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List of Abbreviations

α SMA: Alpha Smooth Muscle Actin Fibers

AGO: Argonaute Protein

AJCC: American Joint Committee on Cancer

AntimiR: Anti-microRNA

APC: Adenomatous Polyposis Coli

AR: Androgen Receptor

ARHGAP19: Rho GTPase Activating Protein 19

ATCC: American Type Culture Collection

BCL: B Cell Lymphoma

BCL9: B-Cell Cll/Lymphoma 9-Like Protein

bHLH: Basic Helix-Loop-Helix

BIC: B Cell Integration Cluster

BIM: BH3-only ligand

BL1: Basal-Like 1

BL2: Basal-Like 2

BMI: Body Mass Index

BRCA1: Breast Cancer Gene 1

cAMP: Cyclic Adenylyl Cyclase Pathway

CCNE1:Cyclin E1

CDK: Cyclin Dependant Kinase

CDKN1C: Cyclin-dependent Kinase Inhibitor 1C

CK: Cytokeratin

CMTM4: CKLF-like Marvel Transmembrane Domain-containing 4

CSCs: Cancer Stem Cells

CSL: CBF1, Suppressor of Hairless, Lag-1

CSPG4: Chondroitin Sulfate Proteoglycan 4

DCIS: Ductal Carcinoma in situ

DDX5: DEAD Homeobox 5

DHH: Desert Hedgehog

Dlls: Deltalike Ligands

DSBs: Double-Stranded Breaks

DUBs: Deubiquitinases

DYRK1A: Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1a

ECM: Extracellular Matrix

EDTA: Ethylenediaminetetraacetic Acid

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

eIF4G3: Eukaryotic Translation Initiation Factor 4-Gamma 3

ELAVL4: ELAV Like Neuron-Specific RNA Binding Protein 4

EMT: Epithelial-Mesenchymal Transition

Eps8: Epidermal Growth Factor Receptor Kinase Substrate 8

ER: Estrogen Receptor

ERK: Extracellular Signal-regulated Kinase

EU: European Union

FBRS: Fibrosin

FBS: Foetal Bovine Serum

FGF: Fibroblast Growth Factor

FGFR: Fibroblast Growth Factor Receptor

FOG: Friend of GATA

FOXOs: Forkhead O Transcription Factors

FZD7: Frizzled Homolog 7

GSI: γ Secretase Inhibitor

HDAC: Histone deacetylase

HER-1: Human Epidermal Growth Factor Receptor 1

HER-2: Human Epidermal Growth Factor Receptor 2

HDI: Histone Deacetylase Inhibitor

HDR: Homology-Directed Repair

Hh: Hedgehog

Hsp90: Heat Shock Protein 90

HuR: Human Antigen R

ICG: Institute for Chemical Genomics

ID4: Inhibitor of DNA Binding 4

IDC: Invasive Ductal Carcinoma

IDC NOS: Invasive Ductal Carcinoma Not Otherwise Specified

IHH: Indian Hedgehog

IL8: Interleukin 8

IM: Immunomodulatory

IR: Ionising Radiation

ITSN2: Intersectin 2

JMJD1C: Jumonji domain containing 1C

KCNK10: Potassium Channel, Subfamily K, Member 10

KLF4: Krüppel-Like Factor 4

KRIT1: Krev Interaction Trapped 1

LAR: Luminal Androgen Receptor

LCIS: Lobular Carcinoma in situ

LRP6: Low Density Lipoprotein Receptor-related Protein 6

M: Mesenchymal

mAbs: Monoclonal Antibodies

MAML: Mastermind-like

Man A: Manumycin A

MAPK: Mitogen-activated Protein Kinase

MAST2: Microtubule-associated Serine Threonine Kinase 2

MEK: MAP/ERK Kinase

MET: Mesenchymal-To-Epithelial Transition

miRNA: MicroRNA

MMP: Matrix Metalloproteinase

MPDZ : Multiple PDZ Domain Protein

mRNA: Messenger RNA

MSL: Mesenchymal Stem-Like

MTDH: Metadherin

mTOR: Mammalian Target of Rapamycin

MYC: Myelocytomatosis Viral Oncogene Homolog

NCD: Non-communicable Disease

NHEJ: Non-Homologous End Joining

NID: Notch Intracellular Domain

NOS: Not Otherwise Specified

NST: No Special Type

Oncomir: Oncogenic miRNA

PARP: Poly ADP-ribose polymerase

PBS: Phosphate Buffered Saline

PDCD4: Programmed Cell Death 4

PFN1: Profilin 1

PHF2: Phd Finger Protein 2

PI3K: Phosphatidylinositol 3-kinase

PIP_3: Phosphatidylinositol (3,4,5)-Triphosphate

PLEKHA1: Pleckstrin Homology Domain-Containing Protein

PR: Progesterone Receptor

PRC2: Polycomb Repressive Complex-2

Pre-miRNA: Precursor miRNA

Pri-miRNA: Primary miRNA

PTCH1: Patched-1

PTHS: Pitt-Hopkins syndrome

PTK: Protein Tyrosine Kinases

PTP: Protein Tyrosine Phosphatases

PTPN12: Protein-Tyrosine Phosphatase, Nonreceptor-Type, 12

qRT-PCR: Quantitative Reverse Transcription PCR

Rho-GEFs: Rho Guanine Nucleotide Exchange Proteins

RISC: miRNA-induced Silencing Complex

RNAi: RNA Interference

RNA-seq: High-Throughput Sequencing

RNP: Ribonucleotide Protein

SDS: Sodium Dodecyl Sulfate

sFRP1: Secreted Frizzled-related Protein 1

SH2: Src Homology 2

SHH: Sonic Hedgehog

shRNA: Short Hairpin RNA

siRNA: Small Interfering RNA

SMAD4: Mothers against decapentaplegic homolog 4

SMO: Smoothened Protein

TBE: Tris/Borate/EDTA

TCF4: Transcription Factor 4

TET2: TET Oncogene Family, Member 2

TF7: Transcription Factor 7

TGFβ: Transforming Growth Factor β

TNBC: Triple-negative Breast Cancer

TNM: Tumour Node Mitotic Index

TP53: Tumour Protein p53

TS: Tumour Suppressor

TSG: Tumour Suppressor Gene

TSHZ3: Teashirt Zinc Finger Homeobox 3

tSp1: Thrombospondin1

UK RCPath: United Kingdom Royal College of Pathologists

UNS: Unstable

UPS: Ubiquitin Proteasome System

UTR: Untranslated Region

VEGF: Vascular Endothelial Growth Factor

WHO: World Health Organisation

Wnt: Wingless Type

YOD1: Ovarian Tumour Deubiquitinating Enzyme 1

ZEB1: Zinc Finger E Box-Binding Homeobox 1

ZEB2: Zinc Finger E Box-Binding Homeobox 2

ZFPM2: Zinc Finger Protein, Multitype 2

ZNF367: Zinc Finger Protein 367

Abstract

Triple-negative breast cancer (TNBC) represents approximately 20% of breast cancers and is characterised by a lack of expression of oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2). TNBC is particularly aggressive and affects younger pre-menopausal women, and is associated with a poor prognosis, particularly in African females. Currently the only treatment available for TNBC is chemotherapy, which tends to result in resistance as well as recurrence after 5 years and ultimately death. Hormone therapy is not a viable treatment strategy due to the tumours lacking surface hormone receptors. The deficit in knowledge relating to this breast cancer type has resulted in a deficiency of effective targeted therapies. In this regard, there is a great need for TNB tumours to be subclassified in order to optimise treatment plans for individual patients. While at present there is no reliable method for categorising TNBC, a method based on gene expression profiles has yielded 6 different subtypes. In the present study a novel approach was taken to evaluate the utility of microRNAs (miRNAs) as markers of hormone insensitive breast cancer. miRNAs are short, single-stranded non-coding RNAs with a regulatory function in cells. The expression of these molecules is often dysregulated in cancer, and their involvement in disease development and progression has been proven in many different cancers. The aim was thus to identify miRNAs which play a role in TNBC pathology and to determine their gene targets in order to discover novel targets for therapy. An *in vitro* cell culture approach was taken to investigate the utility of miRNA analysis in TNBC targeted therapy development and subclassification. Extracted RNA from each cell line was shipped to Seattle, USA for miRNA profiling by two different storage methods. One set of duplicate samples was transported in the conventional frozen, dry ice mode, while the other set of duplicates was transported in a fairly novel "dry" RNAstable mode. The miRNA expression results were compared between the frozen and dried duplicate samples with a standard two-tailed Student's t-test. The test yielded no significant difference between the two storage strategies (p-values < 0.05). These results indicate that RNAstable is a reliable, inexpensive mode of transporting sensitive biological samples. To profile the expressed miRNAs, Nanostring nCounter analysis was performed on two different TNBC cell lines (MDA-MB-231 and MDA-MB-436), as well as on a non-cancerous epithelial breast cell line (MCF-10A) and on an oestrogen-sensitive breast cancer

cell line (MCF-7). The expression results were compared to identify aberrantly expressed miRNAs in the two TNBC cell lines, relative to the two control cell lines. A comparison between the two TNBC cell lines was also considered to appraise a novel method for subclassifying TNBC. A total of 12 significantly altered miRNAs, 9 overexpressed, and 3 underexpressed within the two TNBC cell lines were identified based on selection criteria. miRNAs expressed in the two TNBC cell lines with a fold change of at least 2 relative to the two control cell lines, were considered significant and were selected for bioinformatic analysis. Following this, *in silico* analysis using the DIANA microT v.4 software was used to ascertain significant gene targets of the aberrant miRNAs. Another selection process for miRNA target analysis was carried out to determine the top three most significant gene targets. Subsequently, further analyses of targets were carried out through a comprehensive literature review. The comparison between the two TNBC cell lines yielded a 25% difference in the miRNA expression patterns, suggesting that miRNA expression profiles may well be a novel and reliable method for subclassifying TNBCs. Ultimately, this could elucidate more effective and personalised treatment plans.

Keywords: Triple-negative Breast Cancer (TNBC), Oestrogen receptor (ER), Progesterone receptor (PR), Human epidermal growth factor 2 (HER2), Subclassification, microRNAs (miRNAs), RNAstable, Nanostring nCounter analysis, DIANA microT v.4 software.

1. Literature Review

Non-communicable diseases (NCDs) are the leading cause of death worldwide; in 2008, 29% of all deaths in South Africa were attributed to NCDs, 7% of which were cancer-related (Alwan *et al.,* 2011). Breast cancer is the most prevalent cancer in women, the second most diagnosed cancer worldwide, and has now become the most prevalent cancer in both developed, and developing regions, including South Africa (Ferlay *et al.,* 2010; Parkin *et al.,* 2005). According to the National Cancer Registry of South Africa, 1/29 women are at risk of developing breast cancer in their lifetime. Even with a high incidence, breast cancer has a fairly high survival rate, and thus, a low mortality compared to other cancers. Although it is only fifth in terms of overall cancer deaths, it still accounted for 15% of all deaths in South Africa in 2009 (Ferlay *et al.,* 2010; Mayosi *et al.,* 2009; Parkin *et al.,* 2005).

1.1. Breast cancer classification

Breast cancer is a heterogeneous disease encompassing a range of biological characteristics, clinical behaviors, morphologies, and clinical presentations (Chacón & Costanzo 2010, Chen *et al.,* 2009b; Rakha *et al.,* 2010). Tumours are classified into various groups and classes depending on their anatomical morphology, the extent and location of the cancer, hormone receptor status and how quickly the cancerous cells divide. All of this information is helpful in determining the best route of treatment for particular breast cancers. Breast cancers may broadly be considered as either hormone sensitive or hormone insensitive. In the latter case, such breast tumours lack the expression of oestrogen, progesterone and human epidermal growth factor receptors and are thus termed triple negative breast cancer (TNBC). Due to the lack of hormone receptors, TNBC is non-responsive to hormone therapies and moreover is particularly difficult to characterise due to its diverse features and lack of knowledge (Lavasani & Moinfar, 2012). This is mentioned here, as a particular intention of the present study was at a microRNA level (miRNA), to identify both common and different miRNA features between two differing TNBC cell lines to possibly

determine a novel way of sub-classifying TNBCs. However, prior to discussing this specific approach, the current histopathology based approach to classifying breast cancer is described below.

1.1.1. Histological Classification of Breast Cancer

The histopathological classification of breast tumours is based on the examination of tissue biopsies under a light microscope in order to determine the anatomical pathology.

1.1.1.1. Histopathological Type

Histologic typing of breast carcinomas is essential in the determination of which treatment strategy is appropriate based on the primary tumour origin, whether it is invasive or not, and how the cells appear under a light microscope (Fabbri *et al.*, 2008; Goldhirsch *et al.*, 2009). It is determined by microscopic examination of the cell morphology and architecture of tissues and is an indication of which tissues are affected within a particular organ. The tumour is initially identified as either invasive or non-invasive which is an indication of whether to expect metastasis to other organs and reflects the risk of further tumour development in the same breast, as well as in the other breast.

1.1.1.1.1. The Origin of Breast Tumours

Breast cancers are derived from epithelial cells that are found in the terminal duct lobular unit. Cancer cells which remain within the terminal duct lobular unit and the draining duct are classified as non-invasive (*in situ*), while disseminated cancer cells which have moved out of the basement membrane of the ducts and lobules and into the surrounding normal tissue, are invasive (Dixon, 2012). Some of these non-invasive carcinomas are regarded as "pre-cancerous" lesions which, if not removed, may eventually develop into an invasive carcinoma; ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS), for example (King *et al.*, 2012a). Invasive carcinomas can continue to spread to the lymph nodes and other tissues throughout the body, increasing the risk of death and reoccurrence, exponentially.

Next, the site of origin of the primary tumour is determined. This information is an indicator of how the cancer will behave which is useful for choosing a suitable treatment plan. Ductal carcinomas are the most common breast cancer (81.4%), followed by lobular (6.3%) and unspecified carcinomas (5.5%) (Albrektsen *et al.*, 2010). Connective tissue is rarely the origin of breast tumours and in such instance is termed sarcoma. Figure 1 below summarises the approach to classifying breast tumours.

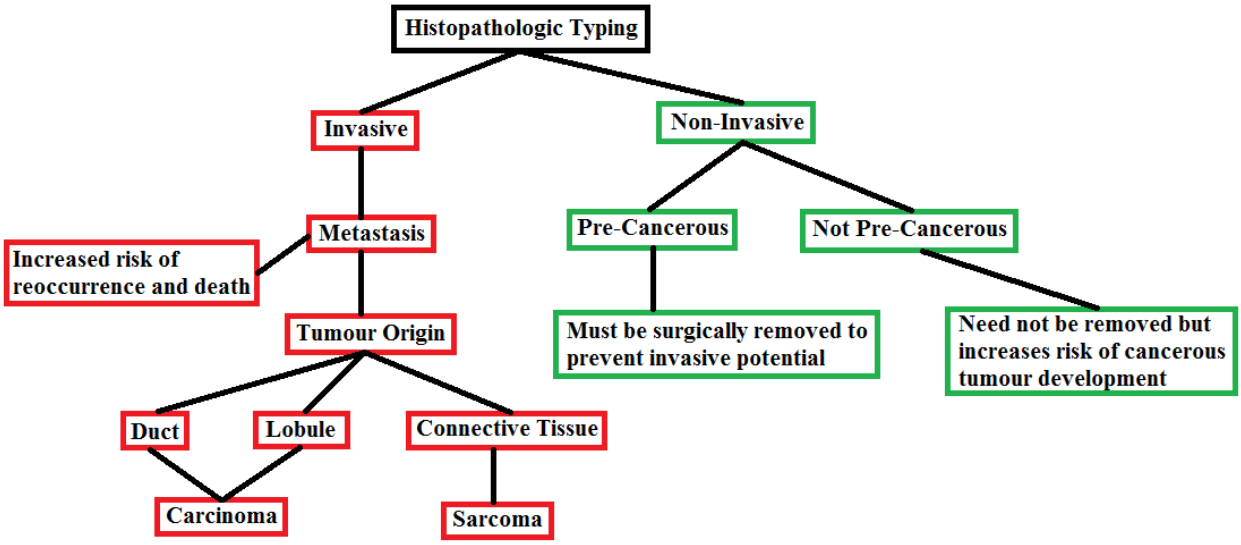


Figure 1: Histopathological typing of breast tumours.

1.1.1.1.2. Tumour Subtyping

Breast tumours are then subtyped and graded based on their appearance under a light microscope. Most invasive breast cancers are classified as invasive ductal carcinoma not otherwise specified (IDC NOS), however about 25% are classified as special types (Weigelt *et al.*, 2008) (Table 1). These special types behave differently to non-special types and have different clinical characteristics, which makes special typing clinically relevant in terms of further characterisation and treatment.

Table 1: Histologic Types of Invasive and *in situ* Breast Cancer. Carcinomas of no special type can be graded to gain useful prognostic information. Adapted from Dixon (2012) and Harris *et al.* (2010).

