proteins packaged within the exosomes and therefore exerting their effect, and in turn increasing the cell proliferation. The increase of proliferation confirms that exosomes have an impact on recipient cell populations, although, it must be emphasised that this is an effect of the 'whole exosome' and not just the miRNA content of the exosomes. Other studies have shown exosome transfer to have an impact on recipient cell populations, with one study highlighting that the phenotypic traits representing the cells of origin, such as increased cellular proliferation, can be transferred to the secondary cells (163).

Migration assays were performed whereby cells were spiked with exosomes and control cells did not receive exosomes. The impact of exosomes on cell migration was investigated. There was no significant difference observed in the migration of the MDA-MB-231 cells which received exosomes and those which did not. The T47D cells did not migrate at all, which is not unusual, as

that particular cell line is known to have very low migratory power. Therefore the purpose here was to ascertain if the addition of exosomes would stimulate migration in the T47D cells, which it did not.

A critical issue in all exosome-related research is the lack of investigation, or indeed disclosure of quantification of exosomes released from cells. NanoSight technology has been reported as the most robust and reliable method for quantification of exosomes in a wide range of preparations. However, as access to such technology is limited, estimations are made based on standardised seeding densities of cells, protein levels, and incubation periods. It has been shown that tumour cells secrete more extracellular vesicles, including exosomes, when compared to normal cells (282). Gercel-Taylor *et al.* (282), utilised the Nanoparticle Tracking Analysis System (NanoSight) to quantitate extracellular vesicles in the circulation of normal healthy individuals, patients with benign ovarian disease, and patients with malignant ovarian cancer. Extracellular vesicles were detectable in the circulation of all patients, although patients with malignant ovarian cancer demonstrated ~3-4 fold more vesicles. Studies have also shown that exosome secretion can be increased in breast cancer cell lines by the introduction of hypoxic environments (177). Hypoxia is a well established feature of tumours, and is most frequently associated with aggressive tumour phenotypes and poor patient outcome (300, 301). As exosomes derived from tumours have the potential to play important roles in both cell-to-cell communication and cancer diagnosis as biomarkers of disease, it is important that the stimuli which promote exosome secretion by tumour cells are understood.

The release of miRNAs and indeed other cellular components into the extracellular space is an area of research which has stimulated much discussion. Improved nomenclature and definition is certainly required as this is a field that is rapidly progressing. Currently microveiscles and exosomes are defined by size as previously discussed. The exosome isolation method utilised in this study excludes apoptotic bodies and most microvesicles by differential centrifugation and micron filtration, while TEM analysis revealed the purified exosome fractions obtained using ultracentrifugation contained vesicles correlating to the correct size and shape of exosomes.

The exact mode of secretion of exosomes from cells and the subsequent release of miRNAs is another critical issue which urgently requires further research. Kosaka *et al.* (93), previously reported that secretion of miRNAs from cells was regulated by neutral sphingomyelinase 2, which is a rate limiting enzyme of ceramide biosynthesis and is known to trigger secretion of extracellular vesicles, including exosomes. Previous reports have shown secreted miRNAs to be bound to the Ago2 protein, which is a main component of the RISC complex (302). However, this protein has not been found in exosomes nor in extracellular vesicles as a whole (129). It is clear that significant research is required imminently to identify proteins which are responsible for the packaging of miRNAs from inner cells into exosomes.

5.6 Conclusion

This study demonstrates that breast cancer cells selectively secrete a range of exosome-encapsulated miRNAS, some of which are implicated in important pathways related to carcinogenesis. This exosomal transfer of miRNAs may play an important role in intercellular communication within the primary tumour microenvironment and indeed may represent a robust source of circulating information from the tumour highlighting their potential in the biomarker setting.

Chapter 6

Investigating miR-504 in breast cancer

6 Chapter 6: Investigating miR-504 in breast cancer

6.1 Introduction

Previous unpublished work by this laboratory highlighted a potential suppressor role for miR-504 in breast cancer, where it was found to be downregulated in a small cohort of patient breast tumours when compared to normal healthy tissues. Therefore, one of the aims of this study was to validate this finding in a separate and larger cohort of patient breast tissue samples. Published studies by other research groups have identified miR-504 to negatively regulate p53 expression, through binding to two sites in the 3'UTR (303). Overexpression of miR-504 was shown to reduce the level and impair the functions of p53, which is the most frequently mutated gene and is known to play a central role in tumour suppression (304). miR-504 is located at Chromosome Xq27.1. Interestingly, frequent DNA amplification of this locus has been reported in several different cancers, including T cell non-Hodgkin's lymphomas and gastric carcinomas (305, 306). Contrary to the previous findings of miR-504's potential oncogenic role, in another study, miR-504 was found to be significantly down-regulated in hypopharyngeal squamous cell carcinoma (HSCC) tissues, suggesting a potential tumour suppressor role (307). In human glioblastomas, miR-504 expression correlates with expression of several EMT, or stem cell-related markers (308). Further to this, miR-504 has been reported to be an obesity-responsive negative regulator of p53 and putative regulator of EMT, whereby findings suggest that obesity may mimic or augment procancer effects related to p53 gene alterations. Therefore, miR-504 may represent a novel molecular target for breaking the link between obesity related hormones and the development breast cancer (309). In a recent study by Soutto *et al.* (310), a novel mechanism was identified by which TFF1 suppresses cell growth and induces apoptosis through activation of p53, by downregulation of miR-504 in gastric cancer cells. With the lack of published reports studying the role of miR-504 in breast cancer specifically, a comprehensive investigation is warranted.

Numerous miRNAs have been identified as oncogenic (oncomiRs) or as tumour suppressors. Tumour suppressing miRNAs are of particular interest

when investigating miRNAs in cancer, as it is essential to establish their mechanism of action. The causative role of specific miRNAs in tumourigenesis remains to be fully elucidated. However, it is understood that miRNAs function in concert with classical tumour suppressors and oncoproteins to regulate key pathways icolved in cellular growth control. Differential expression of miRNAs offers great potential in the field of biomarker discovery for the diagnosis of cancers. However, tumour suppressing miRNAs provide an additional level of utility in terms of cancer therapy. Indeed, the delivery of tumour suppressing miRNAs to a target site, via the exploitation of exosomes, offers immense potential in the field of targeted therapeutics. As shown in the previous chapter, exosomes are natural carriers of RNA, and are readily released by donor cell populations and subsequently taken up by recipient cell populations. This mode of delivery and exchange of genetic information between two cell populations offers a vehicle for the delivery of tumour suppressing miRNAs, whereby a gene silencing effect on recipient cells could be conferred.

6.2 Aims

The aims of this study were as follows:

- To validate miR-504 dysregulation in an individual and larger breast cancer patient cohort
- To investigate the impact of miR-504 replacement/overexpression *in vitro* and *in vivo.*

6.3 Materials and Methods

Following informed patient consent, breast tissues were harvested including matched Tumour (n = 50) and Tumour Associated Normal (TAN) (n = 50) tissue from the same patient, and normal healthy tissues (n = 17). The tissues were homogenised, RNA was extracted, and RQ-PCR was performed targeting miR-504, with miR-16 and let-7a as endogenous controls. The tumour data was further stratified based on epithelial subtype, tumour stage, tumour grade, and menopausal status (Table 6.1).

Number of Tissues	***Total n = 117***		
Tissue Type	**Tumour**	**TAN**	**Normal**
	50	50	17
Epithelial Subtype	***n***		
Luminal A	35		
Luminal B	5		
HER2	6		
Basal	5		
Tumour Grade	***n***		
Grade 0	5		
Grade 1	6		
Grade 2	25		
Grade 3	14		
Tumour Stage	***n***		
Stage 1	13		
Stage 2	23		
Stage 3	11		
Stage 4	3		
Menopausal Status	***n***		
Pre	13		
Peri	4		
Post	33		

Table 6.1 Patient clinicopathological characteristics.

Following lentiviral transduction of T47D cells with a Non Targeting Control (T47D-NTC) and miR-504 mimic (T47D-504) (as discussed in section 2.4), successful transduction was confirmed by RQ-PCR (overexpression of miR-504) and Fluorescent Microscopy (visualisation of the RFP). Proliferation and migration assays were carried out on the T47D-NTC and T47D-504 cells. Expression of potential target genes in the cells was analysed by RQ-PCR. Exosomes were isolated from the conditioned media of both T47D-NTC and T47D-504 cells, miRNA extracted, and miR-504 expression investigated by RQ-PCR. Exosomes were isolated again and were transferred to recipient cell populations, where their effect on miRNA expression, cell proliferation, and cell migration were investigated.

Following ethical approval, *in vivo*, athymic nude mice were inoculated with T47D-NTC cells (control group; n = 5) and T47D-504 cells (test group; n = 5), and tumour formation was monitored over 8 weeks. Tumours were harvested and blood samples were collected. RNA was extracted from the tumours and bloods and miR-504 expression was investigated by RQ-PCR. Gene expression analysis was performed on the tumour tissues by RQ-PCR.

6.4 Results

6.4.1 Investigating miR-504 in patient breast tissues

Following informed patient consent, breast tissues were harvested during surgical resection including matched Tumour (n = 50), and TAN (n = 50) tissues from the same patient. Normal healthy tissues (n = 17) were harvested during breast reduction mammoplasty. The majority of patients were post-menopausal, with predominatly Luminal A subtype tumours, representing a typical patient cohort. miR-504 was detectable in all tissue samples and was found to be significantly down regulated in the tumour tissues (Mean ± SEM; 1.286±0.088 Log_{10} Relative Quantity (RQ)) compared to both the normal (0.391±0.095 Log_{10} RQ) and TAN (0.617±0.088 Log_{10} RQ) tissues (Figure 6.1, $p<0.001$).

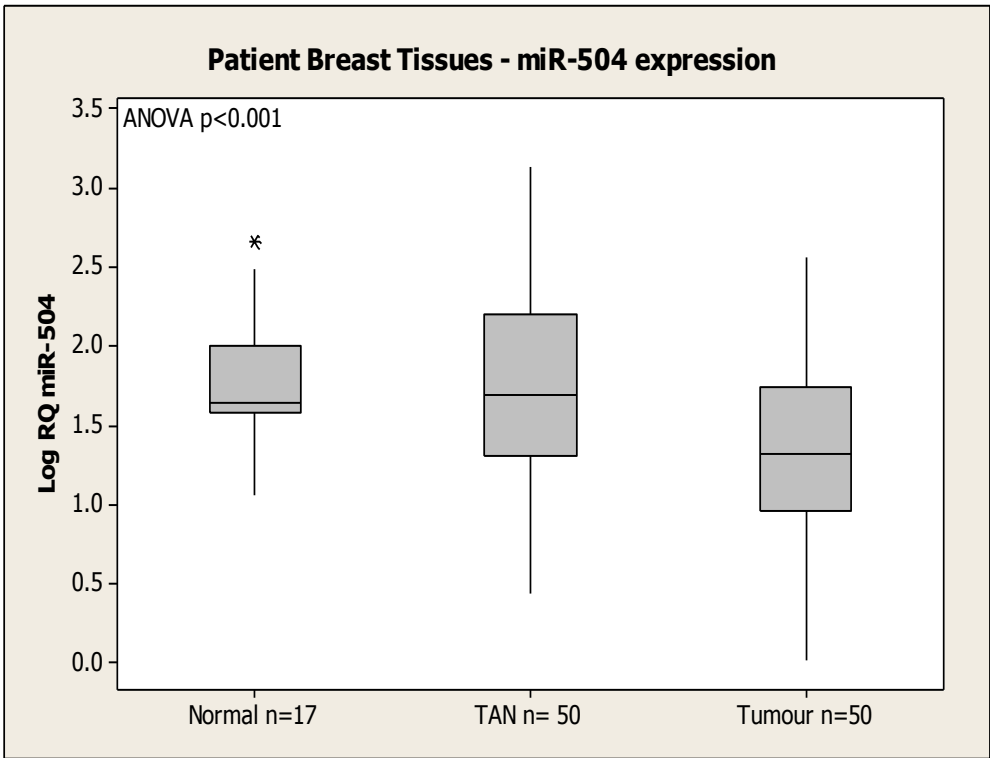


Figure 6.1 miR-504 expression levels in normal, Tumour Associated Normal (TAN) and tumour breast tissue. '*' respresents outliers within the group. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

The tumour tissue data was then further stratified based on epithelial subtype, tumour stage, tumour grade and menopausal status. There was no significant dysregulation observed (Figure 6.2 A-D).

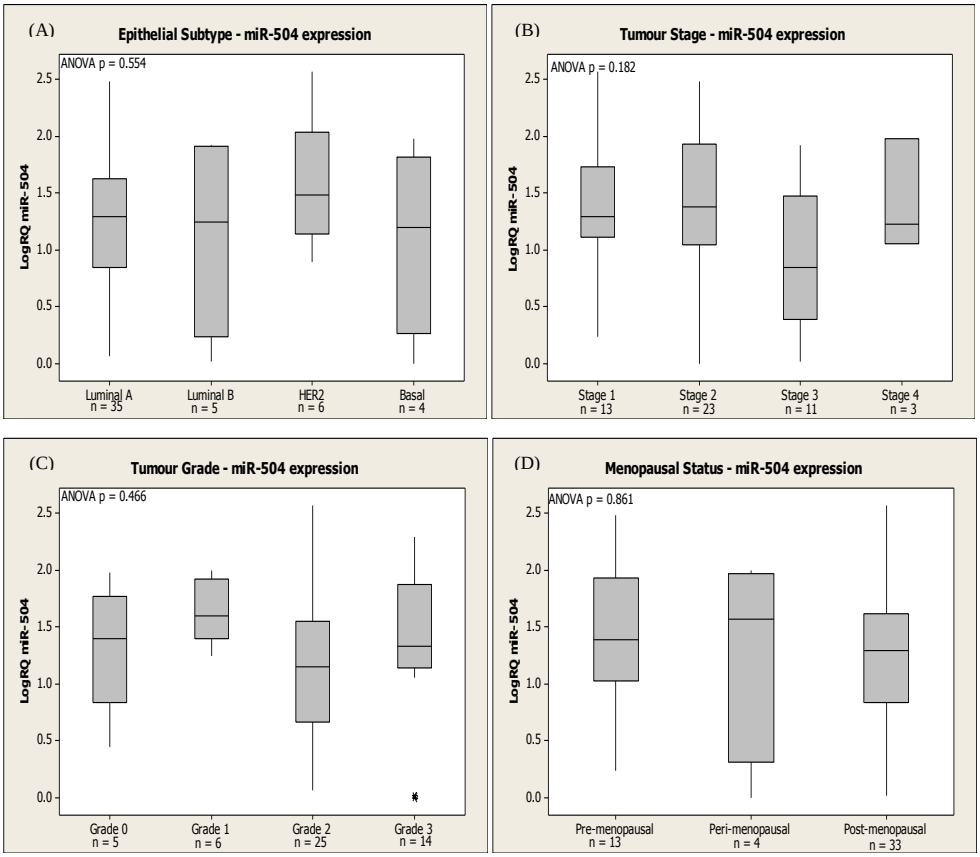


Figure 6.2 miR-504 expression in tumour tissues further stratified based on (A) epithelial subtype, (B) tumour stage, (C) tumour grade, and (D) menopausal status. '*' respresents outliers within the group. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

6.4.2 Confirmation of successful Lenti-viral Transduction

Following lenti-viral transduction of T47D breast cancer cells with a Non Targeting Control (T47D-NTC) and miR-504 mimic (T47D-504), successful over-expression of miR-504 in the T47D-504 cells was confirmed by RQ-PCR in multiple samples. A 2-tailed T-Test revealed significant over-expression of miR-504 ($p<0.001$, Figure 6.3 A). In addition, a Red Fluorescent Protein (RFP) tag was contained within the lentiviral construct, therefore fluorescent microscopy was performed to visualise and confirm successful transduction. The successfully transduced cells were visualised in red (Figure 6.3 B), while the DAPI stained nuclei were visualised in blue (Figure 6.3 C).