Special Types	*In situ* Carcinomas	No Special Type
Tubular Classic Lobular Mucinous/mucoid Medullary Papillary Cribriform	NOS Intraductal Paget's disease and intraductal	Not otherwise specified (NOS/NST) Ductal Inflammatory Lobular Paget's disease and infiltrating Undifferentiated Squamous cell Adenoid cystic Secretory

1.1.1.1.3. Hormone Related Risk Factors

A woman's reproductive history and other hormone-related risk factors have been related to risk of histological type of breast cancer development. Women who give birth to their first-born child before the age of thirty dramatically reduce their risk of developing any histologic type of breast cancer. The risk is further decreased by breast feeding, and increased in women who have never had children or have children later in life (>35 years of age). Early age at menarche also increases risk of tumour development (Beaber *et al.*, 2008; Reeves *et al.*, 2009). The risk of some histologic types of breast cancer seems to increase shortly after childbirth, but decreases significantly thereafter (Albrektsen *et al.*, 2010). The type of hormone-replacement therapy in post-menopausal women affects the risk of which breast cancer type develops and was also correlated to body mass index (BMI) (Li *et al.*, 2006; Reeves *et al.*, 2006; Rosenberg *et al.*, 2006). Oral contraceptives increase the risk of breast cancer development in younger woman (<

20 years) but has no significant effect on risk in post-menopausal women (Gierisch *et al.*, 2013; Marchbanks *et al.*, 2002).

1.1.1.2. Histopathological Grade

In situ ductal carcinoma and all invasive tumours are routinely graded (Fabbri *et al.*, 2008). The grade of a tumour depends on how closely those tumour cells resemble normal breast cells microscopically, and reflects patient prognosis. In order for a tumour to be graded histologically a scoring system must be used; most commonly, the Nottingham Histologic Score System, which is recommended by several international bodies including the World Health Organization (WHO), the American Joint Committee on Cancer (AJCC), European Union (EU), and the Royal College of Pathologists (UK RCPath) (Rakha *et al.*, 2010). Three main factors are considered; firstly, tubule formation, wherein the percent of tumour which forms normal duct/gland structures is assessed. These tissue structures become less orderly in worse or higher grade tumours. Next, nuclear pleomorphism is considered; the nuclear features of tumour cells become larger, darker, and more irregular compared to normal breast epithelial cells. Lastly, the mitotic count is evaluated, that is, how many tumour cells divide in ten microscope fields, the more cells dividing, the worse the prognosis (Table 2) (Ivshina *et al.*, 2006; Rakha *et al.*, 2010). Each of these factors is scored on a scale of one to three, based on their severity, and the scores are then summed to yield the final grade of the tumour (Table 3). There are three variations of histological grade; well differentiated (low grade), moderately differentiated (intermediate grade) and poorly differentiated (high grade) representing good, moderate, and poor prognosis respectively (Fabbri *et al.*, 2008; Galea *et al.*, 1992) (Figure 2). Low grade tumours tend to indicate a good prognosis, with few events in a life time. Moderate grade tumours signify an intermediate outcome in the early years, but a significantly impaired outcome for survival and increased reoccurrence in later years (Rakha *et al.*, 2008a; Rakha *et al.*, 2008b). Higher grade tumours reflect the most abnormal cellular anatomy and indicate a worse prognosis with early reoccurrence and death. As the grade of a tumour represents its "aggressive potential", it is thus important to determine a tumour's grade in order to select the appropriate treatment. Lower grade tumours can be treated less aggressively and have a better prognosis than high grade tumours.

Table 2: A detailed summary of criteria used in the Nottingham Histologic Score System to grade invasive breast tumours histologically. Adapted from Cangiarella *et al.* (2013).

Score	Tubular Differentiation (% of tumour area forming glandular/tubular structures)	Nuclear Pleomorphism	Mitotic Count (Based on a 0.44 mm field diameter; cut-off values to be adjusted with the microscopic field)
1	>75%	Small, regular, uniform cells	0-5
2	10% to 75%	Moderate increase in size and variability	6-10
3	<10%	Marked variation	Over 10

Table 3: Relationship between tumour grade and prognosis in breast cancer patients. Adapted from Fabbri *et al.* (2008) and Galea *et al.* (1992).

	Overall Grade Score	Cellular Differentiation	Prognosis
Grade 1	3/4/5	Well differentiated	Good
Grade 2	6/7	Moderately differentiated	Moderate
Grade 3	8/9	Poorly differentiated	Poor

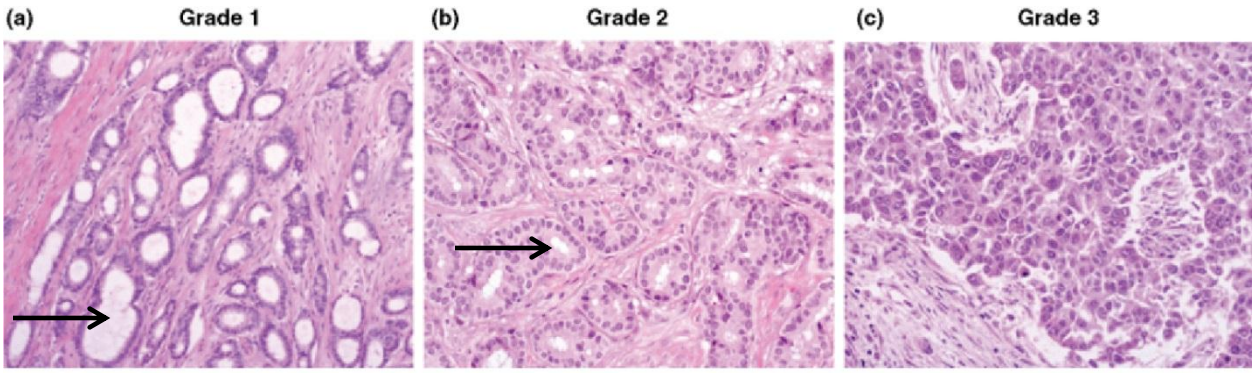


Figure 2: Histological grade of breast cancer as assessed by the Nottingham Histologic Score System. (a) A well-differentiated tumour that demonstrates high homology to the normal breast terminal duct lobular unit, tubule formation present (>75%), a mild degree of nuclear pleomorphism, and low mitotic count. Ducts are still fairly clearly distinguishable (marked with an arrow) (b) A moderately differentiated tumour. Some ducts can still be distinguished (marked by an arrow) (c) A poorly differentiated tumour with a marked degree of cellular pleomorphism, frequent mitoses, and no tubule formation (<10%). No distinguishable ductal structures. Adapted from Rakha *et al.* (2010).

The significance and accuracy of histologic grading of tumours has been tested in several studies and the results were very inconsistent (Anderson, 2002; Davis *et al.*, 1986; Frkovic-Grazio & Bracko, 2002; Le Doussal *et al.*, 1989a; le Doussal *et al.*, 1989b; Parham *et al.*, 1992; Parl & Dupont, 1982; Rank *et al.*, 1987; Schumacher *et al.*, 1993; Stierer *et al.*, 1992a; Stierer *et al.*, 1992b; Zedeler, 1988). It has been suggested that tumours should be graded as well as genetically typed in order to better predict the prognosis of patients and more than one type of classification is needed to truly reflect the severity of the cancer (Pereira *et al.*, 1995). A combination of histologic typing and grading used in conjunction with molecular subtyping, DNA classification and tumour staging would far more accurately determine a patient prognosis, probability of relapse, and provide guidance for the best treatment options.

1.1.2. Tumour Stage

Staging breast tumours allows for quantification of the severity of the cancer in terms of the extent of metastasis and gives an indication of prognosis (Edge & Compton, 2010; Fabbri *et al.*, 2008). There are various methods which can be used to determine the stage of cancer; however, the most widely acceptable is the TNM system (Sobin & Fleming, 1997). This system is based on primary tumour (T), lymph node (N), Metastases (M), and more recently, residual tumour

classification (R) which is a strong indicator of prognosis (Sobin & Fleming, 1997; Wittekind *et al.*, 2002). The T values represent the extent of the primary tumour at its site of origin, the N values represent regional lymph nodes affected, and the M values represent the distant metastases (Edge & Compton, 2010). Complete removal of tumours improves a patient's prognosis and so the residual tumour classification denotes the presence of residual tumour left after treatment is completed (Table 4). Once a tumour has been thoroughly examined, it can be staged accordingly (Table 5). Patients with stage I or II cancer have a low incidence of detectable spread, and in the absence of certain signs and symptoms, they don't require further investigation to identify metastatic disease. However, patients with larger or further advanced tumours should be candidates for further investigation (Dixon, 2012).

Table 4 : Summary of the TNM(R) staging classification system for breast cancer. There are several variants within many of the staging groups described by their specific characteristics not mentioned here. "p" stands for pathologic. Adapted from Witterkind *et al.* (2002) and Fabbri *et al.* (2008). Additional descriptors are used in some cases to further describe special cases of breast cancer; m, y, r, and a. "m" is used to indicate multiple primary tumours at the primary site. "y" indicates the extent of the tumour during or following multimodality treatment. "r" is an indication of recurrent tumours identified after a disease-free period, and is different to the residual tumour symbol "R". "a" indicates a tumour first diagnosed on autopsy (Sobin *et al.*, 2011).

TNM Values	Definition
T values depend on the primary site of origin of the tumour in the breast	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*
T1	Tumour 2cm or less in greatest dimension
T2	Tumour more than 2cm but less than 5cm in greatest dimension
T3	Tumour more than 5cm in greatest dimension

T4	Tumour invades deep extra-dermal structures
N values depend on The number, size and location of breast cancer cell deposits in various regional lymph nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis. Metastasis in movable ipsilateral axillary lymph node(s).
N2	Metastases in ipsilateral axillary lymph nodes fixed or matted, or in clinically apparent ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph-node metastases
N3	Metastasis in ipsilateral infraclavicular lymph node(s), or in clinically apparent ipsilateral internal mammary lymph node(s), and in the presence of clinically evident axillary lymph node metastasis, or metastasis in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph-node involvement
Pathologic Classification (pN)	
pNX	Regional lymph nodes cannot be assessed (e.g., previously removed or not removed for pathologic study)
pN0	No regional lymph node metastasis histologically, no additional examination for isolated tumour cells

pN1	Metastasis in one to three axillary lymph nodes, and/or in internal mammary nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN2	Metastasis in four to nine axillary lymph nodes, or in clinically apparent internal mammary lymph nodes in the absence of axillary lymph-node metastasis
M values depend on the presence of breast cancer cells in locations other than the breast	
MX	Distant metastasis cannot be assessed
M0	No distant metastases
M1	Distant metastases
R values indicate how much of the cancer remains after treatment	
RX	Presence of a residual tumour cannot be assessed
R0	No residual tumour
R1	Microscopic residual tumour
R2	Macroscopic residual tumour

Table 5: Breast Cancer Staging based on all of the considerations involved in the TNM(R) classification system. Stage 0 represents non-invasive carcinoma and a good prognosis, while stage IV represents a dangerous tumour which has metastasised and shows the presence of residual tumour; increasing the chance of reoccurrence and a poor prognosis. Adapted from Dixon, (2012).

Stage	Primary Tumour	Regional Lymph Nodes	Distant Metastases
Stage 0	Tis	N0	M0
Stage I	T1	N1	M0
Stage II	T2	N0	M0
	T3	N0	M0
Stage III	T3/T4	Any N	M0
	Any T	N2/N3	M0
Stage IV	Any T	Any N	M1

1.1.3. Molecular Subtyping of Breast Tumours

Receptor status, along with tumour grade, is used in profiling of breast tumours into molecular subtypes. This is an indication of which hormone receptors are present on the surface of the breast cancer cells and also helps in the process of determining which treatment options are available and suitable depending on receptor status. Targeted therapies have been developed for receptor positive breast cancers whereby a specific receptor or group of receptors is targeted to alter gene expression in those cells to reduce cell proliferation and promote apoptosis. Tamoxifen and Trastuzumab, for example, are targeted therapies which reduce the effect or level of oestrogen and HER-2 in the cancer cells, respectively, and are often used in conjunction with

conventional chemotherapy (Dawood *et al.,* 2010). Triple-negative breast cancer, lacking hormone receptors and targeted treatments, has a comparatively poor prognosis (Liedtke & Kiesel, 2012). Androgen receptor (AR) is expressed in 80-90% of oestrogen receptor positive (ER+) tumours and 40% of triple-negative breast tumours. Activation of androgen receptors in ER+ tumour cells shows a reduction in cell proliferation and, an increase in cell proliferation of ER- cells. Novel interpretation of molecular tumour status may lead to novel diagnostic and therapeutic developments (Hu *et al.,* 2011; Lehmann *et al.,* 2011). A summary of receptor (molecular) status and treatment options is presented below (Table 6).

Table 6: Summary of receptor status in breast cancers and their current treatment options.

Receptor Status	Definition	Treatment
Oestrogen Receptor Positive	Oestrogen sensitive	Hormone therapy (Tamoxifen) and chemotherapy
Progesterone Receptor Positive	Progesterone sensitive	Hormone therapy (Tamoxifen) and chemotherapy
HER-2 Receptor Positive	HER-2 protein over-expression	HER-2-targeted therapy (Trastuzumab) and Chemotherapy
Triple-negative Breast Cancer	Does not have surface ER, PR, or HER-2 receptors, and are thus hormone insensitive	Chemotherapy (no effective targeted therapies yet)

Molecular subtypes of breast cancer are associated with local and regional relapse and overall clinical outcome (Voduc *et al.,* 2010). There are various breast cancer subtypes based on microarray expression profiling analyses; including luminal A, luminal B, HER2-enriched, the "basal-like" subtype, normal-like, and triple-negative breast cancer (TNBC) (see Table 7) (Diaz *et al.,* 2007; Jönsson *et al.,* 2010; Park *et al.,* 2012; Perou *et al.,* 2000; Reis-Filho & Tutt, 2008). The luminal subtypes are characterised by overexpression of hormone receptors (oestrogen receptor, ER, and progesterone receptor, PR) and constitute the majority of breast cancer cases in general; while HER2-enriched breast cancer shows overexpression of the human epidermal

growth factor receptor (HER-2) (Soomro *et al.*, 2011). Normal-like tumours have common characteristics with normal breast tissue like adipose and other non-epithelial tissue components of the tumour micro-environment (Giordano & Normanno, 2009). The basal-like subtype generally, but not always, tends to lack expression of the above mentioned receptors, and the total lack of expression of these protein receptors is termed "triple-negative" breast cancer (Chen *et al.*, 2009b). The different subtypes show distinctive differences in prognosis and response to chemotherapy which is thought to be attributable to differences in their gene pathways and biological pathways (Iwamoto *et al.*, 2011; Parker *et al.*, 2009). Luminal tumours are associated with the most favorable prognosis, while HER-2, basal-like and TNBC have been associated with the worst prognosis (Carey *et al.*, 2006; Nielsen *et al.*, 2004; Rakha *et al.*, 2006). Subtypes also show distinct patterns of metastatic spread with noticeable differences in survival after relapse (Kennecke *et al.*, 2010). Marked differences in DNA methylation patterns have also been noted between different breast cancer subtypes suggesting that methylation patterns play a role in development of specific subtypes (Bediaga *et al.*, 2010; Holm *et al.*, 2010). Several other molecular subtypes of breast cancer have been identified by microarray expression profiling by various research groups, creating controversy in which subtypes are unique entities and which should fall under the same classification (Jönsson *et al.*, 2010; Prat *et al.*, 2010; Prat & Perou, 2011). Thus, there is clearly a need for novel methods and better defined guidelines for subtype classification in breast cancer (Lavasani & Moinfar, 2012; Weigelt *et al.*, 2010).

Table 7: Molecular Subtypes of breast cancer based on receptor status. Adapted from Kwan *et al.* (2009) and Park *et al.* (2012).

Molecular Subtype	Receptor Status
Luminal A	ER + and/or PR +, HER-2 -
Luminal B	ER + and/or PR -, HER-2 +
Basal-like	ER -, PR -, HER-2 -, CK 5/6 +, and/or EGFR +
Triple-negative	ER -, PR -, HER-2 -
HER-2 over-expressing	ER -, PR -, HER-2 +
Normal-like	Unclassified as receptor status unclear

1.2 Triple-Negative Breast Cancer

It is estimated that 10-20% of all women diagnosed with breast cancer have a TNBC phenotype (Boyle, 2012). This type of breast cancer is clinically aggressive with a short time from diagnosis to relapse and relapse to death. Most reoccurrence is between the first and third years and majority of deaths occur in the first five years after treatment (Dent *et al.,* 2007). The high risk of recurrence in TNBC is attributable to an excess of visceral metastases (Dent *et al.,* 2009). Most TNBC tumours are of high histological grade (Grade 3) and show lymph node involvement at diagnosis (Anders & Carey, 2008; Lehmann *et al.,* 2011; Reis-Filho & Tutt, 2008; Soomro *et al.,* 2011). TNBC primarily, but not exclusively, carries the basal-like molecular profile on gene expression arrays and is often associated with BRCA-1 and p53 mutations as well as PTEN loss (Chae *et al.,* 2009; Maegawa & Tang, 2010; Reis-Filho & Tutt, 2008; Rexer *et al.,* 2009). Survival of African and Hispanic females with TNBC is far poorer than that of Caucasian women, even within the same categories and stage, and even when adjusting the differences in access to health-care (Parkin *et al.,* 2008). TNBC tumours are commonly associated with a higher mitotic index, nuclear pleomorphism, and tumour grade (Carey *et al.,* 2006). Currently with a lack of targeted therapies and younger, premenopausal women being affected, it is of utmost importance for TNBC to be further investigated on a molecular level.