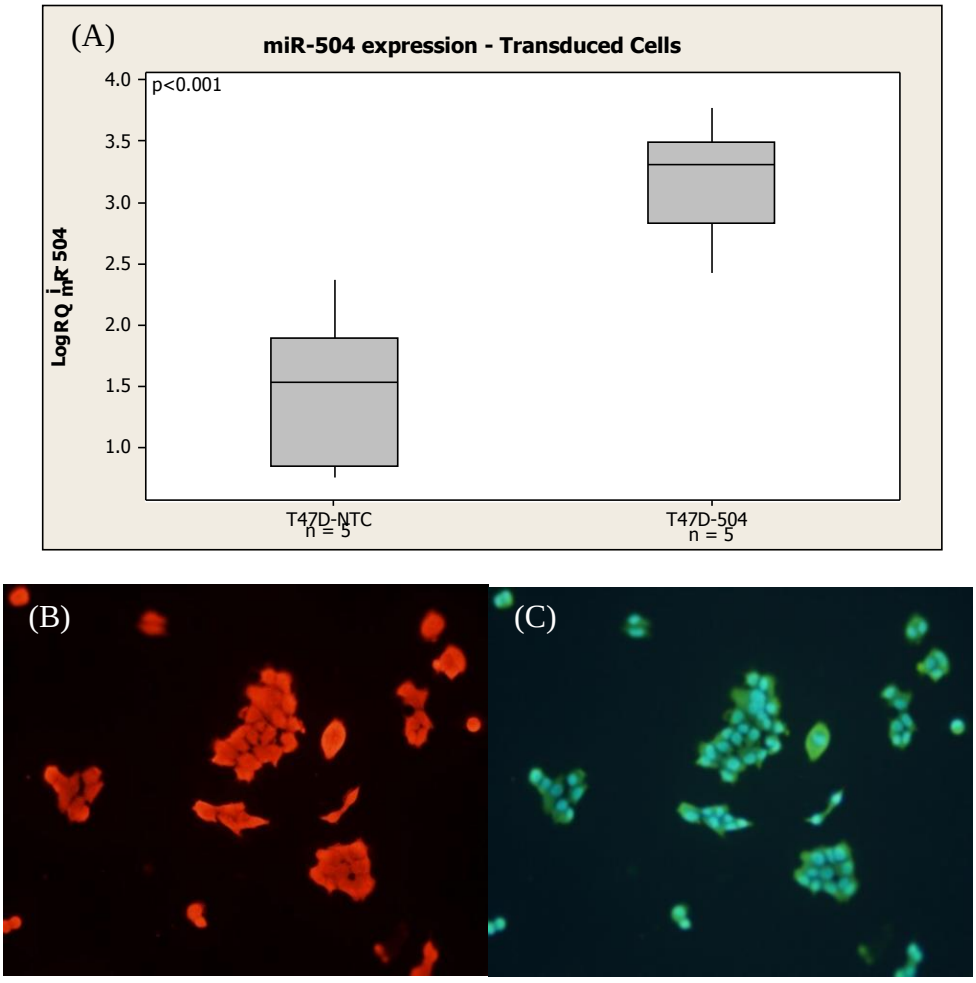


Figure 6.3 Confirmation of successful transduction of miR-504 in the T47D-504 cells. (A) By RQ-PCR, and fluorescent microscopy with (B) the RFP labelled transduced cells shown in red (excitation at 482 nm, emission at 520 nm), and (C) the DAPI stained cell nuclei in blue (excitation at 368 nm, emission at 461nm).

6.4.3 Investigating impact of miR-504 overexpression *in vitro*

6.4.3.1 Effect of miR-504 on cellular proliferation

The effect of miR-504 overexpression on the proliferation of cells was investigated. No significant change was observed when comparing T47D-NTC cells to T47D-504 cells at any of the time points analysed (Figure 6.4).

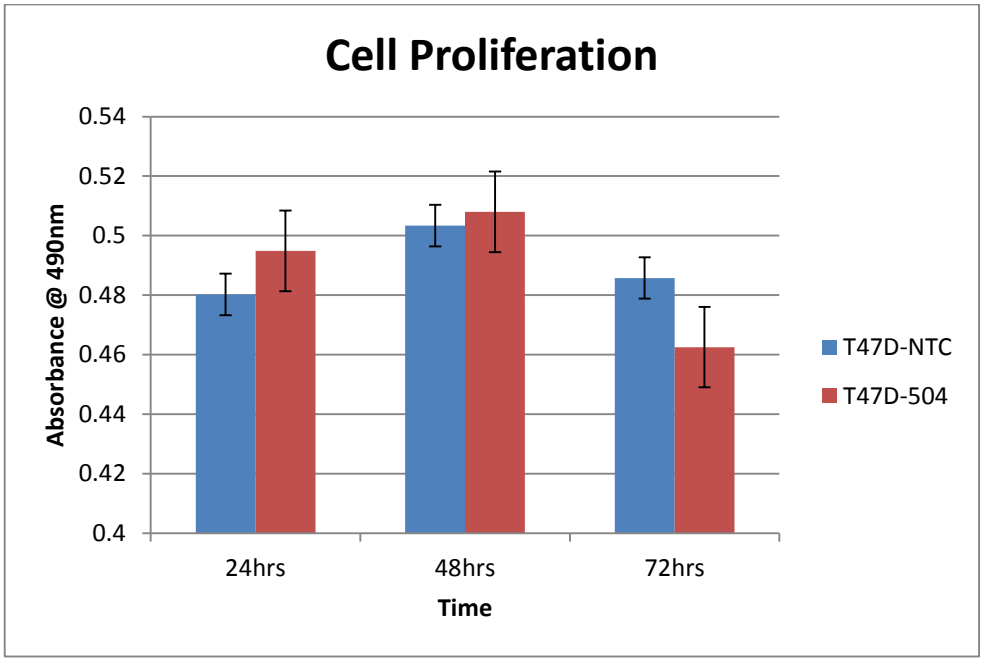


Figure 6.4 Comparison of T47D-NTC and T47D-504 cell proliferation with absorbance read at 490 nm.

6.4.3.2 Effect of miR-504 overexpression on cellular migration

The effect of miR-504 overexpression on cell migration was investigated. T47D-NTC cells and T47D-504 cells were seeded into Corning Transwell Inserts with a pore size of 0.8 µm, and migration in response to FBS was observed. Neither the T47D-NTC cells nor the T47D-504 cells were observed to migrate (Figure 6.6).

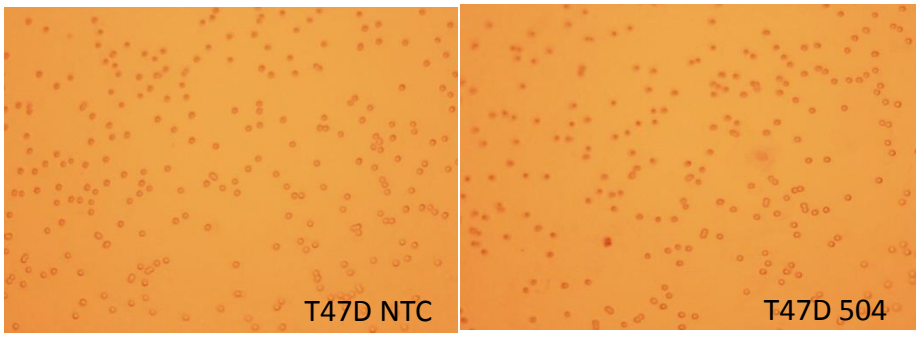


Figure 6.5 Light microscope images of migration membranes showing no cells migrated with the pores clearly visible.

The membrane pores are visible, however no cells have migrated. T47D cells are a non-invasive breast cancer line, therefore only an increase would be observed, if any change at all.

6.4.3.3 Effect of miR-504 expression on gene expression

Gene expression analysis was performed on T47D-NTC cells and T47D-504 cells. Genes analysed included: CDH6, p53, FOXP1, N-Cadherin, TWIST and Snail. CDH6, p53 and FOXP1 are reported to have predicted binding sites in the 3'UTR for miR-504 (303, 311). TWIST and Snail are associated with EMT. Significant upregulation of FOXP1 (ANOVA $p<0.001$, Figure 6.6 B) and TWIST (ANOVA $p<0.05$, Figure 6.6 C) were observed in the T47D-504 cells. P53, N-Cadherin and Snail were not found to be significantly dysregulated (ANOVA $p>0.05$) (Figure 6.6 A, D, and E). CDH6 was not detectable by RQ-PCR in any of the samples and perhaps requires immunohistochemistry. Immunohistochemistry, although is not quantitative, assesses the level of translated protein, which can be more informative than the level of mRNA.

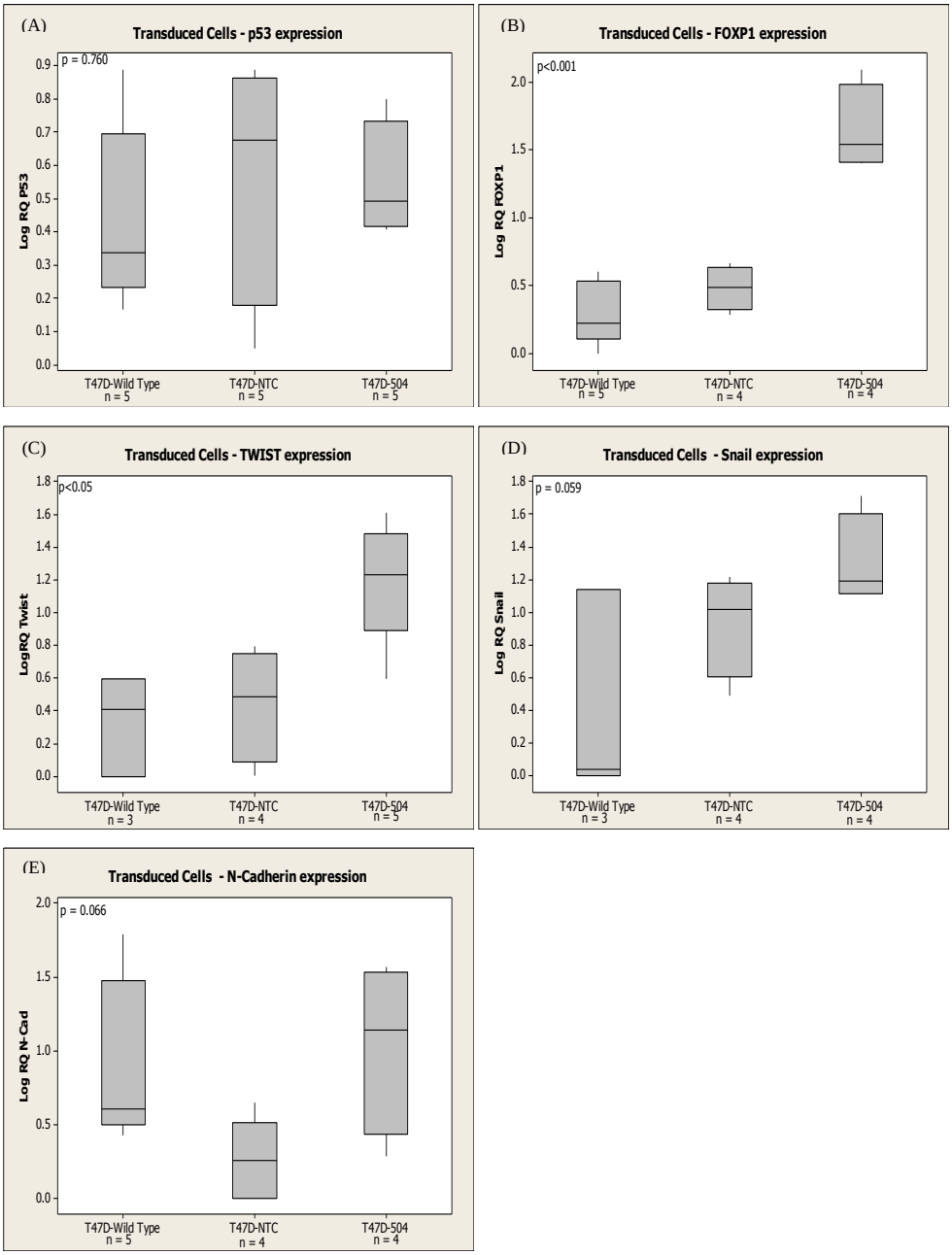


Figure 6.6 Gene expression analysis results from T47D-WildType cells, T47D-NTC cells and T47D-504 cells. (A) p53, (B) FOXP1, (C) TWIST, (D) Snail and (E) N-Cadherin. ANOVA p values show the significance of changes. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

6.4.3.4 Investigating the impact of exosome transfer

Detection of miRNA enrichment in exosomes using RQ-PCR

To determine whether it was possible to engineer exosomes enriched with miR-504, exosomes secreted by T47D-Wildtype cells, T47D-NTC cells and T47D-504 cells were isolated. miRNA was extracted from the exosomes, conditioned media and cells, and RQ-PCR was performed, targeting miR-504, with miR-16 and let-7a as endogenous controls, which was found to be detectable in the exosomes secreted by the T47D-504 cells, and was not detectable in the exosomes secreted by the T47D-Wildtype and T47D-NTC cells (Table 6.2). miR-504 was detectable in the cells of all three populations. The average CT values for each sample are listed below. The lower the CT value, the higher the expression. Samples were considered 'undetected' if they fell beyond the 40 CT Limit of Detection for the assays i.e. the expression was too low to be detected.

	Exosomes	Conditioned Media	Cells
T47D-Wildtype	×	×	32.36
T47D-NTC	×	×	34.98
T47D-504	30.45	32.61	22.07

Table 6.2 Expression analysis of miR-504 in exosomes, conditioned media and cells, collected from T47D-Wildtype, T47D-NTC and T47D-504 cells. ('×' denotes not detected)

Impact on miRNA expression in recipient cells

Exosomes were isolated from the conditioned media of T47D-NTC cells and T47D-504 cells and were transferred to MDA-MB-231 and T47D cells growing in 12-well plates. Following 4 hour and 24 hour incubations, miRNA was extracted from the cells. RQ-PCR was performed targeting miR-504, with miR-16 as an endogenous control. No significant increase in miR-504 expression was observed in any of the cells which received miR-504 exosomes (Figure 6.7). There is an interesting trend however, towards increased miR-504 expression in the cells which received 504-exosomes.

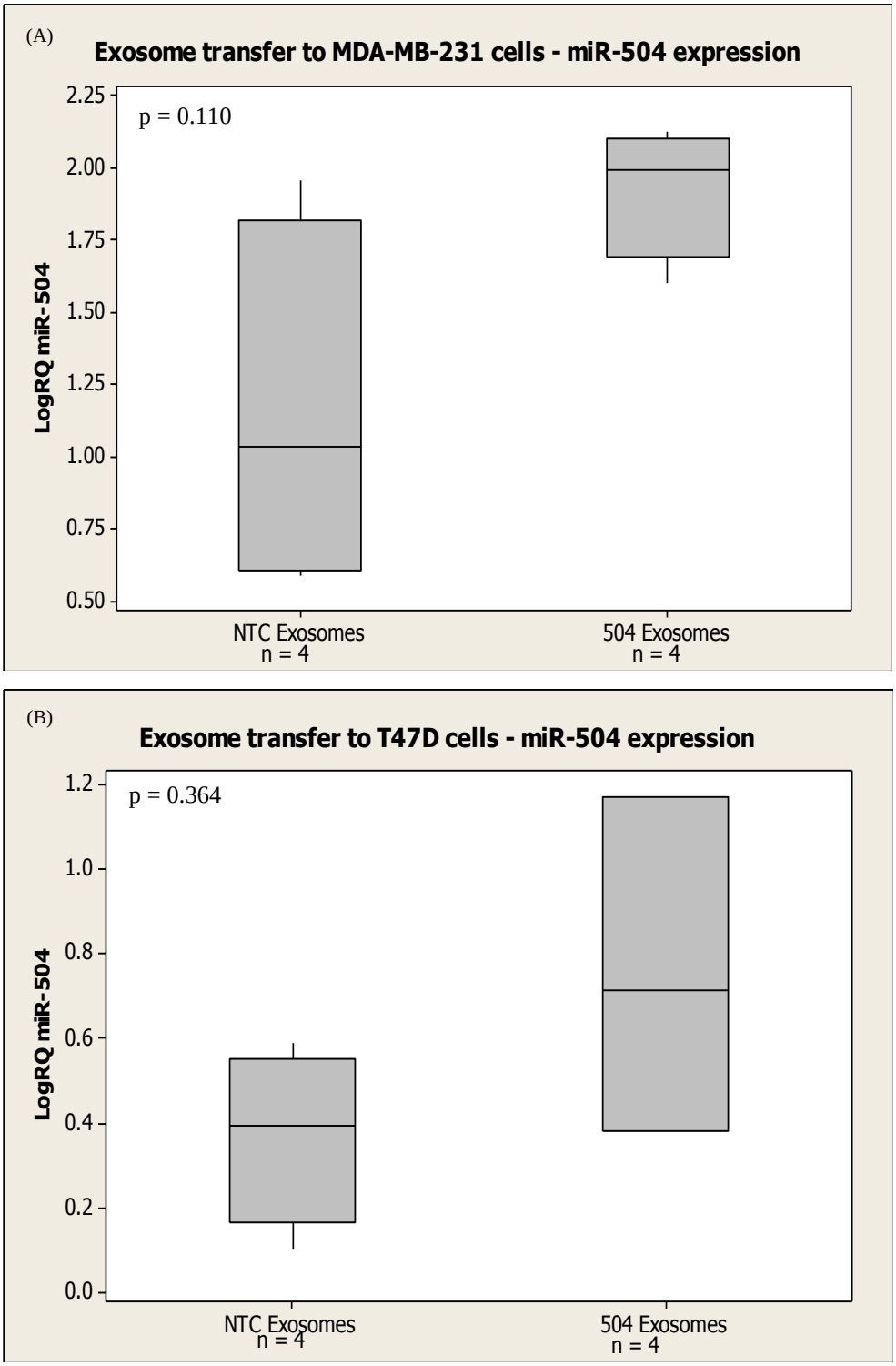


Figure 6.7 miR-504 expression in (A) MDA-MB-231 cells and (B) T47D cells, which received exosomes isolated from T47D-NTC or T47D-504 cells. '*' respresents outliers within the group. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

Impact on proliferation in recipient cells

Exosomes were isolated from the conditioned media of T47D-NTC and T47D-504 cells and were transferred to T47D-Wildtype cells in a 96-well plate. Control cells included T47D cells growing in regular/complete media and T47D cells growing in exosome free media. The proliferation of the cells was assessed at 48 hours and 72 hours. The cells which received the exosomes did display a significant increase (ANOVA: $p<0.05$) in cell proliferation at both time points, although there was no significant difference (2-Tail T-test: $p>0.05$) observed between the cells which received the T47D-NTC exosomes and those which received the T47D-504 exosomes (Figure 6.8).

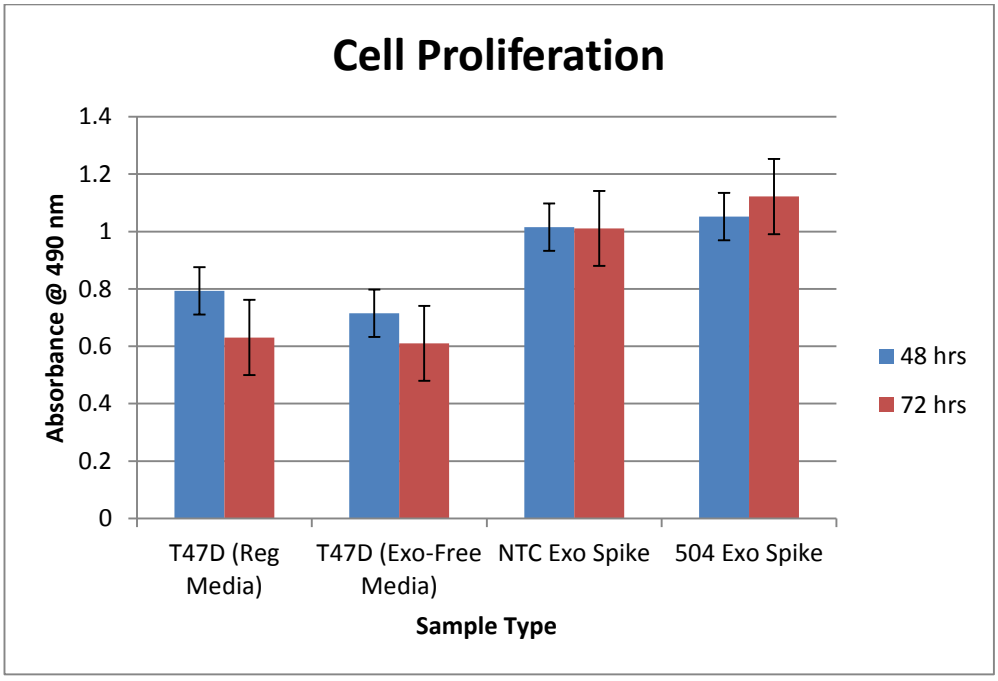


Figure 6.8 Proliferation of cells which received exosomes isolated from T47D-NTC and T47D-504 cells, with absorbance read at 490 nm.

Impact on cellular migration in recipient cells

Exosomes were isolated from the conditioned media of T47D-Wildtype (WT) cell, T47D-NTC cells and T47D-504 cells, and were transferred/spiked into MDA-MB-231 and T47D cells. Control cells received fresh exosome-depleted media. Following exosome uptake, the migratory capacity of the cells was investigated. This assay was performed in duplicate with averages of the duplicate displayed in the graph below. While the MDA-MB-231 cells did migrate, there was no significant difference in the numbers of cells that migrated when comparing those which received T47D-NTC exosomes and those which received T47D-504 exosomes (Figure 6.9). The T47D cells did not migrate at all (results not shown).

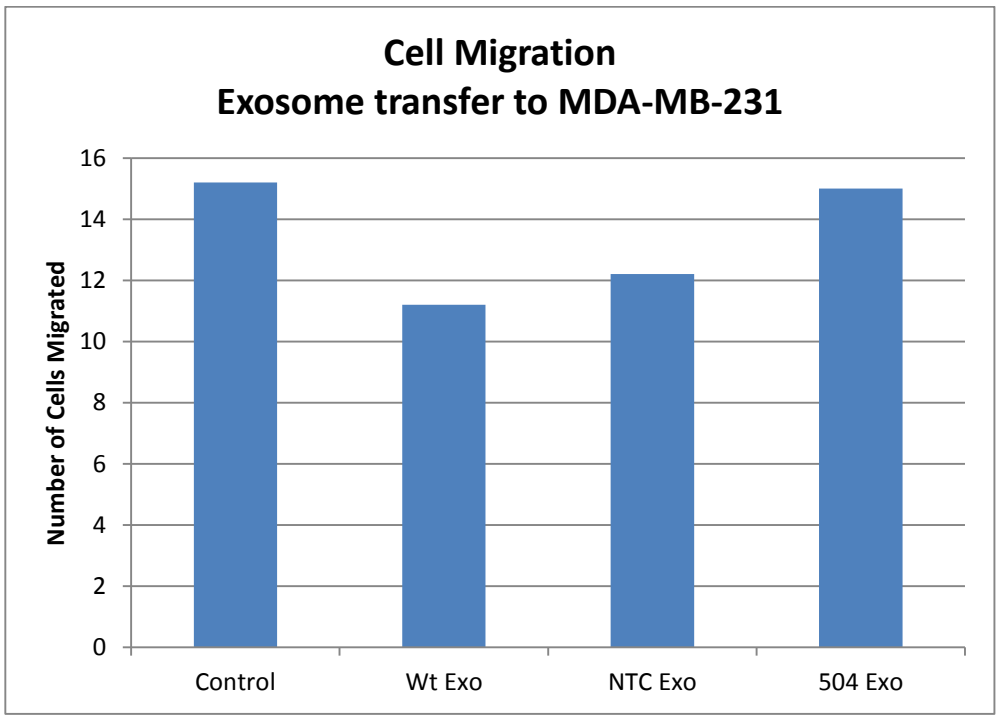


Figure 6.9 Number of cells which migrated in the control cells, and the cells which received T47D-Wildtype (WT) exosomes, T47D-NTC exosomes and T47D-504 exosomes.

6.4.4 Investigating impact of miR-504 overexpression *in vivo*

6.4.4.1 Impact of miR-504 on tumour formation

On the day of tumour induction into the animal model, an aliquot of cells (~ 2.5 x 10^5) from both T47D-NTC and T47D-504 were analysed for over-expression of miR-504. Subsequently, these cells were cultured, routinely harvested, and RQ-PCR was performed on ~ 2.5 x 10^5 cells to investigate stable and consistent over-expression of miR-504. Over-expression was observed at various time points e.g. prior to induction into animal model, on induction day, day 3, 7, 14 and 28. This confirmed the T47D-504 cells were stably over-expressing miR-504 (Figure 6.10).

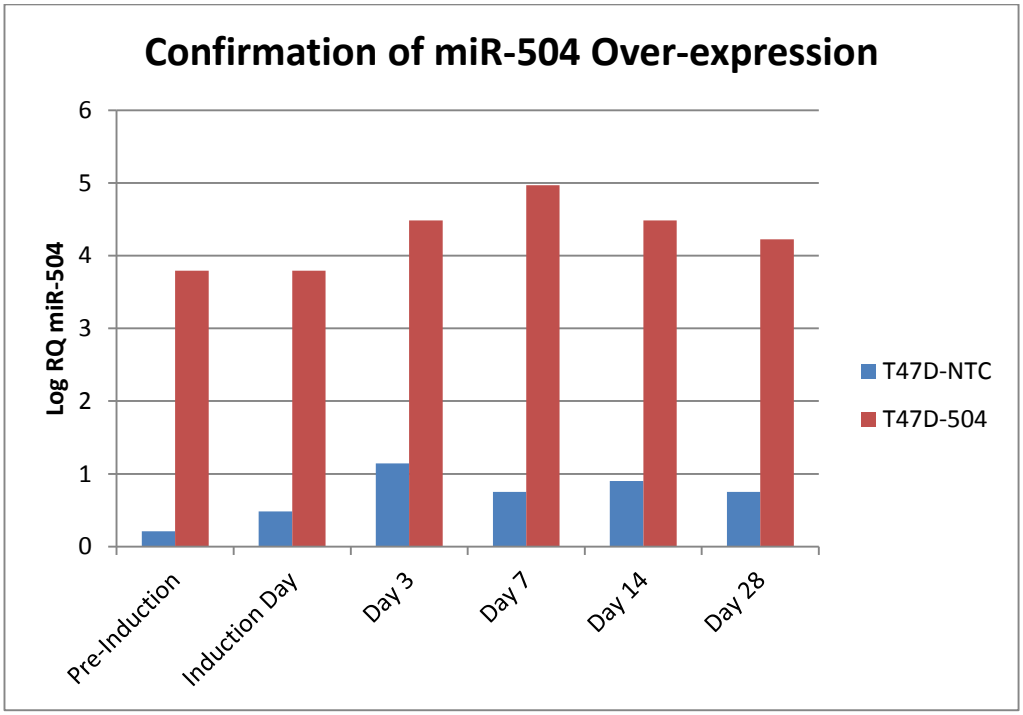


Figure 6.10 Stable and consistent over-expression of miR-504 in transduced cells prior to tumour induction, on induction day, day 3, 7, 14 and 28.

Athymic nude mice were divided into two groups. The control group received T47D-NTC cells, while the test group received T47D-504 cells.

Following 8 weeks, in the control group of animals (n = 5, T47D-NTC), tumours formed in all 5 animals. In the test group of animals (n = 5, T47D-504), tumours formed in 4 out of the 5 animals. Following tumour harvest, the tumours were weighed and photographed (Figure 6.11).

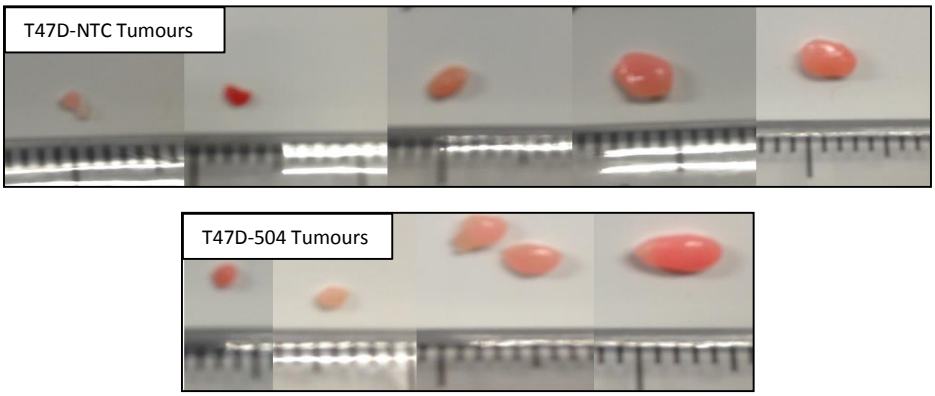


Figure 6.11 Tumours harvested from control T47D-NTC animals (n=5), and from T47D-504 animals (n = 4).

No significant difference was observed between the size of the tumours collected from the control group compared to the test group (Figure 6.11). However, there were variations in the size of tumours harvested within the groups, with some very small and some larger tumours. Interestingly, however, in one animal in the test group, two tumours were observed side by side. Also, one animal in the T47D-504 group failed to form a tumour which must also be noted.

6.4.4.2 miRNA and gene expression analysis in tumours

Following tumour harvest, RNA was extracted from the tissues. Only three tissues from each group yielded sufficient quality and quantity of RNA for further analysis. RQ-PCR was performed targeting miR-504, with miR-16 and Let-7a as endogenous controls. As expected significant upregulation of miR-504 was observed in the tumours harvested from the test group of animals (T47D-504), when compared to the control group of animals (T47D-NTC) ($p<0.05$, Figure 6.12).

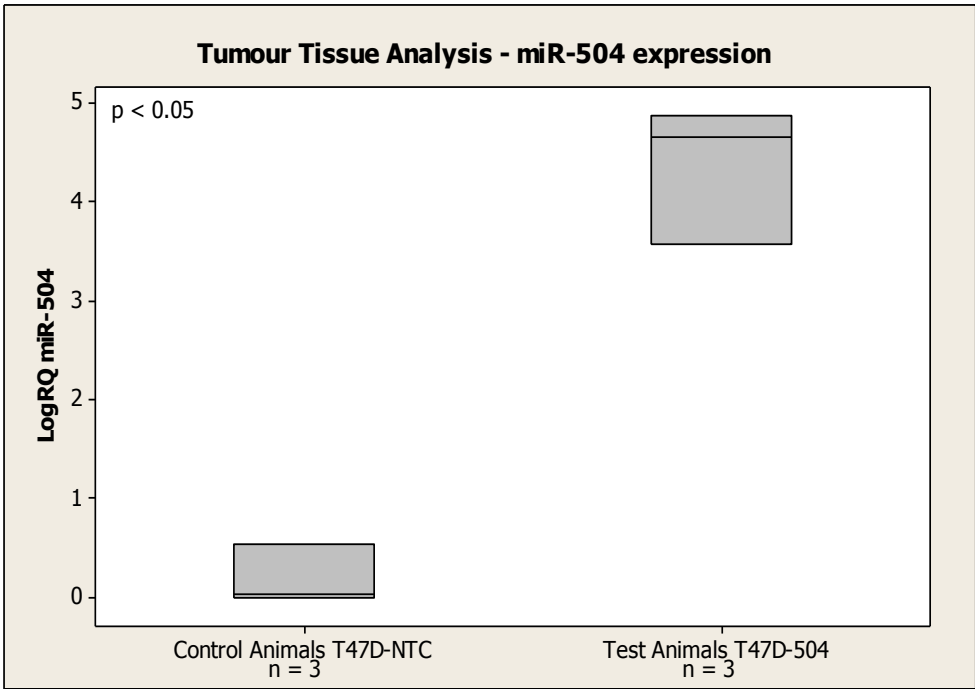


Figure 6.12 Analysis of miR-504 expression in the tumour tissues harvested from the control group (T47D-NTC) and the test group (T47D-504).
'*' respresents outliers within the group. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

This result confirmed that the T47D-504 cells that were inoculated into the test group of animals successfully initiated and developed tumours which significantly overexpressed miR-504.

Gene expression analysis was performed on the tumour tissues harvested from both the control and test groups of animals. Genes associated with metastasis and EMT were chosen for analysis, in addition to those reported in the literature. Genes analysed included: CDH6, P53, FOXP1, N-Cadherin and Twist. No significant dysregulation was observed with any of the genes targeted (Figure 6.13 (A-D)).