Most TNBCs are ductal in origin (grade 3 IDC-NST) and prove difficult to diagnose for specific treatment, however several other aggressive phenotypes can also be present such as metaplastic and medullary, which are associated with a rapid aggressive course and poor prognosis. Less aggressive phenotypes include secretory and adenoid cystic carcinomas (Figure 3) (Hudis & Gianni, 2011; O'Toole *et al.,* 2013; Soomro *et al.,* 2011). There are several different TNBC histologies known to date (Table 8). Histologically, this special type of breast cancer consists of an array of tumour subtypes (Hudis & Gianni, 2011; Soomro *et al.,* 2011). Approximately 80% of TNBC tumours have been classified as basal-like based on expression of basal-like markers and are associated with the worst prognosis (O'Toole *et al.,* 2013). TNBC has been further subdivided into two groups based on gene expression profiling; basal-like and Claudin-low (Prat *et al.,* 2010; Prat & Perou, 2011). A second molecular classification system, also based on gene expression profiling, identified 6 TNBC subtypes. The 6 subtypes were namely Basal-like 1 (BL1), Basal-like 2 (BL2), immunomodulatory (IM); mesenchymal (M); mesenchymal stem–

like (MSL); luminal androgen receptor (LAR); and unstable (UNS) (Lehmann *et al.*, 2011; Masuda *et al.*, 2013). Interestingly, the two TNBC cell lines used in the present study both fell under the MSL group (MDA-2B436 and MDA-2B-231).

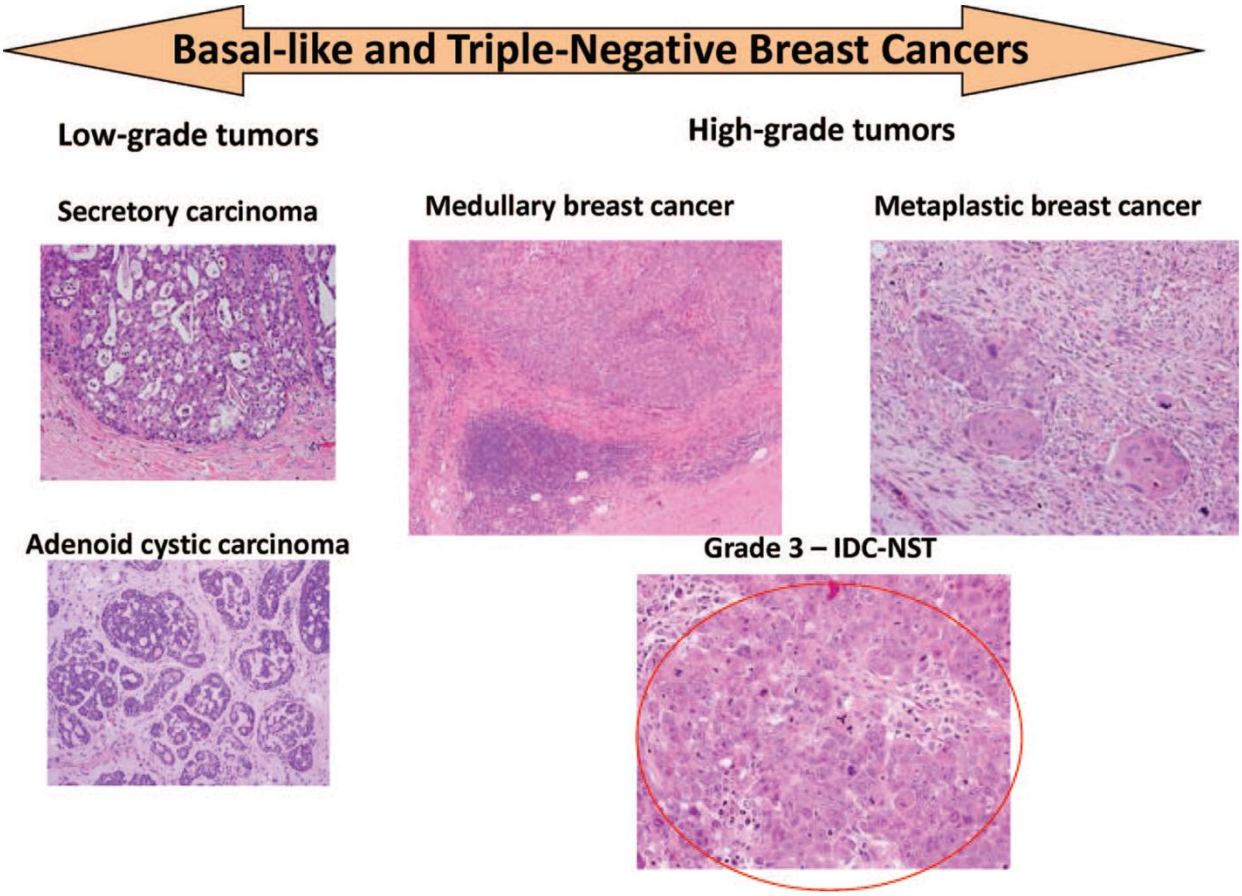


Figure 3: Range of TNBC histologies. Less differentiated cancers are more aggressive, as can be seen in medullary, metaplastic and invasive ductal carcinoma (IDC-NST), with the latter being the most aggressive and least differentiated (Circled). Adapted from Hudis and Gianni, (2011).

Table 8: **TNBC Histologies.**

TNBC Histology	Abbreviation
Adenocarcinoma	AC
Anaplastic Carcinoma	ANC
Acantholytic Squamous Cell Carcinoma	ASCC
Carcinoma	C
Carcinosarcoma	CS
Ductal Carcinoma	DC
Invasive Ductal Carcinoma	IDC
Invasive Galactophoric Adenocarcinoma	IGA
Inflammatory Ductal Carcinoma	INF
Metaplastic Carcinoma	MC
Medullary Breast Cancer	MBC

1.2.1. Prevalence of Triple Negative Breast Cancer in African Females

Breast cancer epidemiology, risk factors, tumour biology, and genetic factors were compared between African-American and Sub-Saharan African women (Fregene & Newman, 2005). Many similarities were observed suggesting a common genetic component, or similar life-styles. The gap between the breast cancer mortality rates in African and Caucasian women emerged in the late 1970's and has continued to widen (Menashe *et al.,* 2009). African women are more than twice as likely as Caucasian women to develop TNBC (Lund *et al.,* 2010). African females also tend to be affected by invasive breast cancer at a younger age; before turning 40, while in contrast, the incidence in Caucasian females increases above that of African females over the age of 40 (Anderson *et al.,* 2008; Clarke *et al.,* 2012). This is likely due to the fact that African females are often afflicted by TNBC which is known to develop in younger premenopausal

women, while Caucasian females develop hormone-sensitive breast cancers which tend to be less aggressive and show a better prognosis (Anders & Carey, 2009; Hudis & Gianni, 2011). African women are more likely to develop aggressive breast cancer pathologies, such as inflammatory, medullary, and papillary carcinomas, and are less likely to have lobular or tubular carcinomas than Caucasian women (Joslyn & West, 2000). Also, African women often present with a higher stage breast cancer which may be attributable to factors including diet, higher BMI, younger age at first birth, less breast feeding, and increased parity, these factors, however, are generally related to hormone sensitive subtypes (Ma *et al.,* 2006; Morris *et al.,* 2007; Porter *et al.,* 2004). It appears that the differences in response and access to innovations in breast cancer screening and treatment may contribute to the racial difference in TNBC survival (Menashe *et al.,* 2009). Parity may play a role in early onset breast cancer; African women are statistically shown to have higher parity at all ages than Caucasian women. Nulliparity and low numbers of full term pregnancies increase the risk of breast cancer in older women; however, after birth there is a period of increased risk. This might contribute to an explanation as to why African females develop invasive breast cancer (TNBC) at an early age (Joslyn et al., 2005).

While the tumours of both African and Caucasian females were found to have similar biology and histological type, tumours in African women were however found to be of higher grade and more commonly hormone-receptor negative (TNBC) (Anders & Carey, 2009; Parkin *et al.,* 2008; Soomro *et al.,* 2011). Elledge *et al.* (1994) found that African women were likely to be younger and have larger tumours, have more lymph node involvement, and have a higher S-phase fraction than Caucasian women. Further to this, it was subsequently reported that African patients had significantly higher Ki-67 proliferation indices, nuclear grade, and P53 expression. There were however no significant differences in p21, bcl-2, and c-erb-B2 between African and Caucasian women. This data indicates that growth signals may differ in aggressive biologic phenotypes of breast cancer (Morris *et al.*, 2007). Since higher expression of P53 was found in Nigerian TNBC patients than in their British counterparts, this suggested that targeting P53 pathways may be a novel treatment option for these women. Overexpressed P53 was also associated with altered levels of the cell adhesion proteins, P-cadherin and E-cadherin, which may explain the higher probability of metastasis in African females (Agboola *et al.*, 2014). In summary, the features of tumours in African women often show over-expression of cell cycle

regulators, cyclin E, P16, and P53, and have a lower expression of cyclin D1. They also have alterations in P53 and c-met (a stem cell factor/ hepatocyte growth factor receptor), and polymorphisms in the UGT1A1 gene (involved in oestradiol metabolism). African tumours also show higher rates of hypermethylation in younger women, (Guillemette *et al.,* 2000; Gukas *et al.,* 2008; Jones *et al.,* 2004; Mehrotra *et al.,* 2004; Nielsen *et al.,* 1997).

TNBC risk was not seen to be related to the same risk factors as mentioned above for all breast cancers including reproductive factors and socioeconomic factors, however, family history showed a greater correlation to TNBC risk than it did for hormone sensitive subtypes (Clarke *et al.,* 2012; Yang *et al.,* 2011b). It is postulated that high prevalence of abdominal obesity among African women may contribute to the strong risk of TNBC. Millikan *et al.* (2008) found that a greater hip-to-waist ratio was associated with an increased risk in TNBC for both pre- and postmenopausal women. They speculated that 68% of cases were preventable by reducing hip-to-waist ratio and promoting breast feeding in African women. Another study found that patients presenting with TNBC were more likely to be obese at diagnosis, if premenopausal, and that they were less likely to have breast fed for longer periods (Kwan *et al.,* 2009). There is evidence to suggest that type 2 diabetes and the metabolic syndrome, both of which have a higher incidence in African women, are associated with an increased risk of breast cancer development and may contribute to the aggressiveness of the particular subtypes seen in these women (Maiti *et al.,* 2010; Rose *et al.,* 2007).

To date there are few clues to identification of genetic or non-genetic factors that may predict the early onset and aggressive nature of TNBC in African women. Evaluation of risk factors, including life style, reproductive history, family history, and socioeconomic status may be helpful in establishing a more defined risk population and education programs could be used to encourage screening. It seems likely that racial differences will be explained by differences in the molecular and genetic changes which cause tumour development and drive clinical behavior in these populations. A better understanding of molecular and genetic alterations may elucidate potential novel prognostic markers for TNBC and could lead to development of targeted therapies. A novel approach to identifying diagnostic miRNAs has been performed in the current study. The link between miRNAs and cancer is discussed below.

1.3 MicroRNAs and Cancer

MicroRNAs (miRNAs) are a class of short single-stranded, non-coding regulatory RNAs, 18-25 nucleotides in length previously thought to be cellular "junk" without any cellular function (Lin & Hannon, 2004). miRNAs are conserved among species and are largely found in the introns of pre-mRNA molecules which are regulated by those miRNAs transcribed with them. This allows for convenient co-expression of a miRNA and a protein (Bartel, 2004). Some miRNAs are found in clusters within the genome which often appear to be related to each other. For example, the Hox cluster miRNAs seem to be involved in development, and the mir5a-mir16 cluster seems to play a role in tumour suppression (Calin *et al.,* 2002). Normal cellular functions of miRNAs include processes such as growth, differentiation, proliferation, metabolism, and apoptosis, generally by negatively regulating gene expression at a post-transcriptional level through the RNA interference (RNAi) pathway (Ambros, 2004; Lowery *et al.,* 2009; Rodriguez *et al.,* 2004). Mature miRNAs are synthesized through a multi-step process involving transcription from specific regions in the genome to produce a primary transcript (pri-miRNA). The pri-miRNA transcript is further processed downstream by enzymes producing the precursor miRNA (pre-miRNA) which is then transported to the cytoplasm for cleavage by Dicer to yield miRNA duplexes; generally only one of the duplex strands acts as the mature miRNA (Nishida *et al.,* 2013; Winter *et al.,* 2009). The mature miRNA is incorporated into a complex of ribonucleotide proteins (RNPs) forming the miRNP, or the miRNA-induced silencing complex (RISC). The Argonaute (AGO) proteins are the primary functional proteins in the RISC, and are responsible for post-transcriptional gene silencing (Figure 4). There are four AGO proteins in mammals (AGO1-AGO4) of which, only AGO2 is able to cleave target sequences as a result of its RNaseH-like domain (Hussain, 2012). The mature miRNA recognises its target mRNA by binding to its 3' untranslated region (UTR) with varying degrees of complementarity (Olena & Patton, 2014). Different degrees of complementarity results in several mechanisms for repression; the majority of animal miRNA targets are regulated by repression of protein translation, either at the initiation stage, or during the elongation stage. There is recent evidence to support the destabilisation of mRNAs in specific cases (Huntzinger & Izaurralde, 2011).

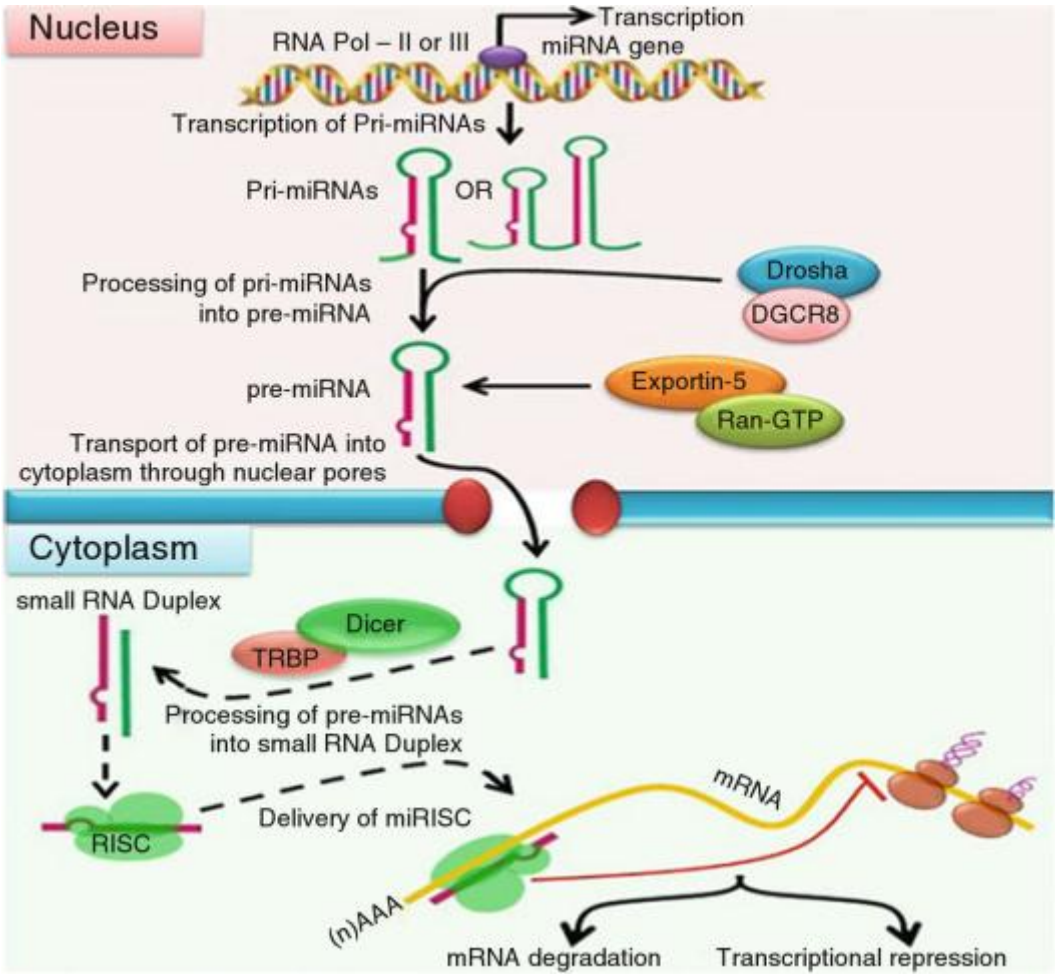


Figure 4: miRNA biogenesis pathway. Pri-miRNAs are processed in the nucleus by Drosha to form pre-miRNAs. Pre-miRNAs are then transported out of the nucleus and are further processed by Dicer and RISC in the cytoplasm to inhibit transcription of complementary mRNAs. Adapted from Shafi *et al.* (2014).

MiRNAs can act as tumour suppressors and oncogenes, and thus aberrant expression of miRNAs in cancers, relative to normal tissues, is thought to be important in tumour progression. Identification of the target genes of the aberrant miRNAs might elucidate their accurate role in cancer biology (Bandres *et al.*, 2007). Recent studies have implicated miRNAs in the regulation of cellular stress responses which are often dysregulated in various cancers (Leung & Sharp, 2010). Shen *et al.* (2013) have also suggested that modulation of miRNA biogenesis is important for stress response in tumour cells and may have clinical implications. miRNA expression is altered in various types of cancer, including breast cancer, where they function as regulators of tumour behavior and progression (Lowery *et al.*, 2009; Sempere *et al.*, 2007). Expression of a

single miRNA potentially impacts the expression of a few to hundreds of proteins with a variety of cellular functions. Conversely, a single mRNA (and its translated protein) might be under stringent, but redundant, control of numerous microRNAs. Mechanistically addressing how cells control microRNA expression requires particular knowledge of their transcription and processing (Karginov *et al.,* 2007; O'Hara *et al.,* 2009).