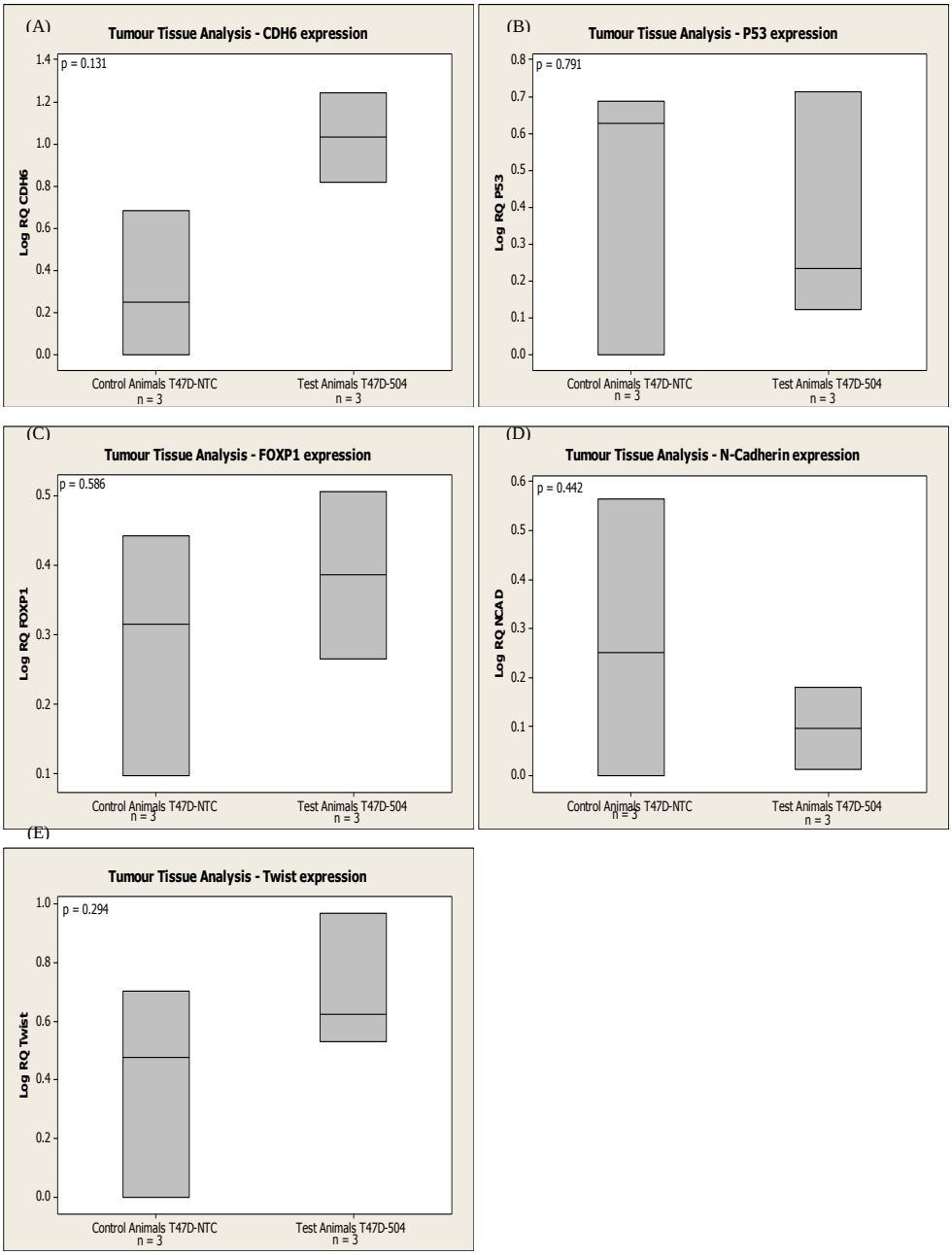


Figure 6.13 Gene expression analysis results from tumour tissues developed using either T47D-NTC or T47D-504. (A) CDH6, (B) P53, (C) FOXP1, (D) N-Cadherin and (E) Twist. '*' respresents outliers within the group. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

6.4.4.3 miRNA expression analysis in blood samples

Terminal cardiac bleeds were carried out on animals from both the control group and the test group. RNA was extracted from the whole blood samples taken from the control and test groups. Only 3 animals from the test group yielded sufficient blood volume and subsequent RNA for further analysis. RQ-PCR was performed targeting miR-504, with miR-16 as an endogenous control. Significant upregulation of miR-504 was observed in the bloods taken from the test group of animals ($p<0.05$, Figure 6.14). However, the lower sample number of bloods in the test group must be noted.

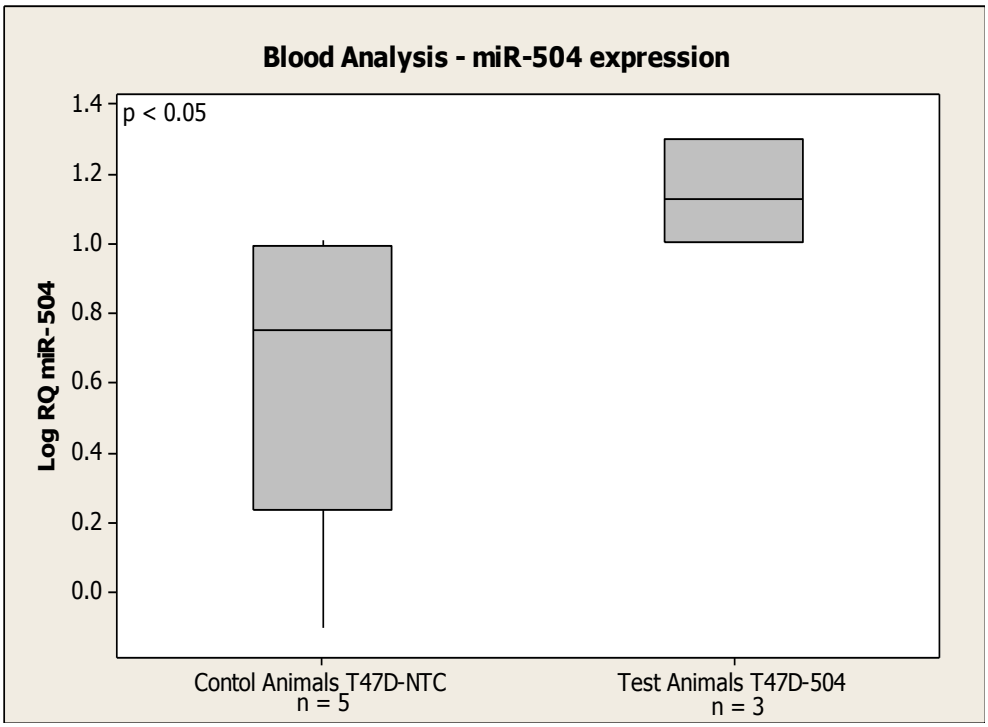


Figure 6.14 Analysis of miR-504 expression in the blood samples collected from the control group (T47D-NTC) and the test group (T47D-504). '*' respresents outliers within the group. The horizontal lines represent the median, while vertical lines represent the minimum and maximum of the data.

This result suggests that the tumours that formed following inoculation of T47D-504 cells into the test group of animals secreted miR-504 into the bloodstream of the animals, resulting in increased circulating levels.

6.5 Discussion

The potential tumour suppressor role of miR-504, highlighted in a pilot study within our group and also in a separate study by Kikkawa *et al.* (307), was further investigated here, in the breast cancer setting. The aim of this study was to investigate miR-504 expression in patient breast tissue samples, and to investigate the impact of the miRNA *in vitro* and *in vivo*.

To begin with, miR-504 expression was investigated in an individual and larger cohort of patient breast tissue samples than the cohort previously examined within our laboratory. The advantage of the cohort used in this study was that the tumour and TAN tissues were matched from the same patient. Therefore, miR-504 expression was examined in the tumour tissue and the associated 'normal' tissue located just a few centimetres away from the tumour. A potential tumour suppressor role of miR-504 was further suggested, as significant downregulation of miR-504 was observed in the tumour tissues, when compared to both their matched TAN tissues, and normal tissues harvested from healthy individuals. The tumour tissue data was further stratified based on clinicopathological details including epithelial subtype, tumour grade, tumour stage and menopausal status. No further dysregulation was observed across these characteristics. These results provide a further contribution to the potent tumour suppressor role of miR-504. Previous studies have reported mixed functions for this miRNA, with one study suggesting a tumour suppressor role for miR-504 in hypopharyngeal squamous cell carcinoma (307), with three studies suggesting oncogenic functions for miR-504 in other cancers (303, 310, 311). Therefore the data presented here, contributes to the investigations, as there are no published reports investigating miR-504 in breast cancer.

Based on these findings, a breast cancer cell line was successfully transduced to stably overexpress miR-504. This was confirmed by targeting miR-504 using RQ-PCR at various time points, in addition to fluorescent microscopy to view the red fluorescent protein included in the lentiviral construct.

The impact of miR-504 overexpression *in vitro* was then investigated. The effect of miR-504 overexpression on the proliferation of cells was examined using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay

(MTT, Promega). No significant difference was observed between the T47D-NTC cells and the T47D-504 cells. However, proliferation assays have generated much discussion in recent years regarding their use as valid assessors of cell proliferation. The cell proliferation assay used in this study, the MTT solution, has come in for criticism in certain settings as it is not believed to be an adequate measurement of the ability of a cell to proliferate, or indeed the rate at which a cell proliferates (312). However, it is believed that the MTT solution is actually a very useful tool in this study as it provides an indication of the impact miR-504 overexpression has on cell proliferation when compared to similar cells without the overexpressing miRNA. Simply, the assay quantifies changes in cell growth between two cell populations. If the aim was to investigate the rate of proliferation of the cells, rather than the difference between two populations, then a dye and flow cytometry based assay would have been employed.

Following this, the effect of miR-504 overexpression on the ability of cells to migrate was examined. T47D-NTC cells and T47D-504 cells were seeded into Corning transwell inserts with a 0.8 µm pore size and the rate of migration between the two cell populations was compared. In this assay, which was performed in duplicate, the results showed that no cells migrated. However, it is known that non-invasive cell lines, such as the T47D cell population, migrate at a very slow rate and often do not migrate at all. Therefore, the results of this migration assay show that miR-504 over expression does not stimulate T47D migration, supporting the idea that miR-504 is not pro-tumourigenic. As it is known that T47D cells do not migrate, by overexpressing miR-504 we could only expect to see an increase in migration, if any change at all.

Previous studies have reported associations of miR-504 with genes such as p53 (307) and FOXP1 (311) suggesting they have predicted binding sites for miR-504 in the 3'UTRs. Therefore, the expression of these genes, and several others associated with migration and adhesion, were investigated in the transduced cells and also the tumour tissues harvested from the animal model. FOXP1 and TWIST were found to be significantly upregulated in the T47D-504 cells when compared to the T47D-NTC cells and T47D-Wildtype cells *in vitro*, while they remained unchanged *in vivo* in the animal tumour tissues.

Neither suggest a role for miR-504 in the regulation of these genes as a decrease would be expected, however, change is not always observed at the RNA level, so the protein level could be informative. No significant alterations were observed with the other genes analysed: p53, N-Cadherin, Snail and Twist. The *in vitro* set-up infers a 'cleaner' effect as it is just the cell line, while *in vivo* other factors can influence the tumour, such as recruited mouse stromal cells etc.

As discussed in the previous chapter, exosomal transfer of miRNAs is a recently recognised form of intercellular communication. The selective packaging and subsequent uptake of exosomal miRNAs is an area of much interest. To determine whether it was possible to enrich exosome content for a particular miRNA, i.e. miR-504, exosomes secreted by T47D-Wildtype, T47D-NTC and T47D-504 cells were isolated, and their miRNA content was extracted and analysed for miR-504 expression. miR-504 was found to be packaged only in the exosomes isolated from the T47D-504 cells. This was an exciting finding, as it reveals the ability to engineer cells to secrete exosomes containing a particular miRNA. This has potentially important implications in terms of targeted therapy. Cells could be engineered to secrete exosomes containing an over-expressed tumour suppressing miRNA, or indeed a whole panel of these miRNAs, and be delivered directly to the tumour site via exosomes. Other studies have shown transfer of enriched exosomes to recipient cells where their effects are conferred. In a study by Kosaka *et al.* (313), it was shown both *in vivo* and *in vitro*, that introduction of miR-16 enriched exosomes into prostate cancer cells resulted in significantly suppressed expression levels of the target genes of miR-16, and consequently inhibited proliferation of the cancerous cells. This demonstrates the functional role of exosomal-miRNA transfer and also highlights the therapeutic potential (171). Based on data in the previous chapter, it has been shown that packaging of miRNAs into exosomes is selective, with some miRNAs not contained in exosomes, although they are at relatively high levels in the cells. It is therefore unlikely that the presence of a particular miRNA in exosomes is based solely on high levels in the cells. The ability to enrich for a particular miRNA needs to be proven individually and further

understanding of all the factors which govern miRNA sorting into exosomes is required.

Following this exciting discovery, the effect of transferring these exosomes to a recipient population was investigated. Again, exosomes secreted by T47D-NTC and T47D-504 cells were isolated, but were transferred to recipient cell populations, where they were subsequently taken up, as demonstrated by confocal microscopy in the previous chapter. miRNA was extracted from the recipient cells and the levels of miR-504 expression were investigated. There was no significant increase in miR-504 expression in the cells which received the T47D-504 exosomes, although a trend towards increased expression was observed. It must be considered, however, that a very large number of exosomes may be required in order to see an increase, the timing allowed for uptake may be important, and indeed it may be more useful to look at the effect on targets genes instead.

In addition to investigating the effect of T47D-504 exosome transfer on miRNA expression, any effect on cell proliferation and migration was also investigated. Exosomes secreted by T47D-NTC and T47D-504 cells were isolated and transferrerd to T47D-Wildtype cells growing in exosome-depleted media in a 96-well plate. Although there was a significant increase in proliferation of the cells which received exosomes when compared to cells which did not, there was no significant increase between those which received exosomes isolated from T47D-NTC cells and those which received exosomes isolated from T47D-504 cells. To investigate migration, exosomes secreted by T47D-NTC and T47D-504 cells were isolated and were transferred to two different cell populations, MDA-MB-231 and T47D. The exosomes were allowed to be taken up by the recipient cells, which were subsequently transferred to Corning Transwell Inserts to investigate their impact on cell migration. The MDA-MB-231 cells were observed to migrate, but no significant difference was observed between the cells which received the T47D-NTC exosomes, and those which received the T47D-504 exosomes.

Lastly, the impact of miR-504 was investigated *in vivo*. T47D-NTC cells were injected into the control group of mice, while T47D-504 cells were injected into the test group of mice. Nude athymic mice were chosen for use in this study for two reasons: first, they are immunocompromised, therefore

allowing efficient growth of human-derived tumours, and secondly, they are phenotypically hairless, therefore allowing tumour growth to be more easily monitored.

Following 8 weeks of tumour growth, the animals were sacrificed and the resulting tumours were harvested. Tumours of varying sizes formed in all 5 of the control group mice, while tumours, again of varying sizes, formed in 4 out of 5 of the test group mice. It must be considered that, perhaps with a larger group of animals, or overexpression of miR-504 in a more invasive cell line, investigations could potentially reveal more clearly any tumour suppressor role for miR-504.

In this study, RNA was extracted from the tumours and blood samples collected from the animals in each group. miR-504 expression was found to be significantly upregulated in the tumours harvested from the test group of animals when compared to those from the control group. This confirms that the T47D-504 cells, which were induced into the test group, developed tumours which maintained over-expression of miR-504 throughout the study. Interestingly, miR-504 expression was also found to be significantly upregulated in the blood samples collected from the test group of animals. This result suggests that the tumours that formed following inoculation of T47D-504 cells into the test group of animals secreted miR-504 into the circulation of the animals, resulting in increased expression.

The *in vivo* model represents a 'cleaner' model when compared to patient tissues samples, as these animals are immunocompromised in a sterile environment, therefore would not infer the same background effects observed with patient samples. The expression of miR-504 in the circulation highlights a potential biomarker role, as the aim would be to look for decreased levels in the presence of a tumour for cancer diagnosis, leading to increased levels during treatment success. Exosome isolation from blood is also an area which requires further investigation, as this could potentially provide a more representative signature of the tumour.