1.3.1. A Novel Approach to Treatment and the Potential Diagnostic Value of miRNAs in TNBC

Biologically relevant miRNAs associated with specific breast cancer phenotypes have been discovered related to hormone receptor status. This indicates a role for these miRNAs in disease classification of breast cancer, and may be useful in subclassifying TNBC. Distribution of miRNAs varies within tumours and thus, even within TNBC tumours, miRNA expression differs according to the region of the tumour being tested (Li *et al.,* 2013; Sempere *et al.,* 2007). It would thus seem to be important to standardise the region of tumours being profiled in order to correctly classify and subclassify TNBCs. Apart from breast cancer, several studies have related dysregulation of particular miRNAs to the development of all different types of cancer (Li *et al.,* 2012; Volinia *et al.,* 2012). The strong connection between miRNAs and cancer development and progression has led to the introduction of small interfering RNAs (siRNAs) being used as a novel approach to treatment (Ashihara *et al.,* 2009). The siRNAs are transfected into affected cells where they bind to complementary mRNA transcripts and inhibit the expression of target proteins either directly or indirectly. The knockout or knockdown of those target proteins opposes tumour progression usually by targeting aberrant cell processes, including amongst others, the cell cycle, apoptosis, angiogenesis, cell proliferation and metastasis. Often miRNAs are downregulated in many tumours, in which case miRNA protein target expression can be induced transiently to increase the levels of those proteins and assist in reversing the abnormal cell behaviours.

The focus of this study was to determine significant miRNA profiles associated with the TNBC type as well as to identify differences between TNBC at a miRNA expression level. This

approach is aimed at the discovery of novel diagnostic markers, and development of miRNA-targeted therapies. There is also an abundant need for TNBC to be more accurately sub-classified in order to determine the optimal targets for individual treatment. Determining miRNA expression in different TNBC cell lines may provide the first step in producing novel subtyping of TNBC which might elucidate mechanisms of tumourigenesis and clinical behavior more specifically. Once a miRNA signature is determined for the TNBC phenotype, it will be possible to determine the functions and effects of the aberrantly expressed miRNAs. Thereafter it will be possible to contemplate future advancements in diagnostics and novel targeted therapy which will aim to reduce the number of TNBC-related deaths, and prolong survival.

Jun *et al.* (2005) discovered that miRNAs partitioned tumours with a single lineage demonstrating that even within a single developmental lineage, distinct patterns of miRNA expression can be observed that reflect the mechanisms of transformation, and further support the idea that miRNA expression patterns encode the developmental history of human cancers. This information has been used to better diagnose various cancer types (Dettmer *et al.,* 2013; Giovannetti *et al.,* 2012; Guo & Friedman, 2013; Solomides *et al.,* 2012). We expect to see a similar finding in TNBC, and suggest that miRNA expression profiles be used to further classify TNBC subtypes. This could elucidate the specific signaling pathways involved in this aggressive subtype of breast cancer and potentially lead to a more accurate diagnosis and thus, a more personalised targeted treatment plan and better overall patient response to therapy and survival. miRNA profiling techniques are discussed below.

1.3.2. MicroRNA Profiling

There are three major approaches currently well-established for profiling miRNA: quantitative reverse transcription PCR (qRT-PCR), hybridization-based methods (for example, microarrays) and high-throughput sequencing (that is, RNA-seq) (Pritchard *et al.,* 2012).

1.3.2.1. Approaches for profiling miRNAs

qRT-PCR relies on reverse transcription of miRNA to cDNA, followed by qPCR with real-time monitoring of reaction product accumulation (Schmittgen *et al.,* 2000). In TaqMan qRT-PCR,

the reverse transcription reactions use stem–loop primers that are specific to the 3′ end of the miRNA for specificity. Amplicons are generated using a miRNA-specific forward primer and a reverse primer. As the DNA polymerase proceeds along the template, the TaqMan probe is hydrolysed and fluorescent dye is freed from the quencher, resulting in light emission (Refer to Life Technologies Taq-man miRNA assays Handbook, 2011). In SYBR-green-based qRT-PCR, miRNA is typically polyadenylated at the 3′ end, and oligo-d(T) is used as a reverse transcription primer. A miRNA-specific forward primer and a reverse primer that anneals to the 3′ portion of the miRNA sequence as well as to the poly(A) tail enable PCR amplification with dsDNA-intercalating SYBR green dye as the detector (Refer to the Qiagen QuantiTect™ SYBR® Green PCR Handbook, 2003) (Figure 5).

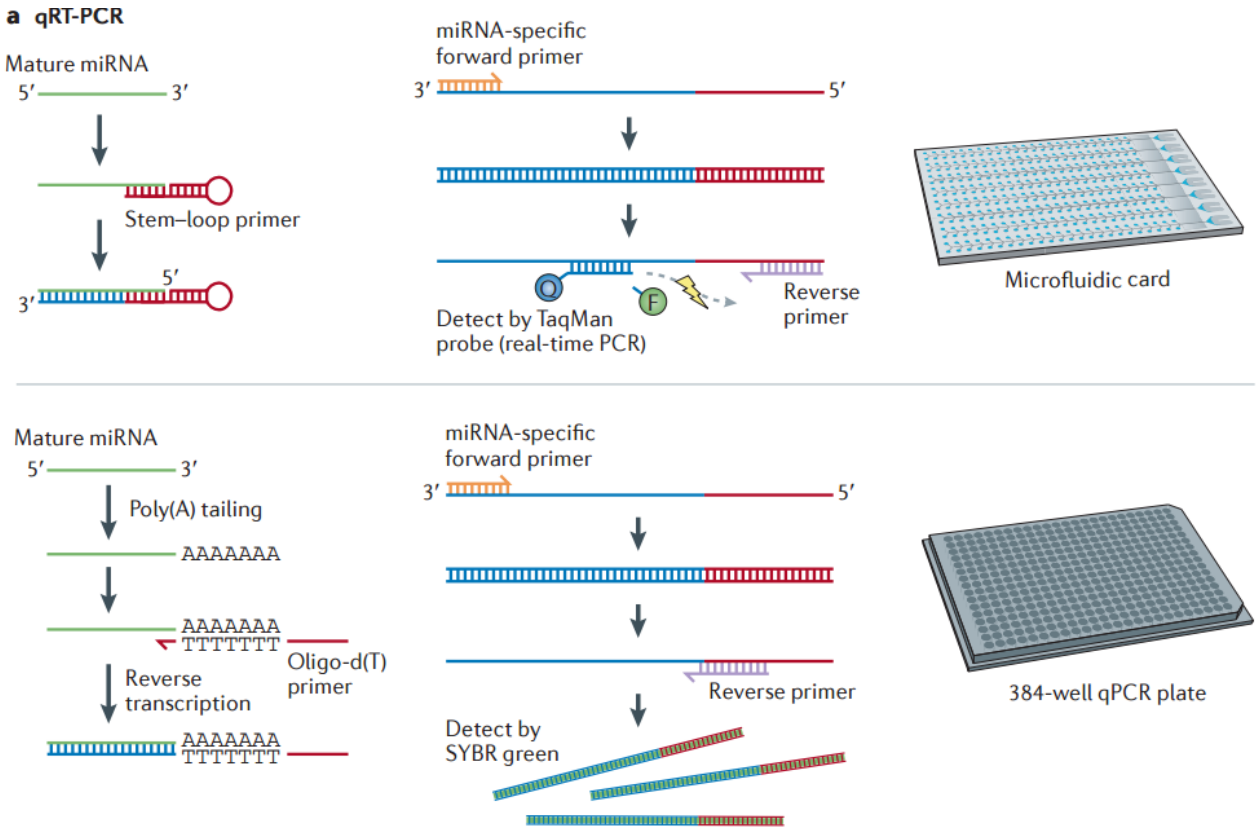


Figure 5: A Representation of qRT-PCR workflow. Adapted from Pritchard *et al* (2012).

Microarrays were developed for the parallel analysis of large numbers of miRNAs. Several variations of microarrays exist with different approaches for fluorescent labelling. DNA-based capture probes (which may or may not incorporate LNA-modified bases) are used to capture

fluorescently tagged miRNAs; this is followed by scanning of slides and quantification of fluorescence (Pritchard *et al.*, 2012; Yin *et al.*, 2008) (Figure 6).

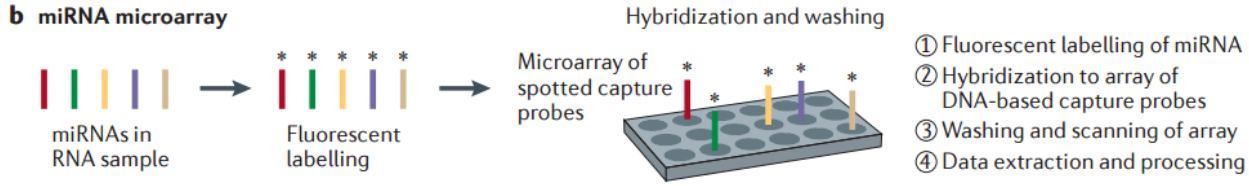


Figure 6: A Representation of miRNA Microarray Analysis Workflow. Adapted from Pritchard *et al.* (2012).

The general approach for RNA-seq begins with reverse transcription of miRNA to a cDNA library, followed by the 'massively parallel' sequencing of millions of individual cDNA molecules from the library. Adaptor ligation then allows the library either to be affixed to a solid phase, as in the Illumina platform, or to beads for emulsion PCR, as in the Roche and ABI platforms (Wang *et al.*, 2009b) (Figure 7). Bioinformatic analysis of the sequence reads identifies both known and novel miRNAs in the data sets and provides relative quantification using a digital approach (that is, the number of sequence reads for a given miRNA relative to the total reads in the sample is an estimate of relative abundance of the miRNA) (Pritchard *et al.*, 2012).

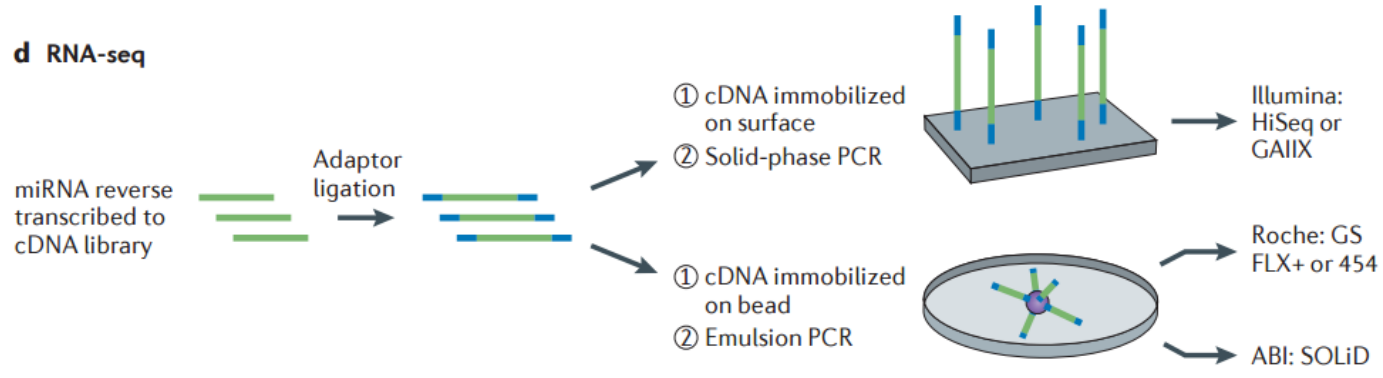


Figure 7: A Representation of RNA-seq Workflow. Adapted from Pritchard *et al.* (2012).

1.3.2.2. Nanostring nCounter Analysis

In the present study, Nanostring nCounter analysis was the selected method for miRNA profiling due to its capability of highly multiplexed, direct profiling of individual molecules in a single reaction without the need for sample amplification. This is a hybridization technique in which a multiplexed probe library is created using two sequence-specific capture probes that are tailored to each miRNA of interest (Brumbaugh *et al.*, 2011). A bridge oligonucleotide guides ligation of a miRNA to a specific barcode. Each color-coded barcode represents a single target molecule. Barcodes hybridize directly to specific target molecules and can be individually counted without the need for subsequent amplification, thus providing very sensitive digital data (Figure 8). Capture and detection is done by two target-specific probes: a 3′ capture probe containing biotin to allow adsorbance to the solid phase via streptavidin and a second 5′ reporter probe with an individual colour-coded sequence (Pritchard *et al.*, 2012) (Figure 9). No pre-amplification or labelling of miRNA is required with this method. A particularly important advantage of this method is the ability to discriminate between similar variants with high accuracy. Current limitations include limited dissemination of the instrument and the need for increased open-source software tools for data analysis.

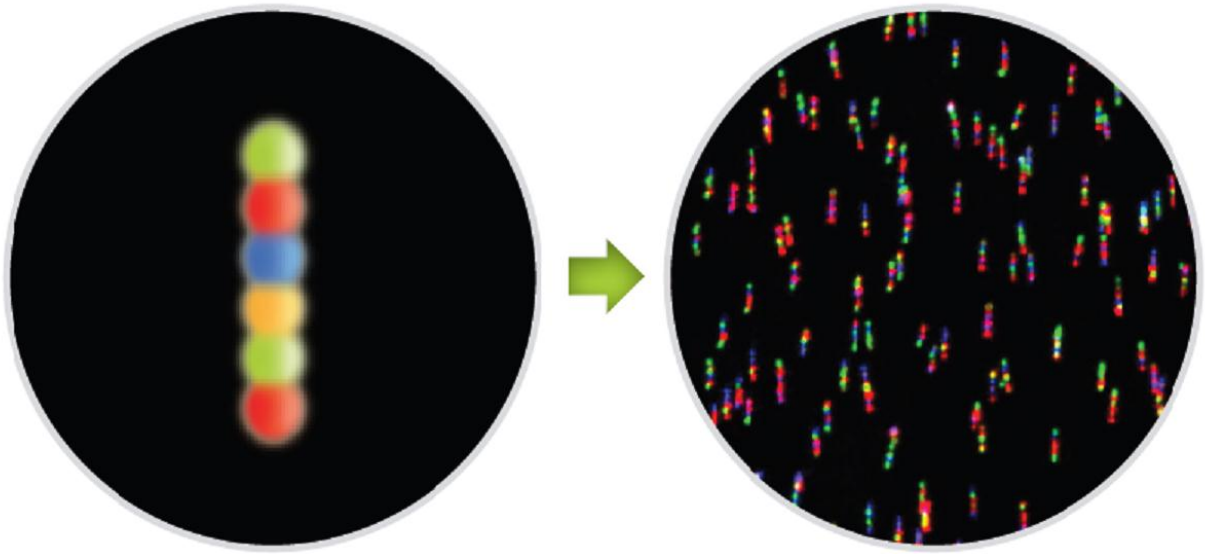
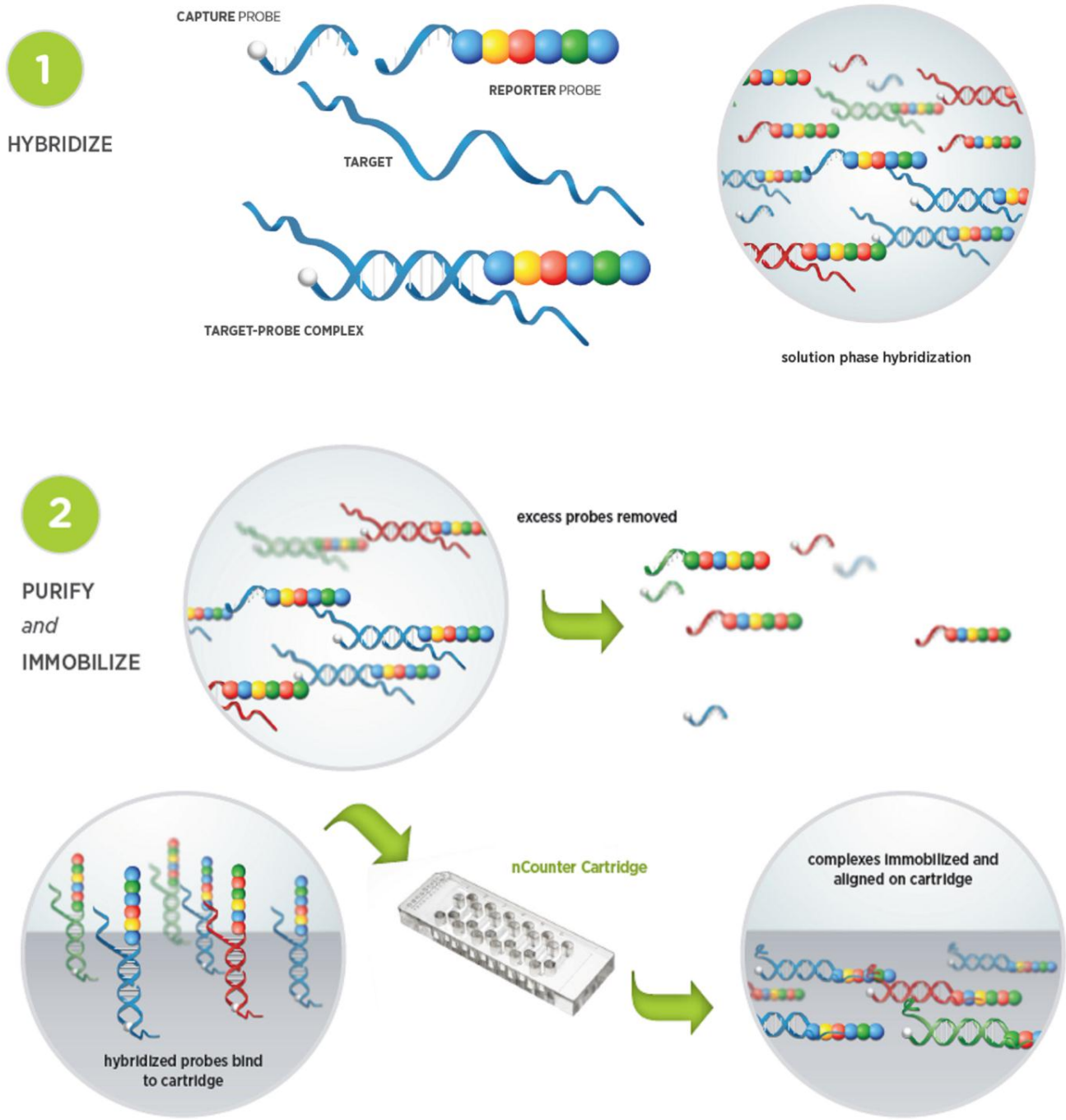


Figure 8: Single molecule barcodes each attach to an individual nucleic acid. Refer to NanoString® Technologies; nCounter® Analysis System information brochure (www.nanostring.com, 2011).