This study found miR-504 to be lost in patient breast tumour tissues, suggesting a potential tumour suppressor role for this miRNA. Although overexpression of miR-504 *in vivo* did not reveal suppressed tumour

formation, it must be considered, that miR-504 is not working on its own when it is lost in cancer. As a result, research is required to investigate other factors which are dysregulated in concert with miR-504. The loss of miR-504 in patient breast cancer tissues raises the question: is miR-504 lost during carcinogenesis, or is it the loss of miR-504 that plays a role in carcinogenesis?

An exciting finding of this study is the proof of principle concept, that it is possible to engineer cells to secrete exosomes containing overexpressed miR-504, which can then be delivered to, and taken up by, recipient cell populations. In the cancer therapy setting, the answer does not lie in interventions to modulate a single miRNA, rather the aim is to modulate a large panel of tumour suppressing miRNAs which can be engineered and delivered to the tumour site. In addition, this new form of therapy would be actively considered in combination with other therapeutic regimens. In this evolving field, recent developments include the manufacturing of miRNA antagonists to target oncomiRs. In parallel however, miRNA mimics of tumour suppressing miRNAs are now being developed. This is where the future of cancer therapy is moving, with interventions to restore lost tumour suppressing activity, rather than applications to antagonise miRNAs. The major advantage to the introduction of miRNA mimics is that the mimics resemble their native molecules, and therefore are not introducing any unwanted or 'foreign' traits. A potential drawback however, may be an impact on safety, as there are potentially lots of target genes associated with the miRNA. Some miRNAs have entered preclinical and clinical studies. However, prior to the development of therapeutic treatments using tumour suppressing miRNAs, more investigative studies are required both *in vitro* and *in vivo*, in order to determine their safety and efficacy. As various methods, such as high-throughput RNA sequencing, reveal new targets at an astonishing rate, the miRNAs studied thus far merely represent a small fraction of those worthy of further investigation. Indeed miR-504 is just one miRNA, and in-depth functional analysis of individual miRNAs is required in order to determine their true relevance in the cancer setting.

6.6 Conclusion

This study found miR-504 to be lost in breast cancer patients, suggesting a potential tumour suppressing role for this miRNA. Overexpression of miR-504 was not found to suppress tumour formation *in vivo*, suggesting other factors may jointly act with miR-504 to suppress tumour formation. This study did, however, reveal the ability to engineer cells to secrete exosomes containing overexpressed miRNAs which can then travel to, and be taken up by, a target cell population. This has potentially important implications in the gene therapy setting.

Chapter 7

Final Discussion and Conclusion

7 Chapter 7: Final Discussion and Conclusion

7.1 Core Findings

The cornerstone of miRNA expression profiling involves identifying patterns of dysregulation in patient samples and to reveal tumour suppressors and tumour promotors/oncomirs. The first results chapter presented here, highlighted two potential oncomirs.

- miR-106a and miR-191 were found to be significantly upregulated in patient breast tumours, suggesting a potential oncogenic role for these miRNAs.
- miR-138 and miR-191 exhibited dual roles, with evidence suggesting potential epithelial subtype specificity also.

As breast cancer is a hormone responsive cancer, it was essential to examine the relationship between circulating hormones associated with the menstrual cycle and circulating miRNA expression levels. In this chapter, a small panel of miRNAs known to be associated with carcinogenesis were investigated.

- With the exception of one miRNA, miR-202, the expression levels were shown to remain stable across the phases of the menstrual cycle.
- Previous studies have shown miR-202 to be significantly upregulated in the circulation of breast cancer patients, therefore the data presented here must be considered when evaluating any dysregulation in the circulation with this miRNA.

Although it is well established that miRNAs are present in the circulation, their specific source and role is unknown and requires elucidation. In addition, there have been relatively few published reports on the isolation of miRNAs from cell-conditioned media to examine their secretion by cells (271, 272). The second results chapter presented here investigated this area.

- In published data, the optimal method of miRNA isolation from conditioned media was identified to be an adaption of the miRVana miRNA isolation kit.
- Furthermore, signals of a range of miRNAs known to be associated with cancer were detected.

- This secretion in the conditioned media was further identified to be selective, as some miRNAs were detectable and some were not.

Following this, exosomes were highlighted as potential delivery vehicles of miRNAs into the circulation. Consequently, the exosome fraction of the conditioned media became the focus in this study, as it was hypothesised that the exosomes would provide a better representation of the parent cell.

- Once the exosomes were isolated, it was crucial to characterise the purified exosome fractions to ensure we had indeed isolated exosomes. This was confirmed by morphological analysis using TEM and further confirmation by Western Blot targeting the exosome-associated protein CD63.
- miRNAs were extracted from the exosomes and miRNA signals were detected in a range of miRNAs, with selective packaging highlighted, as some were present and some were not.

As a result of these finidings, it was essential to identify the full miRNA secretion profile contained within these exosomes and reveal their transfer to, and uptake by target cell populations. In the third results chapter, exosomes were isolated from the conditioned media of a range of breast cancer cell lines and their miRNA content was extracted.

- A microRNA array was performed on the miRNA content of exosomes isolated from the conditioned media of a range of breast cancer cell lines.
- This array targeted all registered miRNAs on the miRBase registry (~2000). Approximately 390 miRNAs were detected in each of the exosome samples secreted from the breast cancer cell lines.
- The miRNAs detected were ranked, based on their expression, and four miRNAs were further validated by RQ-PCR. Each of the miRNAs of interest have previously established associations with a range of cancers, including breast cancer.

Further to this, recent exciting evidence identified exosomes as mediators of intercellular communication through their release from donor cells and subsequent uptake in recipient cells (126).

- This exchange was visualised by transferring fluorescently labelled exosomes to a recipient cell population and viewed using a confocal microscope, further confirming their role as delivery vehicles.
- Following transfer, the impact of exosomes in recipient cell populations was investigated. The impact of exosome transfer on the proliferation of the recipient cells was examined where a significant increase was observed in cells which received exosomes, when compared to cells which did not. This data highlights the impact that the content, and indeed the surface proteins on the exosomes, may potentially have on the proliferation of recipient cells.

In the fourth and final chapter, miR-504, which had previously been identified as a potential tumour suppressor in a pilot study within this laboratory, was further investigated.

- In a larger independent cohort of breast cancer patients, miR-504 was found to be significantly downregulated in tumour tissues when compared to matched tumour-associated normal (TAN) tissues.
- Breast cancer cells were then transduced to overexpress miR-504. The impact of miR-504 overexpression was investigated *in vitro*, where no significant impact on cell proliferation or migration was observed.
- With the exchange of miRNAs via exosome-mediated delivery highlighted in this research, the next avenue of investigation was to determine if the overexpressing miR-504 cells would then engineer exosomes, which contained enriched levels of miR-504.
- This hypothesis was confirmed where miR-504 was found to be detectable in exosomes isolated from the overexpressing miR-504 cells, while it was not detectable in exosomes secreted from control cells.
- These miR-504 enriched exosomes were then transferred to recipient cell populations where their impact was investigated. The levels of

miR-504 expression in recipient cells were not observed to significantly increase, but an interesting trend towards upregulation was observed.

- The transfer of miR-504 enriched exosomes also did not appear to have any significant impact on the cell proliferation or on the cell migration of the recipient cell populations.
- miR-504 was further investigated in an *in vivo* model, where overexpression of miR-504 was not found to suppress tumour formation.
- The tumours harvested revealed significant upregulation of miR-504 in the tumour tissue and in the circulation of the animals which received the miR-504 cells.

7.2 Clinical Relevance

miRNAs have gained much attention in the last decade due to their progressively important implications as master regulators of a wide range of cellular processes in disease, not least in cancer. Critical research has revealed the overwhelming potential of miRNAs not only as novel biomarkers of disease, but also as key players in intercellular communication and indeed as pioneering targets for gene therapy. The data presented here is a contribution to the potential application of miRNAs as biomarkers of disease and therapeutic targets for breast cancer.

This research highlights two potential oncomiRs and one potential tumour suppressor miRNA. This re-enforces the idea that the future of miRNAs as novel diagnostic and prognostic biomarkers of disease will rely not on a single miRNA, but rather on a panel of informative miRNAs. Further to this, some miRNA dysregulation observed in patient tissues in this study was not reflected in the circulation of breast cancer patients in separate studies, highlighting the variability of miRNA expression between the tumour microenvironment and the circulation.

Moreover, with the realisation that miRNAs represent ideal circulating biomarkers of disease, it is crucial that their expression levels are not impacted by normal physiological processes. The research presented here

revealed one miRNA from a panel of six to be significantly dysregulated across the phases of the menstrual cycle, therefore highlighting the need to consider this finding when interpreting any dysregulation of this miRNA in cancer research studies.

Although it is well established that miRNAs are present in the circulation, little is known about their true source, molecular mechanism of release and indeed role in the circulation. Conditioned media, containing all factors secreted by breast cancer cells grown in an *in vitro* setting, provides a valuable representation of the circulation, albeit in a cleaner more controlled setup. With few publications reporting successful isolation of miRNAs from conditioned media at the time, it was clear an appropriate method of isolation must be identified.

Subsequently, exosomes were propelled into the miRNA research field revealing exciting potential in terms of both biomarker discovery and intercellular communication. Exosomes have been shown here to contain selective miRNAs, some of which potentially represent a molecular fingerprint of the parent cell, leading to novel diagnostic biomarkers of disease. Furthermore, this finding confirmed that miRNAs are selectively packaged into exosomes for release into the extracellular environment, revealing huge potential in terms of intercellular communication and indeed exchange of genetic material. This delivery, and indeed transfer of molecular material, creates a new avenue of investigation for therapeutic applications.

To the best of my knowledge, this research is the first to investigate the full miRNA profile contained within exosomes secreted from breast cancer cells and provides a foundation from which other studies can expand. This data highlights the selective nature of the packaging of miRNAs into exosomes for secretion and stimulates further interest into the biological reason behind this selectivity in terms of functional effects downstream.

Tumour suppressing miRNAs are a keen interest of miRNA researchers worldwide and exploitation of their tumour suppressing characteristics is a common goal. As discussed, this research highlights a potential tumour suppressor role for miR-504 as it was found to be lost in the tumour tissues of breast cancer patients. Further to this, we were able to force overexpression

of this miRNA into exosomes for secretion.The proof of principle, whereby cells can be engineered to secrete exosomes enriched with a particular miRNA, raised exciting potential for the delivery of tumour suppressing miRNAs as a therapeutic strategy.

In conclusion, the research presented here provides a valuable contribution to both the miRNA and exosome research fields, highlighting exciting potential in biomarker discovery, intercellular communication, and cancer therapy.

7.3 Future work

Further identification and functional characterisation of miRNAs in cancer is central to the generation of novel therapeutic strategies for this disease. Future work will investigate the release of miRNAs secreted in exosomes from donor cells and their uptake by recipient cells, where an array of biological processes can be impacted, including cell proliferation and differentiation, apoptosis, and the immune response.

Exosomes have been suggested to have many distinct and far-reaching physiological functions, depending on their cellular origin, from immune regulation, to cell migration and differentiation, and other aspects of cell-to-cell communication. Exosomes have also been implicated in the pathogenesis of disease such as tumor development, cardiovascular and neurodegenerative disease. The realization that exosomes may bring about epigenetic alterations by transferring selected RNA molecules between cells has revolutionized our thinking with regards to exosomal signaling. Now that exosomal transfer of miRNAs has been elucidated, a major future challenge will be to reveal the functional relevance of this process and its impact in the clinical setting.

The impact of normal physiological processes on miRNA expression is an area of research which must not be forgotten. This research highlighted one miRNA to be dysregulated across the menstrual cycle phases. Indeed, a much larger panel of miRNAs, and across a variety of normal physiological processes must be investigated in order to reveal any further dysregulation,

as this is a critical point of consideration when evaluating miRNAs in the circulation of breast cancer patients.

This research investigated exosomal miRNAs in an *in vitro* setting using a range of breast cancer cell lines. To further add to this research, the primary breast tumour microenvironment where the predominant cell types are the stromal cells, should be investigated. Stromal cells are known to have an active participation in tumour progression. It is known that in the tumour microenvironment, stromal-epithelial cross-talk is very important. However, in order for stromal-epithelial interactions to emerge as appropriate targets for novel breast cancer therapies, further characterisation of the molecular crosstalk between these two populations is required.

Chapter 8

References

8 Chapter 8 References

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Chapter 9

Appendices

9 Chapter 9 Appendices

9.1 Biobank Ethical Approval and Patient Consent

Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

Merlin Park University Hospital
Ospidéal na h-Oiliscoile, 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,

p.p. Roberte Collins

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

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

Merlin Park University Hospital, Ospidéal na h-Oiliscoile, Páirc Mheirlinne, Galway, Ireland. Tel: 00 353 (0)91 757631

Galway University Hospitals

NUI Galway
OÉ Gaillimh

Health Service Executive

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

9.2 Environmental Protection Agency GMO Licence

Environmental Protection Agency

Register of Genetically Modified Organisms (GMOs)

Users in Ireland

Name and address of user:	Prof Michael Kerin Discipline of Surgery School of Medicine Clinical Sciences Institute National University of Ireland Galway Galway
Location or postal address of the premises to which a record, notification or amended notification relates:	Discipline of Surgery School of Medicine Clinical Sciences Institute National University of Ireland Galway Galway
GMO register No.	G0516-01
Description of each genetically modified organism involved:	Two breast cancer cell lines (T47D, MDA-MB-231) and two colorectal cancer cell lines (HCT116, HT29) stably infected with HIV-1 based Lentivirus vectors containing either microRNA-504 or microRNA-379 will give rise to 8 potential GMMs as follow: • T47D-504; • T47D-379; • MDA-MB-231 504; • MDA-MB-231 379; • HT29-504; • HT29-379; • HCT-116 504; • HCT-116 379.
Purpose of the contained use:	The purpose of the contained use activity is to determine whether the microRNAs have an effect on cancer cell growth, migration/invasion and tumour formation in vitro and in vivo by their re-introduction into cancer cell lines. The GMM cells (not the lentiviral vector) will be injected into nude mice to determine the effect of the modification on tumour establishment, growth and metastasis. These mice are not genetically modified.

Date of receipt of a record, notification or amended notification:	30/Aug/2013 Valid notification received 24/Mar/2014 2013 Annual Report received. No changes to GMO Register

	entry, RA or containment measures applied.
Date and nature of any information provided under article 30(3):	
Date of any request by the Agency under article 10 or 25:	03/Sep/2013 Additional information requested
Date of any response from the user to any request by the Agency under article 10 or 25:	17/Sep/2013 Requested information received
Date of publication of a notice pursuant to article 20 or 21:	
Date of withdrawal of a notification or an amended notification:	
Date and nature of the decision by the Agency on a notification or an amended notification:	17/Sep/2013 Approved. Consent Conditions issued 19 September 2013.
Date and outcome of any review referred to in article 10 or carried out under article 10 or 29	

9.3 Boxplots representing miRNA expression across clinicopathological characteristics in Chapter 3

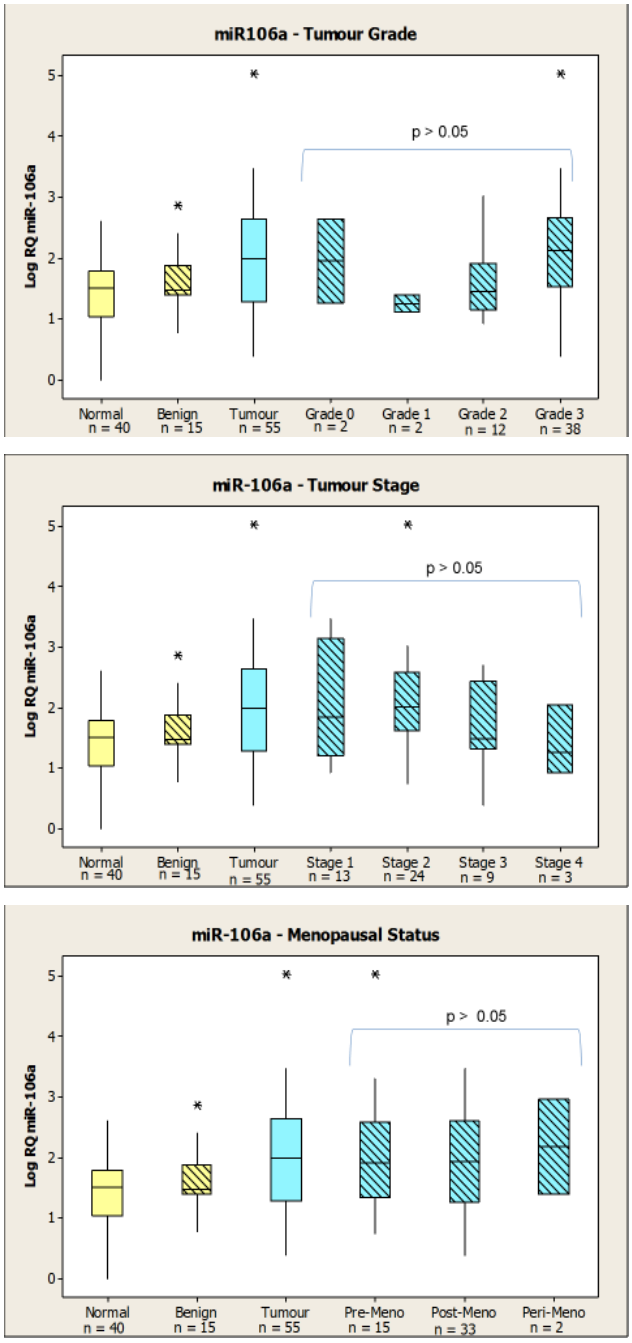

Figure 9.3.1 miR-106a levels in tissue of breast cancer patients further stratified based on (A) tumour grade, (B) tumour stage and (C) menopausal status.