1
HYBRIDIZE
CAPTURE PROBE
REPORTER PROBE
TARGET
TARGET-PROBE COMPLEX
solution phase hybridization
2
PURIFY
and
IMMOBILIZE
excess probes removed
nCounter Cartridge
hybridized probes bind
to cartridge
complexes immobilized and
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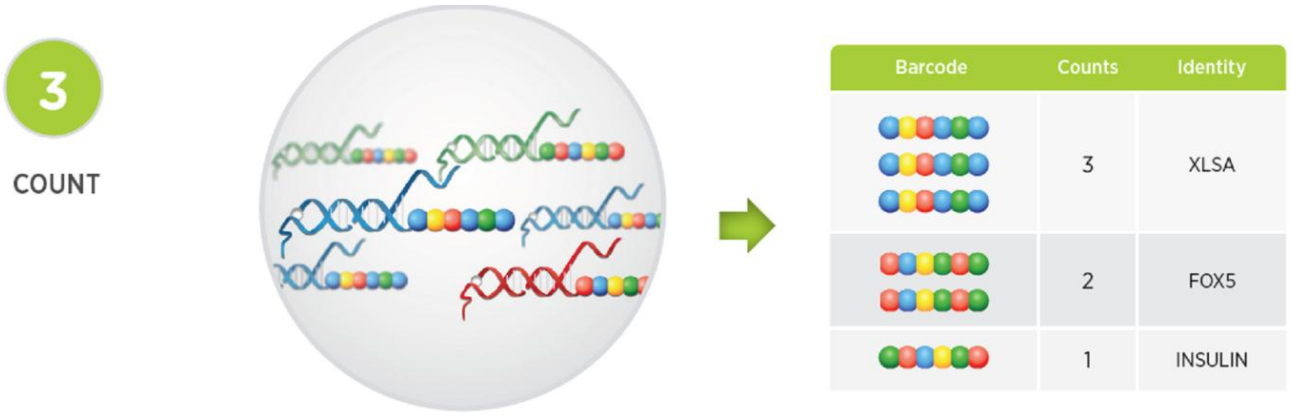


Figure 9: Molecular representation of the Nanostring nCounter analysis system. (1) Hybridisation: barcoded probes hybridize directly to a target molecule in solution. The Reporter Probe carries the signal and the Capture Probe allows the complex to be immobilized for data collection. (2) Purification and immobilisation: After hybridization, samples are transferred to the Prep Station where excess probes are removed and probe/target complexes are bound, immobilized and aligned on the nCounter Cartridge. (3) Count: Sample cartridges are placed in the Digital Analyzer for data collection. Barcodes are counted and tabulated for each target molecule. Refer to NanoString® Technologies; nCounter® Analysis System information brochure (www.nanostring.com, 2011).

In addition to the established miRNA-profiling approaches that are commercially available, there are further emerging methods. These include: splinted ligation, rolling circle amplification, integrated fluidic circuits and detection methods using nanoparticles, antibodies or molecular beacons (Pritchard *et al.*, 2012). A summary of miRNA profiling techniques, along with the advantages and disadvantages of each technique can be seen below (Table 9).

Table 9: A summary of current miRNA profiling techniques. Adapted from Pritchard *et al.* (2012).

Profiling Technique	Advantages	Disadvantages	Examples
Quantitative reverse transcription PCR (qRT-PCR)	Established method, sensitive and specific. Can be used for absolute quantification	Cannot identify novel microRNAs Only medium-throughput with respect to the number of samples processed per day	TaqMan individual assays, miRCURY LNA qPCR, TaqMan OpenArray, TaqMan TLDA microfluidics card, Biomark HD system, SmartChip human microRNA, miScript miRNA PCR array
MicroRNA microarray	Established method. Fairly low-cost and high-throughput with respect to the number of samples that can be processed per day	Typically lower specificity than qRT-PCR or RNA sequencing Difficult to use for absolute quantification Cannot typically identify novel miRNAs	Geniom Biochip miRNA, GeneChip miRNA array, GenoExplorer, MicroRNA microarray, miRCURY LNA microRNA array, NCode miRNA array, **nCounter (not a microarray but hybridization-based),** OneArray, Sentrix array matrix and BeadChips, μParaFlo biochip array
RNA sequencing: high-throughput next-generation sequencing platforms	High accuracy in distinguishing miRNAs that are very similar in sequence, as well as isomiRs. Can detect novel miRNAs	Substantial computational support needed for data analysis Cannot be used for absolute quantification	HiSeq 2000 (or Genome Analyzer IIX), SOLiD, GS FLX+ (454 sequencing)

RNA sequencing: smaller-scale next-generation sequencing platforms	**High accuracy in distinguishing** **miRNAs that are very similar in sequence, as well as isomiRs.** **Can detect novel miRNAs**	**Substantial computational support needed for data analysis** **Cannot be used for absolute quantification**	**Ion Torrent, MiSeq, GS Junior (454)**
RNA sequencing: single-molecule sequencing technologies	Amplification not required. Potential to determine absolute quantification	Expensive, not widely accessible, single-molecule real-time approach not yet demonstrated for miRNAs	tSMS, SMRT

1.4 Aims

Two TNBC cell lines were used in this study; MDA-2B-231 and MDA-2B-436. Both of these cell lines were derived from Caucasian females who were premenopausal. Each of these cell lines were of invasive ducal carcinoma (IDC) histology, and fell under the basal B subtype. The MDA-2B-436's are associated with mutations in *BRCA1* and *TP53*, while the MDA-2B-231's are associated with mutations in *BRAF*, *CDKN2A*, *KRAS*, *NF2*, *TP53*, and *PDGFRA* (Lehmann *et al.*, 2011). According to Lehmann *et al's* (2011) classification of TNBC cell lines, both the MDA-2B-231's and the MDA-2B-436's were classified as the MSL subtype which showed a unique enrichment of genes linked to growth factor signalling pathways, angiogenesis, stem cells, epithelial-mesenchymal transition (EMT), and cell differentiation. This subtype showed an interesting scarcity of proliferation genes however, and displayed a lack of claudins 3, 4, and 7, consistent with the claudin-low 'subtype' of TNBC. An oestrogen-sensitive breast cancer cell line; MCF-7 was used as a control to identify differences between the hormone-sensitive and hormone-insensitive breast cancers. The other control was a normal epithelial breast cell line; MCF-10A. Information on the four cell lines is available in Appendix B.

Due to the combination of a poor prognosis, young age of affected women, and lack of treatment and diagnostic options, it is becoming increasingly important to research alternative diagnostic and therapeutic strategies to prolong survival of affected women. This work aims to assess a novel approach to profile microRNA expression in TNBC cells and to determine how these microRNAs are implicated in disease. By using Nanostring nCounter analysis, this study aims to profile miRNA expression in two TNBC cell lines as well as one oestrogen sensitive breast cancer cell line, and one normal breast cell line. The miRNA expression results, when compared, should elucidate differences in miRNA expression both between TNBC cell lines and the two control cell lines, and between the two TNBC cell lines themselves. miRNAs with significant differences in expression are to be assessed and may lead to an understanding of the basis of TNBC as well as subclassification and potential novel treatment options.

2. Materials and Methods

2.1. Cell Culture:

2.1.1. Cell lines used: All cell lines were originally sourced from ATCC and were human-derived. Dr. R Duarte kindly donated all cell lines; (a) hormone sensitive breast cancer cells (MCF-7), (b) TNBC cell lines (MDA-MB-231 and MDA-MB-436), and (c) 'normal' breast cells (MCF-10A) served as a negative control (Table 10). See appendix B for data sheets.

2.1.2. Establishment of cell cultures: cell lines were thawed rapidly and added to 5 mL of the appropriate cell culture medium (Recipe, Appendix C) and centrifuged at a low rpm (about 800 rpm) for 3 minutes in order to remove the cryogenic medium, and the supernatant was discarded. The pellet was resuspended in appropriate fresh serum-supplemented cell culture medium, mixed by pipetting up and down, and transferred to a sterile filter-cap culturing flask. Cultures were then incubated overnight at 37˚C (5% CO_2). The cultures were checked for confluence under a light microscope 24-48 hours later. If cultures were not yet confluent, the old cell culture medium was pipetted out of the culture flasks and discarded, and subsequently replaced with 5

mL of fresh medium. When the cultures were confluent (75-100% depending on cell line), they were either subcultured or cryopreserved. Once each cell line reached confluence, after about three days, it was subcultured.

2.1.3. Subculture of cell lines: cell culture medium was removed from the culture flasks and discarded. The cells were then washed with 3 mL 1 X PBS and agitated for 30 seconds. The purpose of the PBS wash was to remove and dilute as much of the remaining cell culture medium as possible, since FBS deactivates trypsin. The PBS was pipetted out of the flask and discarded. Next, 3 mL of trypsin was added. Trypsin is an enzyme used to digest the cellular matrix which adheres the cells to the flask, resulting in cellular resuspension. The flask was returned to the incubator at 37˚C (5% CO_2) for a further 3 minutes to allow for trypsinisation to occur. Thereafter, the flask was removed and tapped vigorously on either side to facilitate release of the cells from the flask. The flask was then checked under a light microscope to ensure release of cells. Once the cells were in suspension, 2 mL of the appropriate cell culture medium was added and mixed by gentle shaking, in order to inactivate the trypsin (due to the FBS in cell culture medium). The culture mixture was then pipetted from the flask into a 15 mL centrifuge tube, and centrifuged for 3 minutes at a low rpm (800 rpm), to pellet the cells. The supernatant was discarded and the cells were resuspended in 10 mL of appropriate cell culture medium and distributed evenly into two separate flasks. The flasks were checked under an inverted light microscope to ensure cells were transferred correctly, and were placed into an incubator at 37˚C (5% CO_2).

2.1.4. Cryopreservation: in order to freeze cells, the procedure for subculturing was followed as described above to pellet the cells. The supernatant was discarded and the cells were resuspended in 1 mL of cryogenic medium (Recipe, Appendix C), and transferred to a 2.5 mL cryotube. The vial was then placed inside a polystyrene container inside a -70˚C freezer overnight. The vials were then transferred to a storage box and kept in a -70˚C freezer for future use.

Table 10: A representation of the details regarding the various cell lines used (Adapted from the ATCC cell line information sheets, Appendix B**).**

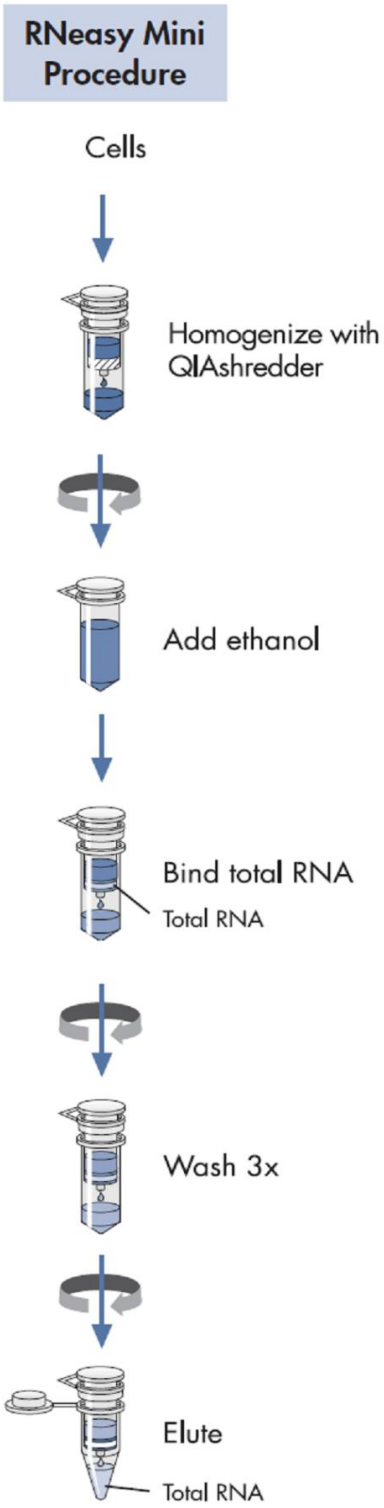
	MCF-10A	MCF-7	MDA-MB-231	MDA-MD-436
Ethnicity	Caucasian			
Morphology	Basal epithelial	Luminal epithelial	Basal epithelial	Basal
Age	36	69	51	43
Hormone sensitivity	Normal	Oestrogen sensitive	TNBC	TNBC
Tissue	mammary gland; breast; fibrocystic disease	mammary gland; breast adenocarcinoma; derived from metastatic site: pleural effusion		

2.2. Cell counting

In order to establish how many cells were present and viable, a BIO-RAD TC10 (BIO-RAD Laboratories, South Africa) automated cell counter system was used, along with BIO-RAD dual chamber counting slides (cat #: 145-0011). Cells were first trypsinised and subjected to three subsequent PBS washes of 5 mL, 3 mL, and 1 mL respectively. They were then resuspended in 1mL PBS and 10 µL of suspended cells was added to 10 µL of Trypan Blue stain (BioWhittaker, cat #: 17-942E) and pipetted up and down several times to mix. 20 µL of the mixture was then pipetted into a chamber of the counting slide and inserted into the cell counter to be counted. Trypan Blue is used to distinguish viable from non-viable cells. Non-viable cells absorb the dye and appear blue, while viable cells remain colourless. The BIO-RAD TC10 automated cell counter is able to detect the both Trypan Blue stained cells and unstained cells to give a reading on viability.

2.3. Total RNA Extraction

This technique combines the selective binding properties of a silica-based membrane with the speed of microspin technology. Biological samples are lysed and homogenised in the presence of a highly-denaturing guanidine-thiocyanate-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then added to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High quality RNA is then eluted in RNase-free water. Two to three flasks of each cell line were combined and used for a single RNA extraction procedure. The RNeasy® Mini Kit (50) from Qiagen (cat. #: 74104), along with QIAshredder™ (cat. #: 79654) was used for total RNA extraction. RNA extraction was carried out as per the manufacturer's instructions after cultured cells had been trypsinised as described above (Figure 10). Subsequent to trypsinisation, each sample underwent three 5 mL PBS washes. The cells were then resuspended in 1 mL PBS and added to a QIAshredder tube for thorough disruption of the cells prior to RNA extraction. The QIAshredder homogeniser consists of a unique biopolymer-shredding system in a microcentrifuge spin-column format. Cell or tissue lysate is loaded onto the QIAshredder homogeniser placed in a collection tube and centrifuged. The homogenised lysate is then collected.



<u>Figure 10:</u> Summary of total RNA extraction procedure as per RNeasy® Mini Kit handbook (QIAGEN, fourth Ed. 2006).

2.4. Spectrophotometry

In order to check the concentration and quality of RNA samples a Nanodrop 1000 ND spectrophotometer was used along with the corresponding computer software. 2 µL of isolated total RNA was placed on the Nanodrop pedestal for analysis. Concentrations above 1000 ng/µL were favored as 100 ng of total extracted RNA was required in a minimum volume of 40 µL for subsequent Nanostring nCounter analysis (Nanostring Technologies Product Data Sheet, 2012). This high concentration of RNA would increase the probability of the isolated RNA being of good quality (fewer degraded strands). In order to assess the purity of the extracted RNA the 260/280 ratio was considered. A 260/280 ratio greater than 2.0 is generally accepted as 'pure' for RNA. A secondary measure of purity is the 260/230 ratio which is usually higher than the 260/280 ratio and is acceptable between 1.8 and 2.2.

2.5. Agarose Gel Electrophoresis

To check the integrity of the isolated RNA a 2% TBE gel (Recipe, Appendix C) was prepared and stained with 9 µL ethidium bromide (Promega Corp. cat#: H5041) per 50 mL gel. 5 µL of each total RNA sample was added to 4µL of loading buffer (New England Biolabs, cat #: B0362S) and incubated at 70° C for 10 minutes in a heating block. The gel was loaded with 9 µL of each sample as per table 11, and electrophoresed at 50 V for about an hour in 1 X TBE Buffer (Recipe, Appendix C). The gels were imaged using a BIO-RAD Gel Doc EZ Imager (BIO-RAD Laboratories, South Africa) under UV transmitted light, along with Image Lab 5.0 (BIO-RAD, 2013) imaging software.

2.6. RNA Sample Preservation and Transportation

Two methods of RNA sample preservation were employed for transportation to the USA for Nanostring nCounter analysis.

2.6.1. Dry Ice Frozen Samples

Two duplicate samples of total extracted RNA from each of the four cell lines were stored in sterile 1 mL microfuge tubes in a polystyrene rack at -70˚C. They were transported on dry ice in a sealed standard polystyrene container by Globe Flight Couriers.