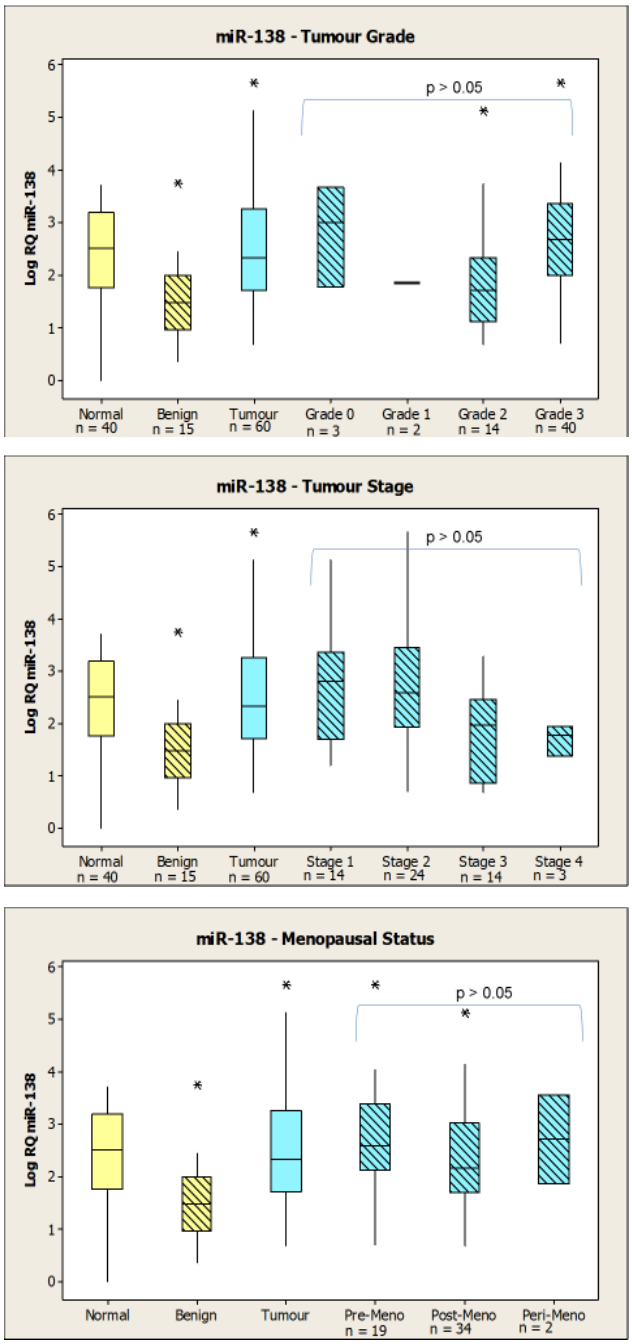

Figure 9.3.2 miR-138 levels in tissue of breast cancer patients further stratified based on (A) tumour grade, (B) tumour stage and (C) menopausal status.

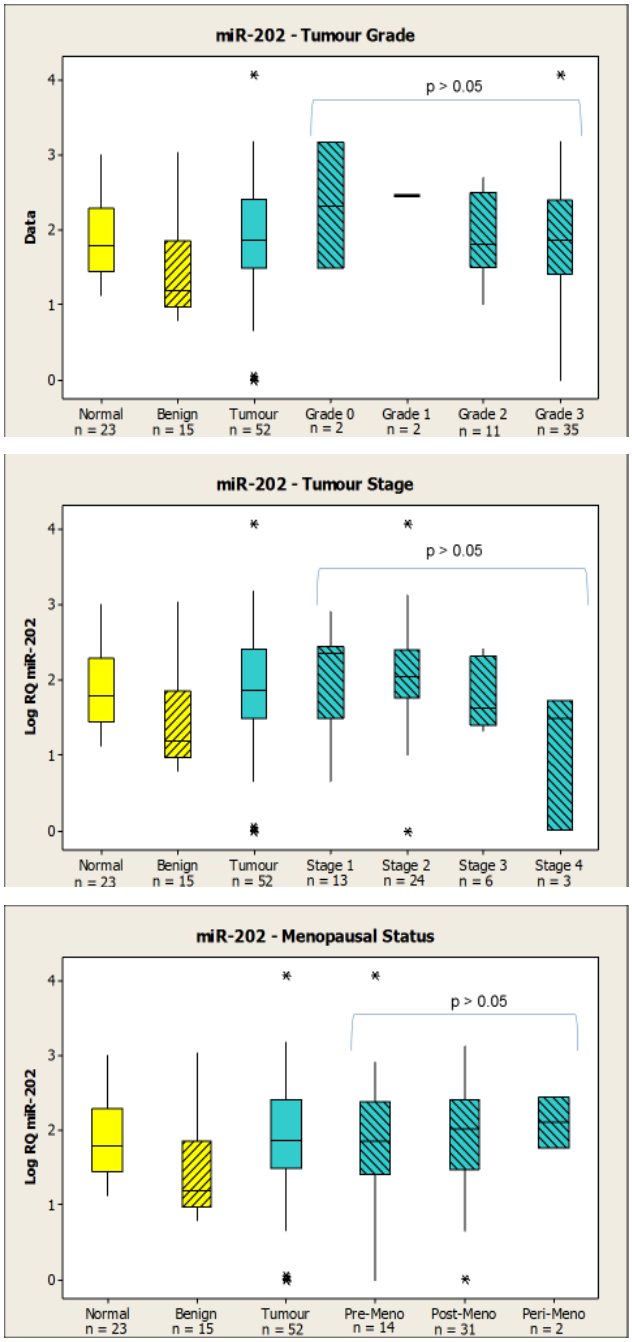

Figure 9.3.3 miR-202 levels in tissue of breast cancer patients further stratified based on (A) tumour grade, (B) tumour stage and (C) menopausal status.

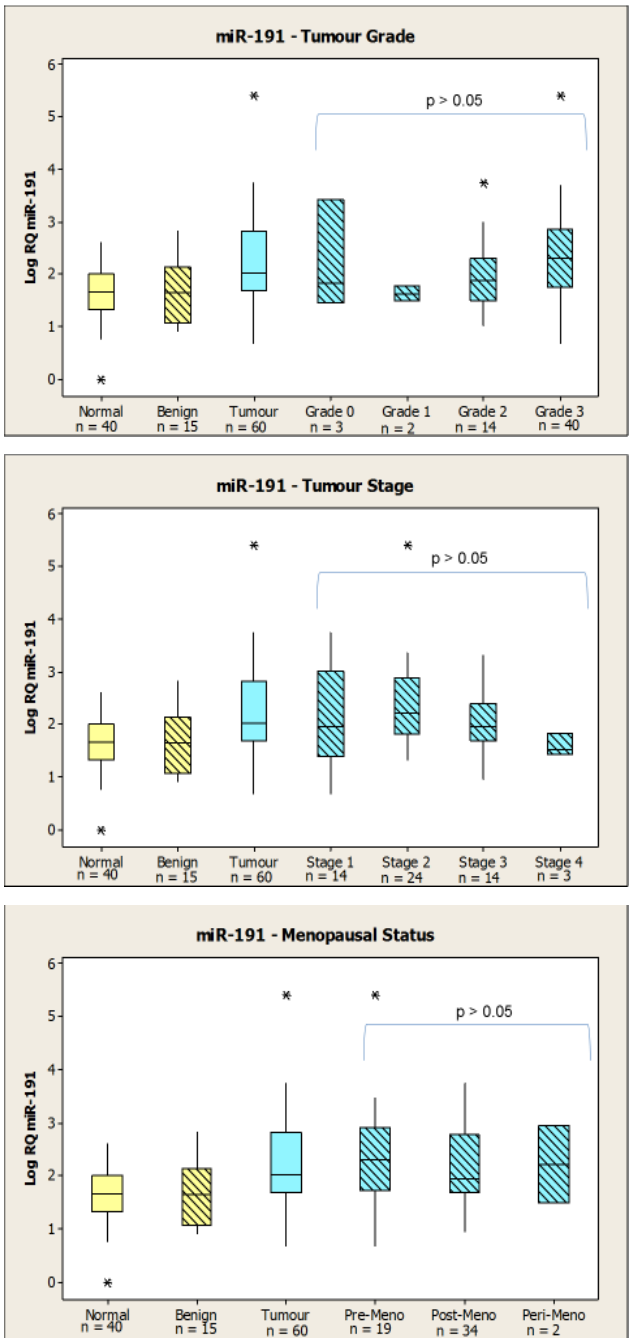

Figure 9.3.4 miR-191 levels in tissue of breast cancer patients further stratified based on (A) tumour grade, (B) tumour stage and (C) menopausal status.

9.4 Array data

ProbeID	Annotation	BT-20	MDA-MB-231	T47D	Sk-Br-3
145633	hsa-let-7d-3p	6.044	6.239	Undetected	6.138
145826	hsa-miR-18b-3p	6.045	6.125	6.081	6.164
148227	hsa-miR-1251-3p	6.047	6.124	6.143	Undetected
148627	hsa-miR-615-5p	6.048	6.332	6.520	6.260
147881	hsa-miR-3115	6.050	6.574	6.489	Undetected
42509	hsa-miR-219a-5p	6.051	6.284	6.046	6.426
147930	hsa-miR-3144-3p	6.052	6.820	6.499	6.386
46450	hsa-miR-548o-3p	6.053	6.311	6.336	6.352
46404	hsa-miR-1244	6.059	6.233	6.332	Undetected
169223	hsa-miR-4680-3p	6.068	6.198	6.164	6.182
42769	hsa-let-7b-3p	6.070	6.188	Undetected	Undetected
46584	hsa-miR-1468-5p	6.071	6.594	6.057	6.285
13132	hsa-miR-519e-5p	6.075	Undetected	Undetected	Undetected
145845	hsa-miR-20a-5p	6.076	Undetected	Undetected	Undetected
11165	hsa-miR-520a-3p	6.080	6.588	6.142	7.068
147694	hsa-miR-4281	6.089	6.515	6.103	6.678
147589	hsa-miR-1193	6.089	Undetected	6.666	6.405
148620	hsa-miR-454-3p	6.092	6.273	Undetected	Undetected
46258	hsa-miR-1184	6.102	Undetected	7.082	Undetected
145715	hsa-miR-648	6.103	Undetected	Undetected	Undetected
46918	hsa-miR-375	6.104	6.378	Undetected	6.202
145980	hsa-miR-939-5p	6.110	6.333	6.543	6.411
14300	hsa-miR-29c-5p	6.111	6.394	6.179	Undetected
17563	hsa-miR-644a	6.118	6.796	6.455	6.341
33596	hsa-miR-126-5p	6.118	6.448	Undetected	Undetected
46929	hsa-miR-548n	6.118	6.505	6.418	Undetected
46381	hsa-miR-1298-5p	6.119	Undetected	Undetected	Undetected
46747	hsa-miR-1263	6.121	Undetected	Undetected	Undetected
169047	hsa-miR-4665-3p	6.121	6.161	6.553	Undetected
148628	hsa-miR-3199	6.126	6.666	6.322	6.103
46633	hsa-miR-1267	6.129	Undetected	Undetected	Undetected
146066	hsa-miR-3116	6.130	6.437	6.315	6.506
31349	hsa-miR-524-3p	6.144	6.394	6.073	6.108
17851	hsa-miR-200c-5p	6.148	6.506	6.405	6.551
28966	hsa-miR-574-3p	6.148	6.193	6.091	6.298
28759	hsa-miR-758-3p	6.149	6.428	6.535	Undetected
147906	hsa-miR-4322	6.152	6.454	6.748	Undetected
169244	hsa-miR-5572	6.153	Undetected	Undetected	6.486
17312	hsa-miR-592	6.154	6.195	6.192	6.992
27551	hsa-miR-612	6.154	6.612	6.475	6.281
148474	hsa-miR-3622a-5p	6.158	6.713	6.726	Undetected
145634	hsa-miR-132-5p	6.162	6.572	6.210	Undetected

10306	hsa-miR-146b-5p	6.162	6.581	6.788	6.337
147731	hsa-miR-3189-3p	6.163	6.556	6.226	6.148
42635	hsa-miR-541-3p	6.164	6.616	6.788	6.279
13177	hsa-miR-143-3p	6.165	Undetected	Undetected	Undetected
42451	hsa-miR-139-3p	6.176	6.744	7.358	6.146
168893	hsa-miR-4505	6.176	Undetected	Undetected	Undetected
42681	hsa-miR-1307-3p	6.176	6.043	6.314	6.325
17882	hsa-miR-20b-3p	6.177	6.056	6.399	6.300
4610	hsa-miR-126-3p	6.177	6.748	6.364	6.217
46406	hsa-miR-500a-3p	6.180	6.428	6.273	6.169
148363	hsa-miR-3652	6.184	6.182	6.734	6.217
169305	hsa-miR-4455	6.186	6.487	6.112	6.216
46419	hsa-miR-1185-5p	6.188	6.554	6.134	Undetected
148379	hsa-miR-3654	6.188	Undetected	8.474	Undetected
145640	hsa-miR-328-3p	6.212	6.219	Undetected	Undetected
17444	hsa-miR-632	6.217	6.527	6.643	6.407
147796	hsa-miR-4327	6.220	Undetected	6.343	6.503
146168	hsa-miR-1912	6.228	6.625	6.665	6.297
148063	hsa-miR-3713	6.232	6.389	6.222	Undetected
42682	hsa-miR-25-3p	6.233	Undetected	6.214	Undetected
145789	hsa-miR-550a-3-5p/hsa-miR-550a-5p	6.238	6.545	6.868	6.280
14328	hsa-miR-124-3p	6.243	6.639	6.890	6.688
169170	hsa-miR-4472	6.246	Undetected	Undetected	6.189
42811	hsa-miR-542-5p	6.248	Undetected	Undetected	6.318
46875	hsa-miR-2276-3p	6.248	7.122	6.764	6.470
46467	hsa-miR-143-5p	6.251	6.601	6.269	6.247
18739	hsa-miR-186-5p	6.253	6.589	6.451	6.488
10916	hsa-miR-1	6.254	6.579	6.427	6.354
147276	hsa-miR-3616-3p	6.257	6.597	6.420	6.204
145999	hsa-miR-517a-3p/hsa-miR-517b-3p	6.260	6.741	6.259	6.580
46944	hsa-miR-1297	6.260	6.842	6.178	6.657
147818	hsa-miR-4270	6.262	6.164	6.379	Undetected
42460	hsa-miR-223-5p	6.264	6.277	Undetected	Undetected
17630	hsa-miR-588	6.271	6.289	6.453	6.375
169239	hsa-miR-4732-5p	6.274	Undetected	Undetected	Undetected
147632	hsa-miR-4297	6.286	6.202	6.147	6.078
46414	hsa-miR-548h-5p	6.287	Undetected	6.196	Undetected
13485	hsa-miR-10a-5p	6.289	6.361	6.387	7.024
168868	hsa-miR-5681b	6.292	Undetected	6.266	Undetected
148504	hsa-miR-874-5p	6.294	6.248	Undetected	Undetected
29379	hsa-miR-452-5p	6.299	6.417	Undetected	6.276
10919	hsa-miR-103a-3p	6.301	6.485	6.105	6.434
42674	hsa-miR-431-3p	6.304	6.678	6.070	6.965
169393	hsa-miR-4747-5p	6.311	6.942	Undetected	Undetected

146005	hsa-miR-3129-5p	6.318	6.921	6.867	6.586
10943	hsa-miR-136-5p	6.319	6.127	6.061	6.167
148624	hsa-miR-942-5p	6.323	6.376	6.483	6.585
46829	hsa-miR-664a-5p	6.329	6.212	6.358	6.137
11038	hsa-miR-299-5p	6.336	6.240	Undetected	6.062
11077	hsa-miR-363-3p	6.336	6.606	6.326	6.819
148466	hsa-miR-3937	6.347	6.449	6.979	6.539
42453	hsa-miR-376b-5p/hsa-miR-376c-5p	6.357	6.160	6.150	Undetected
46803	hsa-miR-503-5p	6.357	6.635	6.401	6.458
42615	hsa-miR-135b-3p	6.359	6.360	Undetected	Undetected
148393	hsa-miR-676-3p	6.363	6.820	6.226	Undetected
17289	hsa-miR-616-5p	6.369	6.694	6.366	6.339
11138	hsa-miR-506-3p	6.373	6.646	6.404	Undetected
146180	hsa-miR-1909-3p	6.375	6.656	6.818	6.971
145843	hsa-miR-330-5p	6.375	6.529	6.935	6.859
147846	hsa-miR-4313	6.379	6.066	6.508	Undetected
46800	hsa-miR-1224-3p	6.395	6.614	6.311	Undetected
46223	hsa-miR-1306-3p	6.397	6.204	6.587	6.886
168698	hsa-miR-3127-3p	6.404	6.320	6.655	Undetected
11085	hsa-miR-373-3p	6.414	6.596	6.580	6.350
17546	hsa-miR-585-3p	6.415	Undetected	Undetected	Undetected
5250	hsa-miR-105-5p	6.418	6.612	6.070	Undetected
147887	hsa-miR-3147	6.423	6.769	7.086	Undetected
46690	hsa-miR-1238-3p	6.428	6.795	6.186	6.359
46479	hsa-miR-1304-5p	6.438	7.149	6.614	6.296
46408	hsa-miR-1322	6.438	6.428	6.414	6.365
168648	hsa-miR-4687-5p	6.443	6.396	Undetected	Undetected
6880	hsa-miR-297	6.443	6.767	7.129	7.203
46860	hsa-miR-1205	6.448	6.832	6.570	6.983
10946	hsa-miR-141-3p	6.451	6.564	6.135	6.617
169120	hsa-miR-4787-3p	6.457	6.361	6.382	Undetected
14313	hsa-miR-499a-5p	6.457	6.829	6.354	7.268
148247	hsa-miR-2355-3p	6.460	6.683	6.451	6.104
169325	hsa-miR-4446-5p	6.462	6.908	6.398	6.350
42899	hsa-miR-377-5p	6.462	6.933	7.039	6.764
147562	hsa-miR-4253	6.470	6.827	7.119	6.712
148663	hsa-miR-557	6.475	6.907	7.190	6.456
148645	hsa-miR-129-5p	6.483	6.770	6.542	6.742
147683	hsa-miR-3188	6.494	6.731	6.662	6.676
17854	hsa-miR-106b-3p	6.499	6.973	Undetected	6.559
148052	hsa-miR-374c-3p	6.500	6.708	6.376	6.633
147891	hsa-miR-3175	6.508	6.402	6.322	7.299
145701	hsa-miR-668-3p	6.518	6.576	6.790	6.613
147742	hsa-miR-4265	6.520	6.951	6.652	6.590

11168	hsa-miR-520d-3p	6.520	6.813	6.485	6.489
42654	hsa-miR-483-5p	6.524	6.439	6.304	6.247
29575	hsa-miR-32-3p	6.538	6.931	6.672	6.344
30687	hsa-miR-93-5p	6.540	7.014	6.413	6.350
17932	hsa-miR-381-5p	6.540	6.936	6.870	6.832
42493	hsa-miR-892b	6.543	6.942	6.444	6.984
46661	hsa-miR-1294	6.545	6.763	6.622	6.881
28884	hsa-miR-876-3p	6.548	6.880	6.179	6.392
168568	hsa-miR-1290	6.581	Undetected	Undetected	6.326
148358	hsa-miR-3657	6.583	6.692	6.497	6.436
169295	hsa-miR-4725-3p	6.586	Undetected	Undetected	Undetected
42718	hsa-miR-130a-5p	6.591	6.923	6.316	6.268
10936	hsa-miR-130b-3p	6.592	6.806	6.693	6.351
148678	hsa-miR-301a-5p	6.622	7.034	6.162	6.059
46791	hsa-miR-1204	6.623	6.459	6.932	6.583
145675	hsa-miR-501-5p	6.623	6.755	6.528	6.804
42965	hsa-miR-424-5p	6.634	6.778	6.613	7.048
42507	hsa-miR-202-5p	6.635	6.791	6.488	7.268
145977	hsa-miR-1247-5p	6.638	6.620	6.451	6.450
148032	hsa-miR-3685	6.644	Undetected	Undetected	Undetected
148187	hsa-miR-410-5p	6.649	7.273	7.239	7.302
21498	hsa-miR-654-3p	6.651	6.932	6.413	6.364
11139	hsa-miR-507	6.658	6.596	6.484	6.624
46326	hsa-miR-1233-3p	6.670	7.040	6.717	7.225
29490	hsa-miR-7-5p	6.694	7.248	7.096	7.428
46215	hsa-miR-1301-3p	6.700	6.835	6.870	6.328
19596	hsa-miR-30d-5p	6.704	7.207	6.897	6.374
146061	hsa-miR-1914-3p	6.719	6.753	6.948	Undetected
42476	hsa-miR-374b-3p	6.721	7.017	6.694	6.292
10977	hsa-miR-183-5p	6.721	7.325	6.905	6.738
42532	hsa-miR-22-5p	6.733	6.254	6.648	6.888
147975	hsa-miR-487a-5p	6.737	6.989	7.016	6.912
147806	hsa-miR-3149	6.739	6.712	6.782	7.297
46866	hsa-miR-1321	6.742	7.171	7.141	7.186
46788	hsa-miR-1299	6.742	7.135	7.120	7.228
148154	hsa-miR-3922-3p	6.742	6.933	6.571	6.161
147186	hsa-miR-200b-3p	6.750	7.025	7.036	6.765
42859	hsa-miR-675-3p	6.759	7.133	6.962	6.503
147871	hsa-miR-3180-5p	6.759	7.016	6.488	6.228
147671	hsa-miR-4323	6.771	7.038	6.831	6.493
17272	hsa-miR-551a	6.776	6.274	6.411	7.047
42609	hsa-miR-135a-3p	6.781	7.216	7.141	6.655
42705	hsa-miR-191-3p	6.782	7.113	6.638	6.626
148317	hsa-miR-3621	6.798	6.537	6.722	6.873
11011	hsa-miR-211-5p	6.807	6.938	7.019	7.312

46695	hsa-miR-1228-3p	6.808	6.740	7.022	6.690
145708	hsa-miR-324-3p	6.820	7.194	7.067	6.564
169171	hsa-miR-4436b-5p	6.824	6.941	6.995	6.677
145717	hsa-miR-516a-3p/hsa-miR-516b-3p	6.828	7.343	7.288	7.449
27533	hsa-miR-320a	6.832	7.049	7.012	6.391
42749	hsa-miR-659-3p	6.844	6.996	6.797	7.597
147334	hsa-miR-3613-5p	6.848	7.199	7.252	7.405
148273	hsa-miR-3150b-3p	6.850	7.285	7.379	6.769
146160	hsa-miR-133b	6.850	6.726	6.447	6.822
145962	hsa-miR-639	6.859	6.954	6.953	6.947
11104	hsa-miR-422a	6.878	7.500	7.213	6.718
19585	hsa-miR-148b-3p	6.882	7.463	6.948	7.543
169272	hsa-miR-4419b	6.890	Undetected	8.618	10.287
17752	hsa-let-7f-5p	6.890	7.580	7.584	7.468
42870	hsa-miR-616-3p	6.892	7.274	6.965	6.616
46541	hsa-miR-1225-5p	6.899	6.830	7.226	6.866
147821	hsa-miR-3169	6.899	7.350	7.442	7.671
168935	hsa-miR-4687-3p	6.905	Undetected	Undetected	7.163
17280	hsa-miR-15b-5p	6.908	6.837	6.550	6.881
148621	hsa-miR-892a	6.915	7.609	6.920	6.533
46832	hsa-miR-1202	6.916	6.434	7.431	7.098
168802	hsa-miR-4516	6.920	Undetected	Undetected	Undetected
42732	hsa-miR-532-3p	6.925	7.495	7.209	6.496
145678	hsa-miR-150-5p	6.936	6.945	6.632	6.784
148243	hsa-miR-3689a-3p	6.936	7.144	7.171	7.032
10997	hsa-miR-19a-3p	6.939	6.919	6.493	6.619
145846	hsa-let-7e-5p	6.941	6.946	6.705	6.289
14271	hsa-miR-539-5p	6.941	7.247	7.634	7.083
46750	hsa-miR-1254	6.956	6.925	6.996	6.904
42592	hsa-miR-338-3p	6.956	7.281	7.019	7.038
46810	hsa-miR-1827	6.967	6.795	7.196	7.262
147606	hsa-miR-4259	6.971	7.241	7.259	6.869
147614	hsa-miR-4299	6.977	Undetected	Undetected	6.265
11007	hsa-miR-206	6.986	7.315	7.400	6.903
27536	hsa-miR-190a-5p	6.999	7.242	7.121	7.473
46689	hsa-miR-1229-3p	7.005	7.236	6.389	7.395
42672	hsa-miR-323b-5p	7.005	7.427	7.283	7.106
11058	hsa-miR-325	7.012	7.522	7.435	7.524
42839	hsa-miR-135a-5p	7.014	7.343	7.128	7.524
146006	hsa-miR-670-5p	7.017	7.027	7.269	Undetected
146052	hsa-miR-1471	7.022	7.353	7.464	6.809
46675	hsa-miR-1181	7.028	7.554	7.228	6.742
147591	hsa-miR-4283	7.029	7.079	6.936	6.435
146178	hsa-miR-502-3p	7.031	7.506	7.026	6.942

17506	hsa-miR-24-3p	7.057	7.337	7.168	6.987
11111	hsa-miR-432-5p	7.068	7.367	7.295	6.824
148482	hsa-miR-874-5p	7.069	7.258	6.755	6.475
27838	hsa-miR-302d-3p	7.070	7.546	7.512	7.536
42848	hsa-miR-1180-3p	7.070	7.565	7.580	7.372
17898	hsa-miR-99b-3p	7.070	7.592	7.317	7.112
146115	hsa-miR-940	7.085	7.649	7.286	6.995
46822	hsa-miR-1178-3p	7.086	7.464	7.094	6.418
17961	hsa-miR-629-5p	7.088	7.822	7.782	7.513
46462	hsa-miR-1224-5p	7.090	7.382	7.600	7.283
145838	hsa-miR-125b-1-3p	7.095	6.512	6.282	6.333
14962	hsa-miR-581	7.098	7.666	7.424	7.224
10964	hsa-miR-155-5p	7.114	7.413	7.385	7.450
42523	hsa-miR-26b-3p	7.117	7.430	7.397	7.769
147897	hsa-miR-3136-5p	7.124	7.577	7.305	7.445
46440	hsa-miR-1287-5p	7.126	7.267	7.558	7.385
148361	hsa-miR-3911	7.154	7.273	7.404	7.034
145705	hsa-miR-431-5p	7.161	7.621	7.365	7.572
145690	hsa-miR-512-5p	7.163	7.371	7.247	7.030
148641	hsa-miR-518b	7.165	7.009	7.482	7.139
17349	hsa-miR-595	7.203	7.767	7.620	7.573
148282	hsa-miR-3714	7.205	7.801	7.839	7.225
11022	hsa-miR-221-3p	7.206	7.677	7.523	7.552
17490	hsa-miR-571	7.210	7.424	7.342	7.181
42591	hsa-miR-634	7.211	7.223	7.745	6.733
168919	hsa-miR-4456	7.215	Undetected	6.732	6.532
46752	hsa-miR-1270	7.224	7.515	7.684	7.385
46416	hsa-miR-1293	7.236	7.758	7.768	7.799
147203	hsa-miR-302a-3p	7.238	7.347	7.351	7.305
148371	hsa-miR-3620-3p	7.244	7.660	6.611	6.453
148228	hsa-miR-3656	7.245	7.028	7.772	7.440
168708	hsa-miR-296-5p	7.252	7.573	7.819	6.621
11141	hsa-miR-509-3p	7.255	7.633	7.731	7.820
17336	hsa-miR-618	7.270	7.311	7.045	7.021
30033	hsa-miR-877-5p	7.275	7.535	7.535	7.740
148065	hsa-miR-3689b-3p/hsa-miR-3689c	7.280	7.713	7.717	7.605
4040	hsa-miR-9-5p	7.290	7.791	7.612	7.612
46917	hsa-miR-205-5p	7.292	7.389	7.561	7.815
9938	hsa-let-7i-5p	7.302	7.134	6.979	6.913
147981	hsa-miR-4325	7.308	7.681	7.447	7.737
27568	hsa-miR-744-5p	7.308	Undetected	Undetected	Undetected
145753	hsa-miR-484	7.315	7.867	7.645	7.603
46789	hsa-miR-513b-5p	7.318	7.747	7.578	7.960
146069	hsa-miR-1915-5p	7.321	7.876	7.527	7.673

11024	hsa-miR-223-3p	7.330	7.746	7.528	7.538
42898	hsa-miR-124-5p	7.342	7.768	7.365	7.249
42729	hsa-miR-34c-3p	7.355	7.673	7.175	7.988
42929	hsa-miR-25-5p	7.378	Undetected	Undetected	Undetected
146140	hsa-miR-1976	7.386	7.890	7.630	7.817
147942	hsa-miR-4268	7.396	6.533	6.188	6.445
11005	hsa-miR-204-5p	7.398	7.769	7.350	7.342
148049	hsa-miR-3924	7.406	7.578	7.626	6.877
148206	hsa-miR-3664-5p	7.410	7.958	7.201	6.360
145647	hsa-miR-584-5p	7.417	7.728	7.629	7.428
46443	hsa-miR-193a-5p	7.438	7.757	7.896	7.846
10990	hsa-miR-196a-5p	7.441	7.823	7.666	7.560
27720	hsa-miR-15a-5p	7.451	7.806	7.294	7.110
17302	hsa-miR-578	7.477	7.919	7.712	7.849
11140	hsa-miR-508-3p	7.484	7.927	7.712	7.738
168943	hsa-miR-4769-3p	7.486	7.777	7.373	6.439
27565	hsa-miR-423-5p	7.500	6.487	6.338	7.473
168619	hsa-miR-1260b	7.500	7.678	Undetected	7.824
147667	hsa-miR-3182	7.512	7.623	Undetected	8.552
11182	hsa-miR-98-5p	7.532	7.943	7.921	7.983
169083	hsa-miR-371b-3p	7.534	7.974	7.931	6.919
168882	hsa-miR-664b-3p	7.536	Undetected	Undetected	Undetected
27740	hsa-miR-574-5p	7.549	7.834	8.023	8.082
169375	hsa-miR-660-3p	7.573	Undetected	Undetected	Undetected
10954	hsa-miR-147a	7.584	7.878	7.931	7.990
42581	hsa-miR-513a-5p	7.608	7.717	7.401	7.908
46556	hsa-miR-623	7.615	8.061	7.942	7.752
46565	hsa-miR-1207-5p	7.625	7.726	7.935	7.634
46624	hsa-miR-1236-3p	7.632	8.003	7.827	7.936
147631	hsa-miR-4258	7.639	7.760	7.821	7.325
42810	hsa-miR-149-5p	7.642	7.917	7.775	7.781
46738	hsa-miR-1182	7.644	8.068	7.873	7.573
46221	hsa-miR-519d-3p	7.648	8.060	7.971	7.590
46634	hsa-miR-1281	7.651	7.912	7.725	7.863
46222	hsa-miR-1228-5p	7.652	8.157	8.020	7.810
46266	hsa-miR-1825	7.660	7.998	7.647	7.319
10947	hsa-miR-142-3p	7.671	8.013	7.886	7.970
145820	hsa-let-7c-5p	7.683	7.948	7.775	7.663
46705	hsa-miR-548k	7.694	8.153	7.895	8.247
148577	hsa-miR-3943	7.695	7.971	7.848	7.386
147935	hsa-miR-3125	7.716	8.163	7.831	7.731
32946	hsa-miR-486-5p	7.720	8.059	7.954	7.883
42892	hsa-miR-450b-3p	7.736	8.009	7.912	8.153
46806	hsa-miR-1227-3p	7.739	7.979	7.738	7.707
168950	hsa-miR-4646-3p	7.752	8.038	7.983	7.428