2.6.2. RNAstable Samples

Two duplicate samples of total extracted RNA from each of the four cell lines were transported in a novel 'dry' fashion. RNAstable obtained from Biomatrica (cat. #: 93221-001) is a product used for the long-term stabilisation of biological samples at room temperature, RNA in this case. This technique is based on the principle of anhydrobiosis, wherein the preservation solution forms a glass-like shell over the RNA, essentially "shrink-wrapping" it and thus allowing for long periods free of degradation. As sample preservation is achieved without degradation, this enables laboratories to decrease their reliance on freezers and reduce shipping costs. Complete sample recovery is easy and achieved simply by the addition of sterile water. Rehydrated samples are ready for immediate use without the need for further purification (Refer to Biomatrica RNAstable Handbook, 2012). Isolated total RNA samples were dried and stabilized in duplicate using an RNAstable kit (Biomatrica; cat #: 93221-001), and stored in a foil bag with a desiccator, for purposes of transportation to Seattle, USA, for miRNA profiling.

The provision of both dried and frozen RNA samples allowed for the comparison of the two storage techniques. Each cell line was sequenced in duplicate for both frozen and dried samples; these duplicates were first averaged to create two test groups being "frozen" and "dried". Next a standard two-tailed Student's T-test was performed between like-cell lines, assuming equal variances. The null hypothesis stated that there would be no difference in miRNA expression between frozen and dried samples from the same cell line. The T-tests produced *p*-values which were then used to determine whether there was a significant difference in miRNA expression between frozen and dried samples (Table 12). Once it was determined that frozen samples were not significantly different from dried samples, the average was determined for each cell line in order to simplify the data for further data analysis (See Appendix A, Table A1 and A2).

2.7. Profiling of Isolated miRNAs

A comparison between the frozen and RNAstable samples, as well as between TNBC cell lines and the two control cell lines, and between the two TNBC cell lines was performed through miRNA expression analysis. The nCounter® Analysis System from Nanostring Technologies was used according to manufacturer's instructions, and carried out at Nanostring Technologies in Seattle, USA (Figure 11).

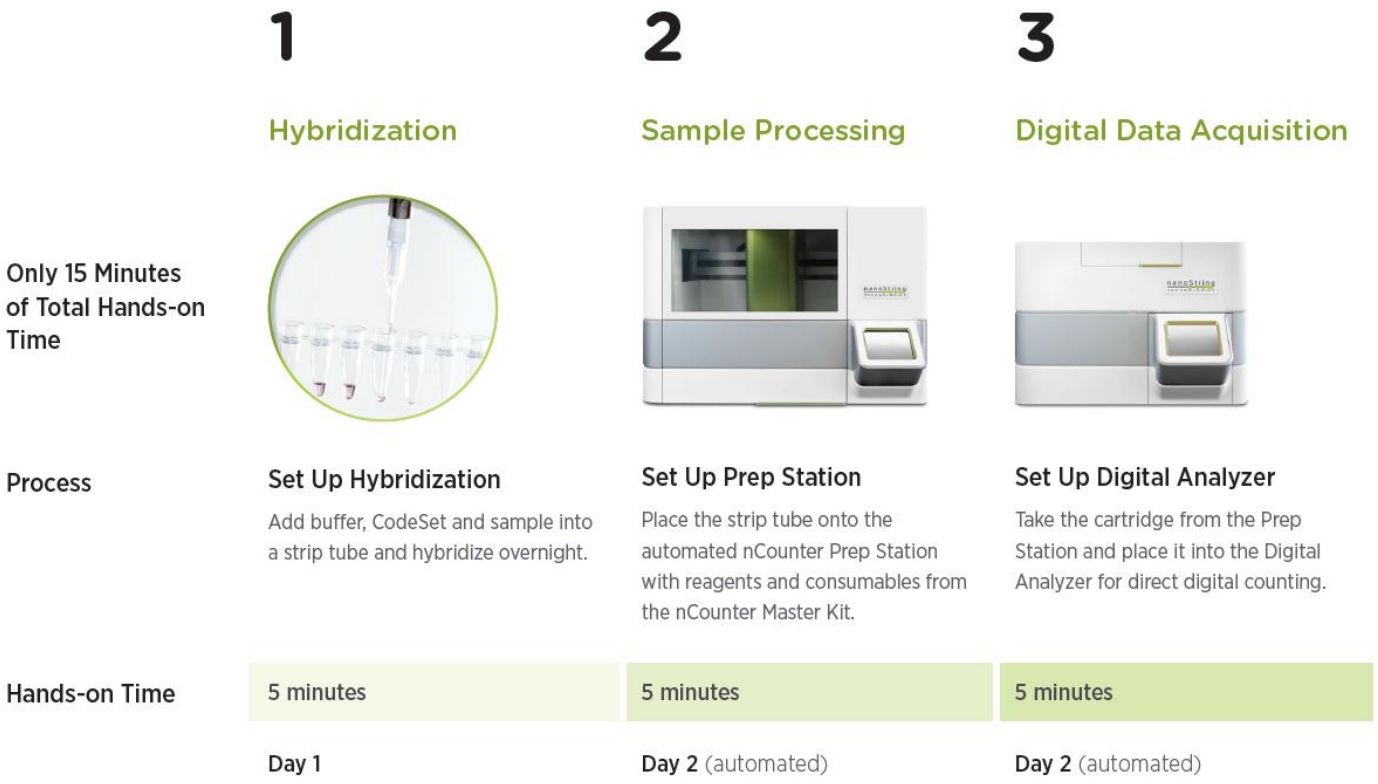


Figure 11: Summary of Nanostring nCounter analysis workflow (Nanostring nCounter analysis Handbook, 2011).

2.8. *In-silico* Prediction of miRNA Target Genes

Normalised Nanostring nCounter analysis results for each of the four cell lines was averaged to yield a single data set for each cell line. Microsoft Excel 2010 was used to determine which

miRNAs were overexpressed or underexpressed in the TNBC cell lines, relative to oestrogen-sensitive breast cancer and normal breast cells (Figure 12). Nine miRNAs were significantly overexpressed and three were significantly underexpressed in TNBC relative to the control cell lines.

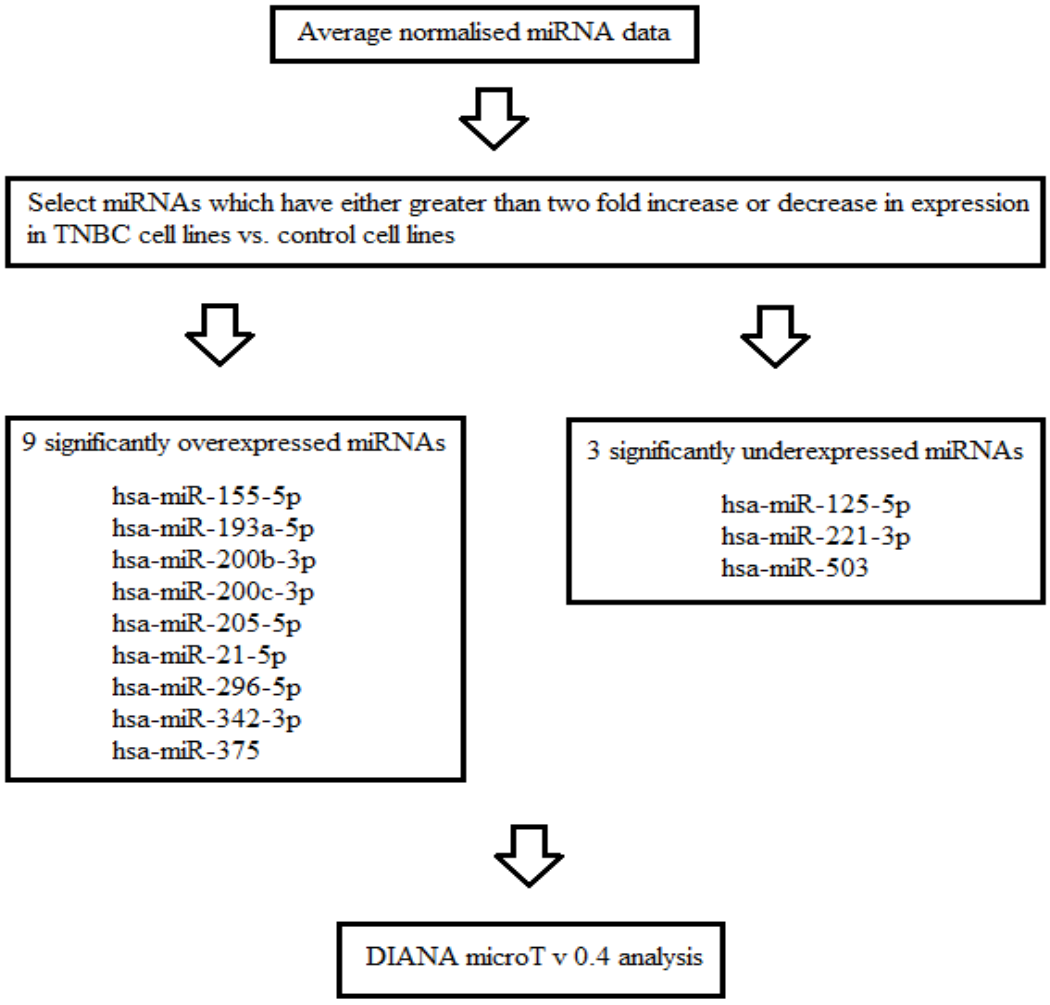


Figure 12: Summarised workflow for miRNA selection for DIANA microT software analysis target prediction.

The human targets of the miRNAs of interest were determined by DIANA-microT Web Server software version 4 (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microtv4). A search of each miRNA was performed to yield a list of probable targets (Figure 13). The threshold miT score was set at 0.7.

The first selection criteria used was the verification of the target by at least two other bioinformatics software programmes and experimental verification. The tab labelled "Also Predicted" in the DIANA-microT search results showed which targets were predicted by other target-predicting software. To further verify the accuracy of this tab, a search in the Pictar and TargetScan bioinformatics programmes was performed. The results obtained showed that the DIANA-microT results were valid and were thus used exclusively thereafter. Due to a difference in target prediction between different algorithms, three different prediction algorithms were used to decrease the possibility of false target prediction. Targets which have been experimentally confirmed also took preference. Targets which were not validated by at least two other methods were excluded.

The miTG score was the second selection criteria used to exclude targets from the list; only targets with an miTG score greater than 0.9 were considered significant targets. The miTG score shows the probability of the target prediction, the higher the score, the greater the probability of accurate gene target prediction. miTG scores of greater than 0.9 were thus favored. If there were several miTG scores above 0.9, the precision score was used as subsequent exclusion criteria. This is a supplementary measure for each miRNA:miTG interaction which ranges from 0 to 1 and corresponds to the probability that this interaction is correctly predicted. The closer the score is to 1, the more accurate the prediction. Precision scores above 0.9 were particularly favored. Targets failing to meet any of the above criteria were excluded, unless there were no targets that met the selection criteria. In those cases, targets were selected at our discretion.

The Ensembl gene ID for predicted targets was entered into the OMIM database to determine their full names and functions, and possible implications in cancer and other diseases. The number of predicted targets per miRNA is represented in the results section below (Table 16). Figure 13 below indicates the steps taken to obtain a miTG score, followed by a search for targets in the OMIM database.

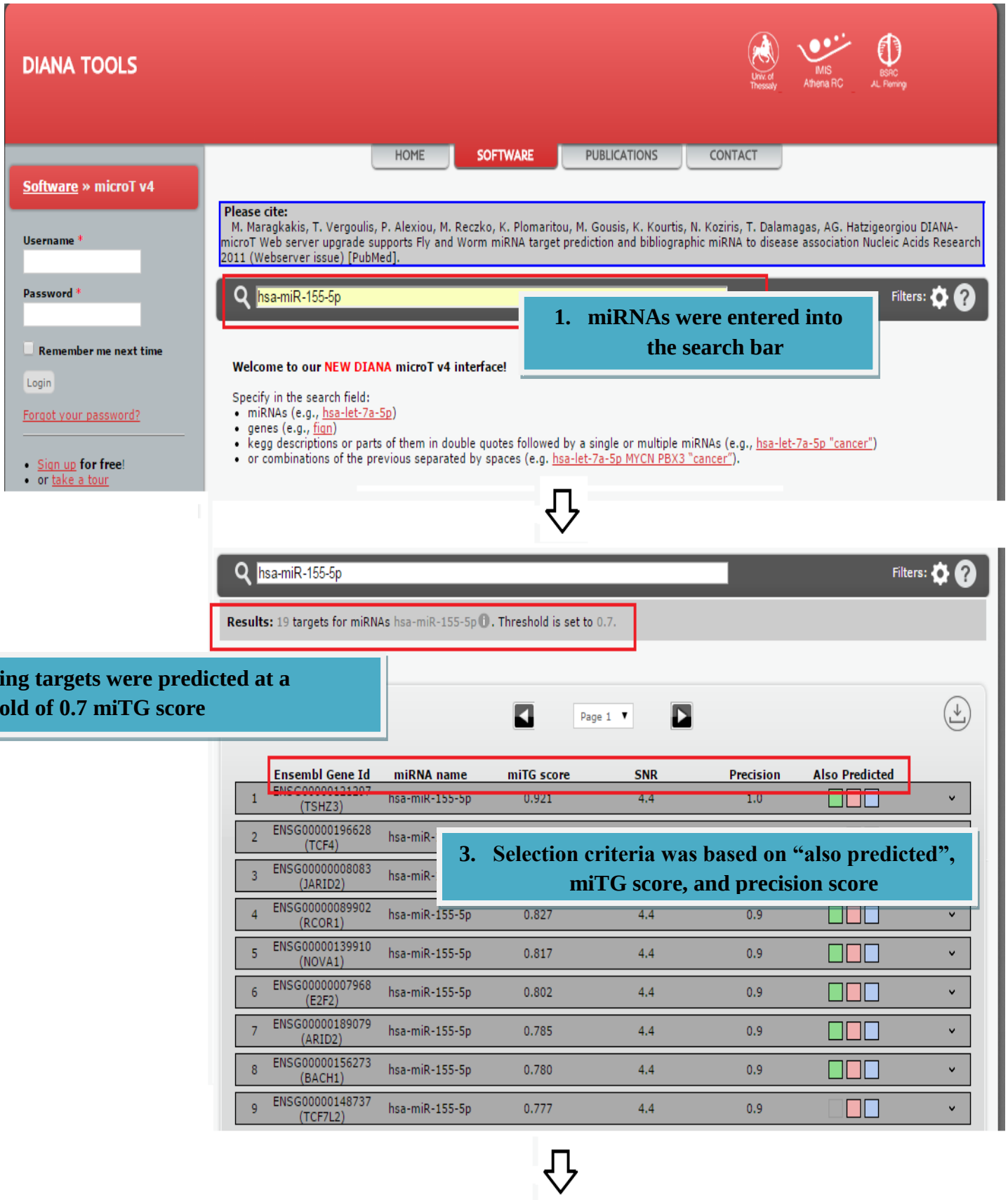

DIANA TOOLS
HOME
SOFTWARE
PUBLICATIONS
CONTACT
Software » microT v4
Username *
Password *
Remember me next time
Login
Forgot your password?
Sign up for free!
or take a tour
Please cite:
M. Maragkakis, T. Vergoulis, P. Alexiou, M. Reczko, K. Plomaritou, M. Gousis, K. Kourtis, N. Koziris, T. Dalamagas, AG. Hatzigeorgiou DIANA-microT Web server upgrade supports Fly and Worm miRNA target prediction and bibliographic miRNA to disease association Nucleic Acids Research 2011 (Webserver issue) [PubMed].
hsa-miR-155-5p
Filters:
1. miRNAs were entered into the search bar
Welcome to our NEW DIANA microT v4 interface!
Specify in the search field:
miRNAs (e.g., hsa-let-7a-5p)
genes (e.g., fign)
kegg descriptions or parts of them in double quotes followed by a single or multiple miRNAs (e.g., hsa-let-7a-5p "cancer")
or combinations of the previous separated by spaces (e.g. hsa-let-7a-5p MYCN PBX3 "cancer").
hsa-miR-155-5p
Filters:
Results: 19 targets for miRNAs hsa-miR-155-5p. Threshold is set to 0.7.
2. Resulting targets were predicted at a threshold of 0.7 miTG score
Page 1
Ensembl Gene Id
miRNA name
miTG score
SNR
Precision
Also Predicted
1 (TSHZ3) hsa-miR-155-5p 0.921 4.4 1.0
2 ENSG00000196628 (TCF4) hsa-miR-
3 ENSG00000008083 (JARID2) hsa-miR-
3. Selection criteria was based on “also predicted”, miTG score, and precision score
4 ENSG00000089902 (RCOR1) hsa-miR-155-5p 0.827 4.4 0.9
5 ENSG00000139910 (NOVA1) hsa-miR-155-5p 0.817 4.4 0.9
6 ENSG00000007968 (E2F2) hsa-miR-155-5p 0.802 4.4 0.9
7 ENSG00000189079 (ARID2) hsa-miR-155-5p 0.785 4.4 0.9
8 ENSG00000156273 (BACH1) hsa-miR-155-5p 0.780 4.4 0.9
9 ENSG00000148737 (TCF7L2) hsa-miR-155-5p 0.777 4.4 0.9

Figure 13: Summarised workflow for predicted gene target analysis. (1) miRNAs were entered into the DIANA-microT database. (2) The threshold miTG score was set at a default of 0.7. (3) Selection criteria were used to select the top 3 most significant gene targets. (4) Gene targets were searched for on the OMIM database. (5) The correct search result was selected. (6) Information on the given gene target was analysed to power further literature analyses of gene targets.

3. Results

3.1. RNA Analysis

Total extracted RNA samples were of good purity and integrity and were thus acceptable for Nanostring nCounter analysis. The 260/280 and 260/230 ratios were both generally above 2.0 and concentrations were well above the required 100 ng/ 40 µL (Table 11). The agarose gel electrophoresis reflected that the RNA was of good quality as the two human ribosomal RNA bands were present (28S and 18S), along with the 5S subunit band (Figure 14). The 28S bands were all about three times the intensity of the 18S bands, which was expected due to the difference in length of the RNA strands. Some Smaller RNAs were also present represented by the smaller bands on the gel, indicating some degradation of RNA. However, for purposes of miRNA analysis, this mild sample degradation is not significant. The TNBC cell line MDA-MB-436 consistently yielded lower concentrations of total RNA and thus has lighter band intensity (see Fig 14).

Table 11: Total RNA samples as sent for miRNA profiling. The purple shading represents the samples transported on dry ice while the blue shading represents the samples transported in RNAstable tubes.

Cell line	Lane	Blind Sample Number	Concentration (ng/µL)	260/280 Reading	260/230 Reading
MCF10A	1	**1**	2886.5	2.00	2.18
	2	**2**	2047.0	2.07	2.17
	3	**9**	1148.0	2.09	2.27
	4	**10**	1253.9	2.08	2.04
MCF7	5	**3**	1223.8	2.09	1.76
	6	**4**	954.3	2.09	1.94
	7	**11**	1190.2	2.09	2.14
	8	**12**	1128.2	2.11	2.20
MDA-2B-231	9	**5**	1562.2	2.08	2.21
	10	**6**	1584.6	2.09	2.28
	11	**13**	1220.3	2.11	2.26
	12	**14**	1518.0	2.10	1.79
MDA-2B-436	13	**7**	1072.1	2.10	2.19
	14	**8**	1047.6	2.09	2.17
	15	**15**	1093.1	2.09	2.27
	16	**16**	760.4	2.10	2.17

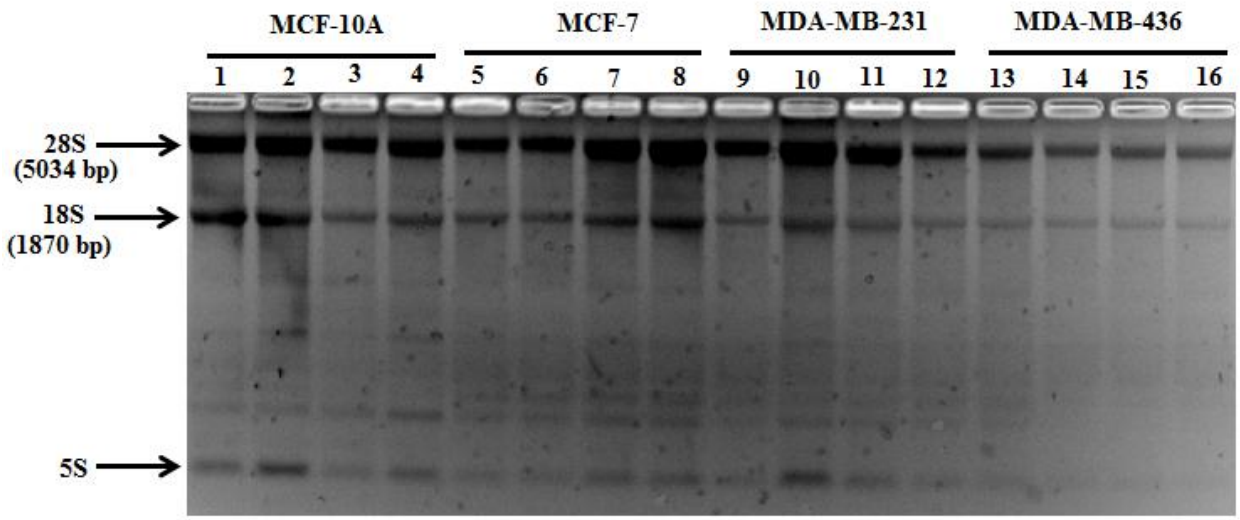


Figure 14: Agarose gel electrophoresis of total RNA extracted from four breast cell lines (MCF-10A, MCF-7, MDA-MB-231, and MDA-MB-436); each performed in quadruplicate stained with ethidium bromide. Three characteristic bands were visible in all samples indicating that RNA integrity was of appropriate standard to proceed with miRNA profiling. The intensity of the 28S band should be about three times the intensity of the 28S band due to it being about three times the length.

3.2. miRNA Profiling

Raw, normalised data was received from Nanostring nCounter analysis in the form of an Excel spread sheet. The data was a representation of the degree of expression of 800 different miRNAs known in humans. The normalised data was used for subsequent analysis.

3.3. RNA Sample Preservation

Statistical analysis revealed that there was no significant difference within each cell line. A single factor ANOVA test was performed in Microsoft Excel between the four duplicate samples for each of the four cell lines. The level of significance was set at 5% ($\alpha = 0.05$) and the *p*-values can be seen in Table 12 below. T-tests were then performed on the average frozen sample expression and the average RNAstable preserved sample miRNA expression (See Table 12

below). There was no significant difference in expression profiles between the two preservation methods as reflected by the p-values represented in Table 13. This allowed for the miRNA expression in the four duplicates (two frozen, two RNAstable) to be averaged for further analysis.

Table 12: Statistical significance of variance within sample duplicates of each cell line based on a single factor ANOVA test. In all cases the *p*-value was greater than 0.05 indicating that there is no significant difference within duplicates of each cell line.

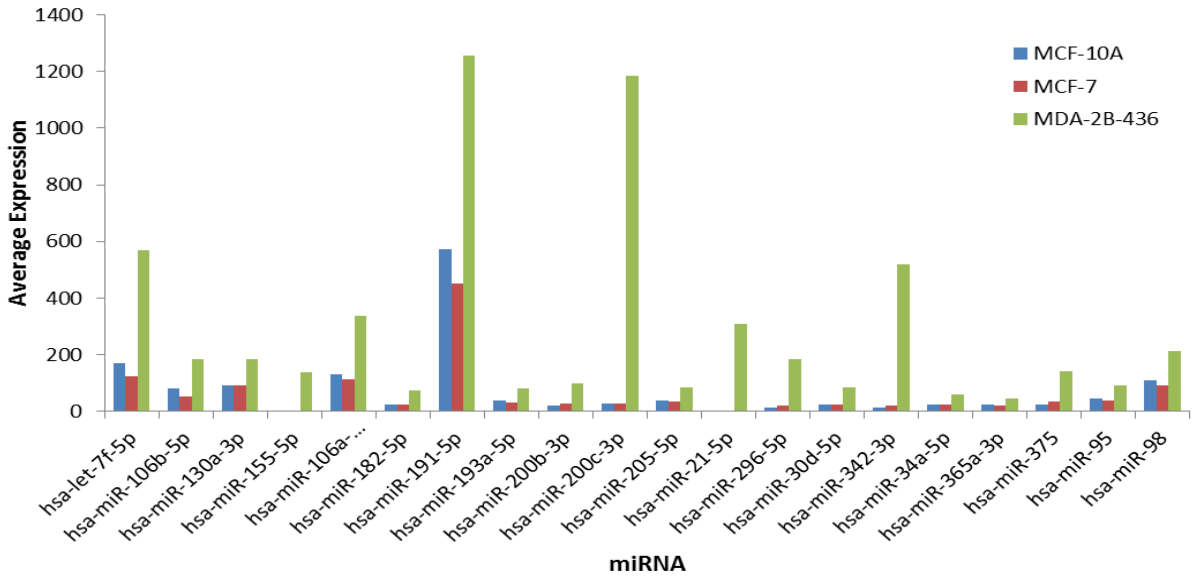
Cell Line	p-value (α = 0.05)
MCF-10A	0.979102
MCF-7	0.950912
MDA-MB-231	0.727886
MDA-MB-436	0.88333

Table 13: Statistical significance of variance between frozen samples and dried samples. A standard two-tailed T-test was performed on frozen *vs.* dried samples. In all cases the *p*-value was greater than 0.05 indicating that there is no significant difference between frozen and dried samples miRNA sequencing results.

Cell Line	*p*-value (α = 0.05)
MCF-10A	0.836
MCF-7	0.694
MDA-2B-231	0.451
MDA-2B-436	0.590

3.4. miRNA Analysis

Average miRNA expression profiles were subjected to a selection process for *in silico* target prediction. Each TNBC cell line was compared to the normal breast call line (MCF-10A) and the hormone sensitive cell line (MCF-7) respectively. Only miRNAs which were either upregulated or downregulated in TNBC cell lines by at least two-fold compared to the other cell lines were chosen for a second round of selection. Finally, the miRNAs which were commonly upregulated in both TNBC cell lines when compared to both normal and hormone sensitive were identified and represented in Table 13 below. Overall, a total of 12 miRNAs of interest were identified; 9 upregulated (Figure 15), and 3 downregulated (Figure 18). These miRNAs were subsequently subjected to bioinformatics in order to determine their specific targets.



<u>Figure 15:</u> Significantly overexpressed miRNAs in TNBC cell line MDA-2B-436 relative to normal breast and hormone-sensitive breast cancer cell lines.

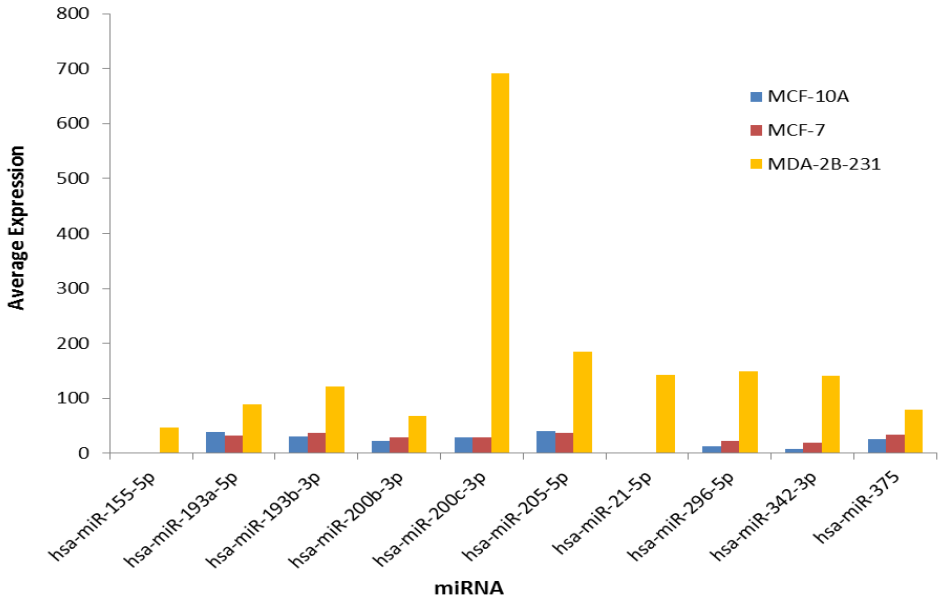


Figure 16: Significantly overexpressed miRNAs in TNBC cell line MDA-2B-231 relative to normal breast and hormone-sensitive breast cancer cell lines.

Table 14: MiRNAs common to both TNBC cell lines (MDA-2B-231 and MDA-2B-436), with at least a two-fold increase in average expression, in comparison to normal breast cells (MCF-10A) and hormone sensitive breast cancer cells (MCF-7), respectively.

miRNA	Fold Increase			
	MCF-10A vs MDA-2B-231	MCF-7 vs MDA-2B-231	MCF-10A vs MDA-2B-436	MCF-7 vs MDA-2B-436
hsa-miR-155-5p	24.64	25.17	72.15	73.71
hsa-miR-193a-5p	2.31	2.74	2.17	2.57
hsa-miR-200b-3p	2.93	2.36	4.29	3.44
hsa-miR-200c-3p	24.16	23.67	41.39	40.56
hsa-miR-205-5p	4.63	4.99	2.18	2.35
hsa-miR-21-5p	75.19	76.81	163.18	166.7
hsa-miR-296-5p	11.29	6.51	13.9	8.02
hsa-miR-342-3p	2.21	7.11	39.78	26.27
hsa-miR-375	3.11	2.31	5.65	4.19

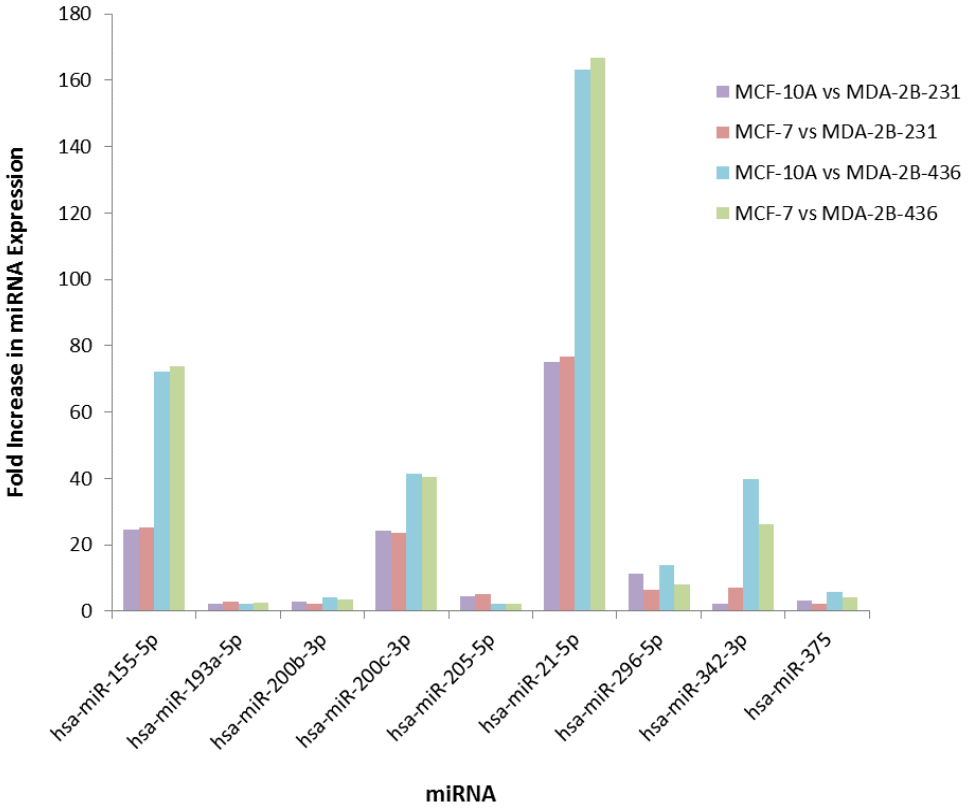
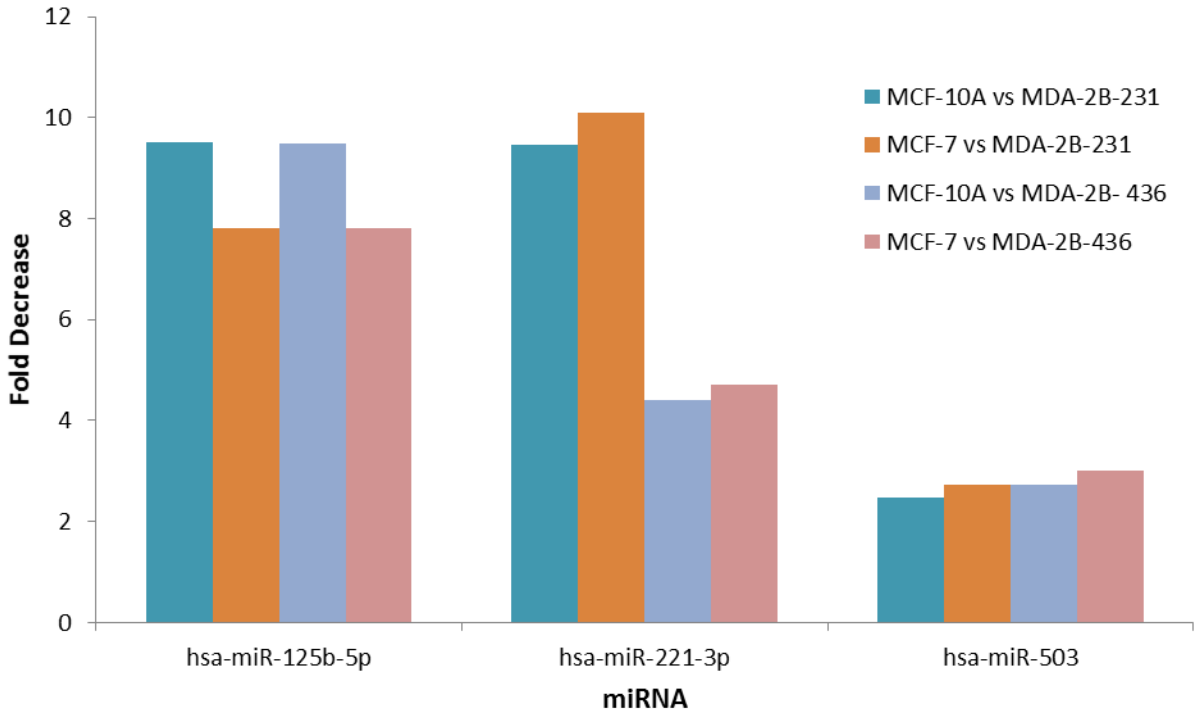


Figure 17: Significantly overexpressed miRNAs in TNBC cell lines relative to hormone-sensitive breast cancer and normal breast cell lines. Only miRNAs with an increase in expression over two-fold in both TNBC cell lines are represented.