147701	hsa-miR-491-3p	7.767	8.371	7.687	7.938
168869	hsa-miR-5010-3p	7.767	8.238	8.009	7.682
46850	hsa-miR-1237-3p	7.768	8.245	8.028	8.008
42504	hsa-miR-593-3p	7.772	8.158	8.022	7.978
46259	hsa-miR-885-5p	7.795	8.188	7.944	7.572
42490	hsa-miR-505-5p	7.803	8.217	8.037	7.759
46368	hsa-miR-1282	7.808	8.157	7.979	7.917
42661	hsa-miR-492	7.840	Undetected	Undetected	Undetected
14301	hsa-miR-361-5p	7.841	8.225	8.124	8.219
169316	hsa-miR-3976	7.871	Undetected	Undetected	Undetected
42832	hsa-miR-638	7.877	Undetected	6.098	6.391
148481	hsa-miR-3646	7.884	Undetected	Undetected	Undetected
148413	hsa-miR-3614-3p	7.909	8.335	7.917	7.611
169188	hsa-miR-4443	7.929	7.664	7.430	8.068
169009	hsa-miR-548ap-5p/hsa-miR-548j-5p	7.946	Undetected	Undetected	Undetected
11245	hsa-miR-433-5p	7.952	7.504	7.497	7.666
168944	hsa-miR-4707-5p	7.969	Undetected	Undetected	7.299
147755	hsa-miR-378c	7.975	8.505	8.330	8.403
147664	hsa-miR-4311	7.979	8.366	8.199	8.014
11078	hsa-miR-365a-3p/hsa-miR-365b-3p	7.988	8.325	8.179	8.274
168911	hsa-miR-4682	7.991	Undetected	Undetected	Undetected
148622	hsa-miR-877-3p	8.010	8.349	8.311	8.012
146103	hsa-miR-1913	8.034	8.455	8.269	8.062
42641	hsa-miR-145-5p	8.038	8.569	8.185	8.258
146064	hsa-miR-718	8.068	8.442	8.366	8.228
17853	hsa-miR-30d-3p	8.069	8.511	8.421	8.269
169182	hsa-miR-4728-3p	8.073	8.444	8.287	7.778
46345	hsa-miR-1207-3p	8.073	8.531	8.412	8.280
146116	hsa-miR-2116-3p	8.079	8.518	8.207	8.132
46731	hsa-miR-4657	8.084	Undetected	7.016	Undetected
169395	hsa-miR-4484	8.107	Undetected	Undetected	Undetected
147907	hsa-miR-4312	8.123	8.512	8.363	8.118
145973	hsa-miR-664a-3p	8.126	8.505	8.265	7.953
42442	hsa-miR-498	8.146	7.587	9.660	7.445
148682	hsa-miR-483-3p	8.154	7.964	8.098	8.068
147165	hsa-let-7b-5p	8.179	8.566	8.527	8.383
169282	hsa-miR-4290	8.195	7.970	7.894	7.913
147722	hsa-miR-4306	8.196	6.204	6.622	6.730
169380	hsa-miR-3124-3p	8.240	Undetected	Undetected	6.316
168638	hsa-miR-4530	8.255	Undetected	Undetected	7.503
146072	hsa-miR-1469	8.265	6.063	Undetected	6.970
46427	hsa-miR-1248	8.275	8.666	8.491	8.478
42866	hsa-miR-451a	8.288	8.412	Undetected	7.633
169323	hsa-miR-4723-3p	8.333	8.786	8.557	8.315

147751	hsa-miR-4274	8.337	8.779	8.645	8.337
42557	hsa-miR-624-5p	8.351	8.829	8.623	8.505
46210	hsa-miR-1249	8.379	8.813	8.732	8.493
42969	hsa-miR-10b-3p	8.412	8.891	8.663	8.583
42696	hsa-miR-943	8.450	8.883	8.824	8.571
147604	hsa-miR-4285	8.455	Undetected	Undetected	7.832
169399	hsa-miR-4750-5p	8.510	Undetected	6.035	7.022
148156	hsa-miR-3686	8.524	7.650	Undetected	8.052
147595	hsa-miR-3178	8.526	Undetected	Undetected	6.339
169211	hsa-miR-5704	8.532	8.983	9.027	8.302
168878	hsa-miR-5100	8.577	9.326	7.610	8.803
147767	hsa-miR-4279	8.653	8.826	8.809	8.704
148038	hsa-miR-3679-3p	8.658	9.194	8.989	8.689
169082	hsa-miR-1275	8.667	Undetected	Undetected	8.214
148687	hsa-miR-1908-5p	8.700	6.463	6.601	7.622
169015	hsa-miR-4454	8.754	9.602	8.815	9.311
168844	hsa-miR-4532	8.782	Undetected	Undetected	7.718
169034	hsa-miR-642b-5p	8.825	9.021	9.236	8.765
169031	hsa-miR-4726-5p	8.868	Undetected	Undetected	7.675
42502	hsa-miR-204-3p	9.107	8.008	7.802	9.132
168637	hsa-miR-3940-5p	9.160	Undetected	6.716	7.799
168870	hsa-miR-1246	9.541	8.938	8.402	9.378
169285	hsa-miR-4467	9.750	6.203	7.387	8.429
169313	hsa-miR-4800-3p	10.128	Undetected	Undetected	9.094
168978	hsa-miR-371b-5p	10.132	6.321	7.540	8.708
169024	hsa-miR-3960	10.354	7.804	8.260	8.865
169028	hsa-miR-4708-3p	10.652	7.171	8.444	8.995
169050	hsa-miR-4787-5p	10.729	7.933	8.539	8.775
169110	hsa-miR-4497	10.978	7.089	8.030	9.300
147979	hsa-miR-3150a-3p	Undetected	Undetected	6.360	6.054
148643	hsa-miR-642a-5p	Undetected	6.295	6.122	6.060
168989	hsa-miR-4447	Undetected	Undetected	Undetected	6.077
148661	hsa-miR-486-3p	Undetected	Undetected	6.059	6.079
145638	hsa-miR-29a-5p	Undetected	6.099	Undetected	6.081
46398	hsa-miR-513c-5p	Undetected	6.157	Undetected	6.088
147556	hsa-miR-4254	Undetected	6.128	6.069	6.094
169135	hsa-miR-3173-5p	Undetected	Undetected	Undetected	6.096
148331	hsa-miR-3655	Undetected	Undetected	6.591	6.097
17946	hsa-miR-192-3p	Undetected	6.372	Undetected	6.099
145741	hsa-miR-545-3p	Undetected	Undetected	Undetected	6.103
11144	hsa-miR-512-3p	Undetected	6.167	6.115	6.106
17353	hsa-miR-609	Undetected	6.304	6.057	6.111
46274	hsa-miR-3187-3p	Undetected	6.110	Undetected	6.116
146179	hsa-miR-2113	Undetected	Undetected	Undetected	6.121
147162	hsa-let-7a-5p	Undetected	Undetected	Undetected	6.128

168672	hsa-miR-1587	Undetected	Undetected	Undetected	6.151
42869	hsa-miR-936	Undetected	6.069	6.155	6.153
148562	hsa-miR-128-1-5p	Undetected	Undetected	6.098	6.153
145637	hsa-miR-187-3p	Undetected	6.148	6.080	6.158
148380	hsa-miR-3913-5p	Undetected	6.148	6.217	6.169
147816	hsa-miR-3162-5p	Undetected	6.583	6.370	6.174
146008	hsa-miR-26b-5p	Undetected	6.056	Undetected	6.184
42941	hsa-miR-218-1-3p	Undetected	Undetected	Undetected	6.184
42865	hsa-miR-181a-5p	Undetected	Undetected	Undetected	6.194
147523	hsa-miR-4263	Undetected	Undetected	Undetected	6.203
46531	hsa-miR-1231	Undetected	6.434	6.531	6.207
147600	hsa-miR-4292	Undetected	6.392	6.268	6.219
27672	hsa-miR-615-3p	Undetected	6.105	6.117	6.231
147772	hsa-miR-4303	Undetected	Undetected	Undetected	6.233
46501	hsa-miR-1305	Undetected	Undetected	Undetected	6.235
42956	hsa-miR-545-5p	Undetected	Undetected	Undetected	6.247
147915	hsa-miR-3174	Undetected	6.143	6.322	6.261
42617	hsa-miR-541-5p	Undetected	6.142	Undetected	6.276
145821	hsa-miR-518c-5p	Undetected	Undetected	Undetected	6.287
146010	hsa-miR-2116-5p	Undetected	Undetected	6.323	6.296
42570	hsa-miR-194-3p	Undetected	6.103	6.239	6.317
147809	hsa-miR-514b-3p	Undetected	Undetected	Undetected	6.323
148514	hsa-miR-365a-5p	Undetected	6.349	6.040	6.323
145859	hsa-miR-33a-5p	Undetected	Undetected	Undetected	6.332
147786	hsa-miR-3198	Undetected	6.371	6.800	6.376
11044	hsa-miR-302c-3p	Undetected	6.182	6.135	6.386
42567	hsa-miR-590-3p	Undetected	Undetected	Undetected	6.387
17493	hsa-miR-622	Undetected	Undetected	6.213	6.392
11154	hsa-miR-517c-3p	Undetected	Undetected	Undetected	6.396
10925	hsa-miR-10b-5p	Undetected	6.212	6.074	6.467
11074	hsa-miR-34c-5p	Undetected	6.088	Undetected	6.486
146131	hsa-miR-2117	Undetected	6.449	6.143	6.576
10923	hsa-miR-107	Undetected	Undetected	Undetected	6.577
32731	hsa-miR-190b	Undetected	6.501	6.055	6.581
17810	hsa-miR-29b-1-5p	Undetected	6.256	6.143	6.600
19591	hsa-miR-199b-5p	Undetected	6.625	6.201	6.605
14285	hsa-miR-487b-3p	Undetected	Undetected	Undetected	6.606
42970	hsa-miR-744-3p	Undetected	Undetected	Undetected	6.623
17835	hsa-miR-450a-5p	Undetected	Undetected	6.325	6.635
146020	hsa-miR-449c-3p	Undetected	Undetected	Undetected	6.655
168644	hsa-miR-4775	Undetected	Undetected	Undetected	6.664
28047	hsa-miR-890	Undetected	Undetected	Undetected	6.726
46744	hsa-miR-526b-5p	Undetected	6.541	Undetected	6.736
145889	hsa-miR-196b-5p	Undetected	6.046	6.079	6.838
169412	hsa-miR-1260a	Undetected	Undetected	6.943	8.598

169189	hsa-miR-4795-5p	Undetected	Undetected	8.793	10.457
147613	hsa-miR-3145-3p	Undetected	Undetected	6.046	Undetected
145751	hsa-miR-23b-5p	Undetected	Undetected	6.060	Undetected
21702	hsa-miR-219a-1-3p	Undetected	Undetected	6.061	Undetected
147894	hsa-miR-3137	Undetected	Undetected	6.061	Undetected
147493	hsa-miR-3944-3p	Undetected	Undetected	6.093	Undetected
42666	hsa-miR-26a-2-3p	Undetected	6.173	6.097	Undetected
11083	hsa-miR-371a-3p	Undetected	6.174	6.101	Undetected
147709	hsa-miR-3185	Undetected	Undetected	6.105	Undetected
17641	hsa-miR-573	Undetected	6.291	6.118	Undetected
146152	hsa-miR-3179	Undetected	Undetected	6.131	Undetected
147898	hsa-miR-2861	Undetected	Undetected	6.138	Undetected
148637	hsa-miR-198	Undetected	Undetected	6.142	Undetected
148351	hsa-miR-3945	Undetected	Undetected	6.155	Undetected
147795	hsa-miR-3186-3p	Undetected	6.061	6.165	Undetected
42734	hsa-miR-2110	Undetected	Undetected	6.165	Undetected
42480	hsa-miR-485-5p	Undetected	Undetected	6.166	Undetected
148688	hsa-miR-765	Undetected	Undetected	6.174	Undetected
147823	hsa-miR-3146	Undetected	Undetected	6.177	Undetected
42852	hsa-miR-760	Undetected	Undetected	6.193	Undetected
146085	hsa-miR-3170	Undetected	Undetected	6.212	Undetected
46880	hsa-miR-1183	Undetected	6.088	6.213	Undetected
145716	hsa-miR-671-5p	Undetected	Undetected	6.255	Undetected
169102	hsa-miR-4639-3p	Undetected	Undetected	6.277	Undetected
147570	hsa-miR-4271	Undetected	6.092	6.288	Undetected
147798	hsa-miR-3141	Undetected	Undetected	6.292	Undetected
46275	hsa-miR-1251-5p	Undetected	6.210	6.293	Undetected
148654	hsa-miR-184	Undetected	Undetected	6.439	Undetected
46872	hsa-miR-1262	Undetected	6.179	6.478	Undetected
147654	hsa-miR-3138	Undetected	6.254	6.487	Undetected
147760	hsa-miR-4316	Undetected	Undetected	6.620	Undetected
169379	hsa-miR-4694-3p	Undetected	Undetected	6.687	Undetected
46558	hsa-miR-1268a/hsa-miR-1268b	Undetected	6.670	6.713	Undetected
169018	hsa-miR-4640-5p	Undetected	Undetected	6.753	Undetected
147376	hsa-miR-3679-5p	Undetected	6.142	6.772	Undetected
169130	hsa-miR-4764-3p	Undetected	Undetected	7.046	Undetected
147360	hsa-miR-95-5p	Undetected	6.047	Undetected	Undetected
146114	hsa-miR-1538	Undetected	6.069	Undetected	Undetected
169220	hsa-miR-4697-3p	Undetected	6.079	Undetected	Undetected
146138	hsa-miR-1909-5p	Undetected	6.080	Undetected	Undetected
42827	hsa-miR-652-3p	Undetected	6.086	Undetected	Undetected
168890	hsa-miR-1306-5p	Undetected	6.090	Undetected	Undetected
146122	hsa-miR-2053	Undetected	6.105	Undetected	Undetected
148454	hsa-miR-3682-3p	Undetected	6.107	Undetected	Undetected

46411	hsa-miR-1203	Undetected	6.109	Undetected	Undetected
146121	hsa-miR-1470	Undetected	6.110	Undetected	Undetected
42874	hsa-miR-16-2-3p	Undetected	6.134	Undetected	Undetected
148650	hsa-miR-516a-5p	Undetected	6.140	Undetected	Undetected
145974	hsa-miR-200b-5p	Undetected	6.145	Undetected	Undetected
145970	hsa-miR-129-2-3p	Undetected	6.145	Undetected	Undetected
147768	hsa-miR-4257	Undetected	6.184	Undetected	Undetected
45936	hsa-miR-1292-5p	Undetected	6.208	Undetected	Undetected
17613	hsa-miR-645	Undetected	6.210	Undetected	Undetected
21501	hsa-miR-891b	Undetected	6.248	Undetected	Undetected
147902	hsa-miR-3193	Undetected	6.279	Undetected	Undetected
46231	hsa-miR-519b-3p	Undetected	6.299	Undetected	Undetected
46361	hsa-miR-1278	Undetected	6.353	Undetected	Undetected
17298	hsa-miR-548b-3p	Undetected	6.439	Undetected	Undetected
45775	hsa-miR-1279	Undetected	6.452	Undetected	Undetected

9.5 Copies of Communications arising from this work