Table 15: MiRNAs common to both TNBC cell lines (MDA-2B-231 and MDA-2B-436), with at least a two-fold decrease in expression, in comparison to normal breast cells (MCF-10A) and hormone sensitive breast cancer cells (MCF-7), respectively.

miRNA	Fold Decrease			
	MCF-10A vs MDA-2B-231	MCF-7 vs MDA-2B-231	MCF-10A vs MDA-2B-436	MCF-7 vs MDA-2B-436
hsa-miR-125-5p	9.52	7.82	9.49	7.80
hsa-miR-221-3p	9.47	10.10	4.41	4.70
hsa-miR-503	2.46	2.72	2.72	3.00



<u>Figure 18:</u> Significantly underexpressed miRNAs in TNBC cell lines relative to hormone-sensitive breast cancer and normal breast cell lines. Only miRNAs with a decrease in expression over two-fold in both TNBC cell lines are represented.

Due to the vast number of data points representing the underexpressed miRNAs in the respective TNBC cell lines, the full table is provided in appendix A (Table A4).

A comparison between the two TNBC cell lines showed a difference of 25% between the TNBC cell lines, represented in a pie chart below (Figure 19). Only differences of above two-fold upregulation and downregulation were considered as significant differences. 1.75% of miRNAs in the MDA-2B-436 cell line showed higher expression than their corresponding miRNAs in the MDA-2B-231 cell line, while 23.25% of miRNAs showed lower expression (at least 50% less than in the MDA-2B-231 line) (Figure 12) (Refer to Appendix A, Table A17).

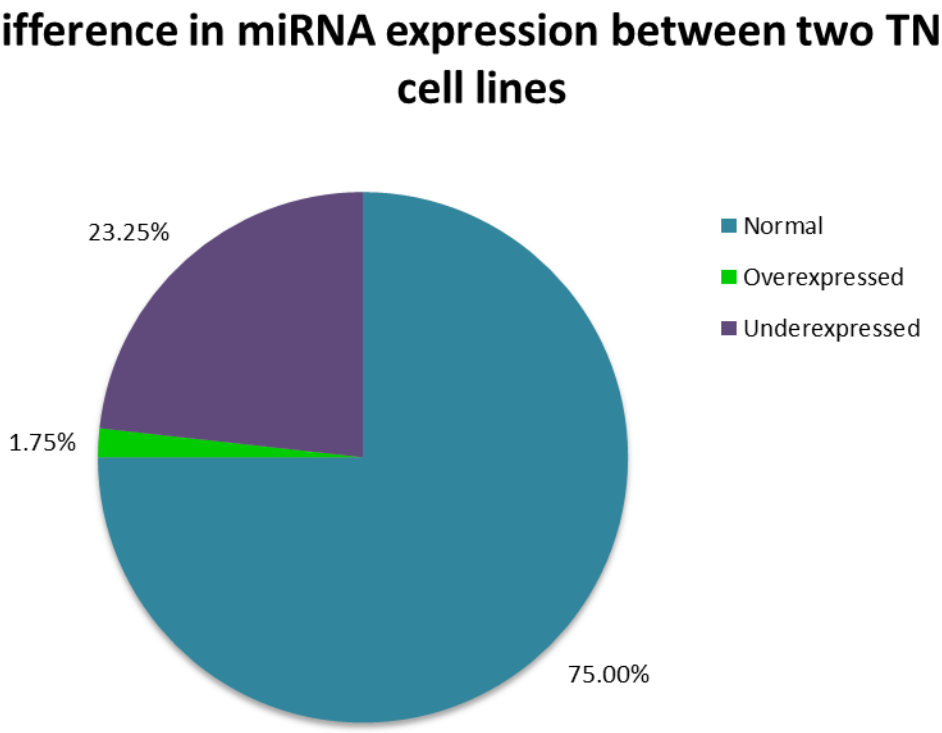


Figure 19: Difference in miRNA expression between two TNBC cell lines. A quarter (25%) of all miRNA expression was found to be different between the two TNBC cell lines (MDA-MB-231 and MDA-MB-436). Only fold changes above two were considered significantly different.

3.5. *In Silico* Target Prediction

DIANA-microT v 4.0 web server results for the 9 overexpressed and 3 underexpressed miRNAs of interest have been tabulated below (Table 16). Only the top three most significant targets have been considered for this study. Some miRNAs had fewer than three predicted targets, while some miRNAs had no targets matching the selection criteria. The targets for such miRNAs were selected at our discretion, based on interest and probability to be true targets. The search results for DIANA microT are summarised below in Table 16. Further details on target significance, including miTG scores, precision scores, and how many other algorithms predicted them, can be found in Appendix A (Tables A5-16).

Table 16: **miRNA Predicted Targets selected for further analysis.**

miRNA	Gene Targets	Cell Processes/ Cancer Pathway Involvement
hsa-miR-155-5p	Teashirt Zinc Finger Homeobox 3(TSHZ3)	ER- Breast cancer Cell Survival
	Transcription Factor 4 (TCF4)	Wnt signalling
hsa-miR-193a-5p	Intersectin 2 (ITSN2)	EGFR signalling
hsa-miR-200b-3p	Zinc Finger E Box-Binding Homeobox 2 (ZEB2)	EMT
	Zinc Finger Protein, Multitype 2 (ZFPM2)	TSG
	Zinc Finger E Box-Binding Homeobox 1 (ZEB1)	EMT
	Protein-Tyrosine Phosphatase, Nonreceptor-Type, 12 (PTPN12)	Growth Factor Signaling Pathway
hsa-miR-200c-3p	Zinc Finger E Box-Binding Homeobox 2 (ZEB2)	EMT
	Zinc Finger Protein, Multitype 2 (ZFPM2)	EMT
	Zinc Finger E Box-Binding Homeobox 1 (ZEB1)	TSG

hsa-miR-205-5p	CKLF-like marvel transmembrane domain-containing 4 (CMTM4)	Mitosis, Apoptosis
	DEAD Homeobox 5 (DDX5)	EMT, cell survival, and proliferation
hsa-miR-21-5p	Zinc Finger Protein 367 (ZNF367)	Metastasis
	Yod1 Ovarian Tumour Deubiquitinating Enzyme 1 (YOD1)	Apoptosis, Proliferation
	Pleckstrin Homology Domain-Containing Protein, Family A, Member 1 (PLEKHA1)	EMT
hsa-miR-296-5p	Fibrosin 1 (FBRS)	Proliferation, Inflammation
	Cyclin-Dependent Kinase 16 (CDK16)	Unknown in TNBC
	Lysophospholipase II (LYPLA2)	
hsa-miR-342-3p	Rho GTPase Activating Protein 19 (ARHGAP19)	Migration, Proliferation, Differentiation, Cell Cycle
	Jumonji Domain Containing 1C (JMJD1C)	TS

hsa-miR-375	ELAV Like Neuron-Specific RNA Binding Protein 4 (ELAVL4)	Notch Pathway
	Eukaryotic Translation Initiation Factor 4 Gamma, 3 (eIF4G3)	Cell Survival, Proliferation
hsa-miR-125b-5p	Potassium Channel, Subfamily K, Member 10 (KCNK10)	Proliferation, Migration, Apoptosis, Angiogenesis
	TET Oncogene Family, Member 2 (TET2)	EMT
hsa-miR-221-3p	Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1a (DYRK1A)	MAPK Pathway, Differentiation, Apoptosis
	Phd Finger Protein 2 (PHF2)	TS
	Cyclin-Dependent Kinase Inhibitor 1C (CDKN1C)	Cell Cycle
hsa-miR-503	Cyclin E1 (CCNE1)	Cell Cycle

Table 17: Number of predicted targets for miRNAs with significant differences in expression in TNBC (http://diana.imis.athenainnovation.gr/DianaTools/index.php?r=microtv4).

miRNA	NOT*	NOT > 2*	NOT miTG>0.9*	NOT PS>0.9*
hsa-miR-155-5p	19	18	2	11
hsa-miR-193a-5p	1	0	0	0
hsa-miR-200b-3p	92	4	6	30
hsa-miR-200c-3p	91	3	7	30
hsa-miR-205-5p	30	18	1	0
hsa-miR-21-5p	8	6	0	2
hsa-miR-296-5p	4	0	0	0
hsa-miR-342-3p	13	0	2	0
hsa-miR-375	5	2	0	0
hsa-miR-125b-5p	29	22	0	0
hsa-miR-221-3p	15	13	1	2
hsa-miR-503	5	1	0	0

*__NOT__ = Total Number of predicted targets, *__NOT > 2__ = Number of Targets predicted by more than 2 other methods, *__NOT miTG>0.9__ = Number of predicted targets with an miTG score above 0.90, *__NOT PS>0.9__ = Number of predicted targets with a precision score above 0.9.

4. Discussion

4.1. RNA Analysis and Transportation

There was clearly no significant difference in the level of miRNA expression between frozen and dried samples for each cell line as the *p*-values for each t-test were greater than 0.05. It can be assumed that this result indicates that the quality of RNA in frozen and dried samples is comparably similar, and thus there was a similar amount of sample degradation in all samples. A previous study reported that spectrophotometry and Bioanalyzer analysis showed no difference in RNA integrity between frozen (- 80℃) and RNAstable dried samples stored over various time

periods (Hernandez *et al.,* 2009). Another study indicated that there were no significant differences in RNA quality after being stored in RNAstable, and may signify superior stability, even with temperature fluctuations, for up to 92 days compared to conventional storage methods (Minogue *et al.*, 2014; Stevens *et al.,* 2012).

Other techniques involving dried RNA samples have also been developed. The use of FTA® paper (commercially available reagent-loaded papers similar to filing cards) has been used in several molecular biological investigations (Abdelwhab *et al.,* 2011; Jones *et al.,* 2012; Kraus *et al.,* 2011) and is relatively user-friendly whereby dried samples undergo an *in situ* wash allowing for impurities to be washed off the paper while the nucleic acid remains. The nucleic acid may then be used for further investigation. The advantages of this mode of storage and transportation is much the same as using RNAstable, whereby samples can be stored and transported at room temperature (Smith & Burgoyne, 2004). Solid chromatography strips, pretreated with SDS and EDTA (along with Tris-HCl) have been used to collect rotavirus RNA from stool samples. It was observed that this technique was effective at inactivating the virus as well as RNases which allowed for the safe and effective storage of dried samples at room temperature for a limited amount of time (Rahman *et al.,* 2004). Similar to RNAstable, a microfluidic SlipChip device has been developed for the drying of biological specimens. The device incorporates chemical stabilization matrices based on those found in nature. It is extremely user-friendly and can store samples at room temperature for up to a week without significant degradation (Begolo *et al.,* 2013; Byrnes *et al.,* 2013). Another product from Biomatrica; SampleMatrix, also dries biological samples. This product has been shown to effectively store DNA samples up to a year with no substantial differences in quality to frozen samples stored over the same time period (Lee *et al.,* 2012).

There was no significant difference in miRNA expression between frozen and dried samples used in Nanostring nCounter analysis. This indicates that either method of transportation of samples may be used without significant risk of RNA degradation. RNAstable does however add the benefit of practicality in terms of storage and transportation of RNA, along with being a more cost-effective method of transportation and reduces the risk of other uncontrollable variables which may affect RNA integrity such as freezer failure, space limitations, temperature

fluctuations, and delays in transportation. RNAstable is a good option for resource-limited researchers and holds great aspirations for the future of biological sample storage and transportation.

4.2. *In silico* miRNA Target Prediction

The DIANA-microT web server uses a considerably short 'match length' for pairing miRNAs with gene targets (6-9 nt). This may lead to many false positive results which is why it was favorable to only consider targets which had been experimentally validated where possible. Although some of the possible novel targets identified in the present study may not have been experimentally validated yet, they were nevertheless considered of interest and are discussed below. Bioinformatics is based on identifying conserved regions within genomes which may further limit target prediction as some unconserved yet functional sites would not be identified as possible targets at this stage. New methods of target prediction would potentially elucidate novel targets and lead to targeted therapies, as well as diagnostic and prognostic marker discovery. Given the vast number of results generated for the 12 miRNAs of interest, only up to the three most significant targets were chosen for further analysis. It would however, be preferable to analyse all possible targets and their potential roles in TNBC physiology in future studies.

4.3. Treatment of TNBC: the way forward

After initial response to chemotherapy, many patients with TNBC experience reoccurrence of drug-resistant metastatic disease (Bhola *et al.,* 2013). A lack of targeted therapies for TNBC is largely due to the lack of expression of hormone receptors; however, by divulging the roles of other mutated genes within this cancer, it may be possible to develop novel targeted therapy options for TNBC treatment. Currently there is no single proven effective agent targeted at a

driving vulnerability in TNBC, however there are a number of potential therapies under investigation (Hudis & Gianni, 2011). TNBCs are generally unresponsive to current targeted therapies and remain the most difficult subtype to treat (O'Toole *et al.*, 2013). The majority of BRCA1-associated breast cancers are triple negative and are of higher grade and later stage as per the TNM staging system. These cancers were also more likely to be associated with mutations in TP53 (Lakhani *et al.*, 2005). TNBCs have also been associated with high expression of basal-like cytokeratins (CK5/6, 14, 17, 18) as well as P-cadherin and HER1/EGFR (Foulkes *et al.*, 2003). The PI3K pathway has been identified as a key player in TNBC pathogenesis, and development of pathway-specific inhibitors may produce a novel approach to targeted therapy (Gordon & Banerji, 2013). Although chemotherapy is highly effective for treatment of TNBC, the prognosis remains poor as recurrence is common (Soomro *et al.*, 2011). These markers may be useful in development of new potential targets for diagnosis, predictive testing and therapy (Gusterson *et al.*, 2005).

A number of current approaches being investigated as potentially effective targeted therapies for TNBC are summarized in Table 18. In addition, to place the miRNA analysis and proposed functions into context, key pathways are described further below. Following on this section, selected overexpressed miRNAs and their predicted functions are considered.

4.3.1. DNA Repair: BRCA1

Chromosomal double-stranded breaks (DSBs) can occur as a result of DNA metabolism or external factors such as ionising radiation (IR) (Moynahan *et al.*, 1999). Usually these potentially detrimental DSBs are repaired naturally in the cell by one of two mechanisms. The first process, non-homologous end joining (NHEJ) is error-prone because the alignment between the two DNA segments being fused is not dependant on the original DNA sequence in the sister chromatid (Weinberg, 2007). The second pathway, homology-directed repair (HDR), uses homologous recombination to repair the DSB using a template from the undamaged sister chromatid, thereby allowing for a more accurate DNA sequence (Figure 20). BRCA1 is an important protein known to facilitate HDR, and mutations in the BRCA1 gene are associated with an 80% risk of breast cancer development in women (Weinberg, 2007).

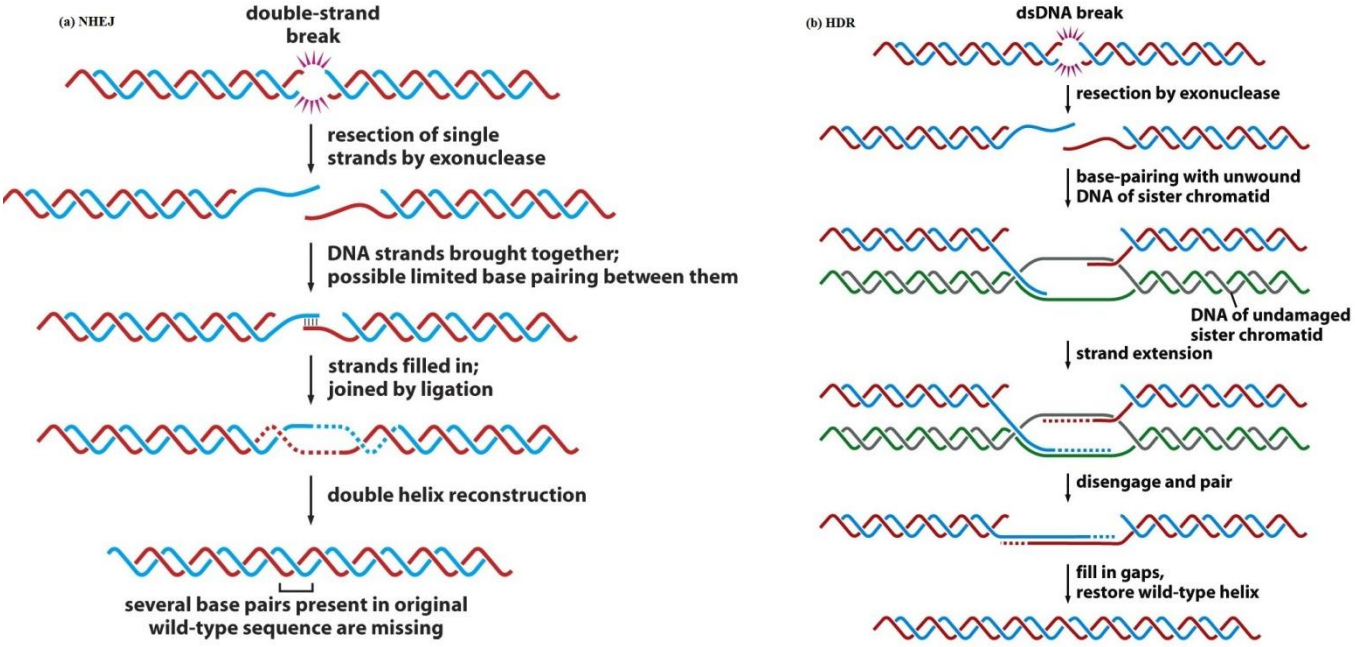


Figure 20: Double stranded DNA break repair machanisms. (a) NHEJ is less accurate and may result in novel expression patterns. (b) HDR is more accurate as the undamaged sister chromatid is used as a template for repairing the damaged DNA. Adapted from Weinberg, (2007).

BRCA1 is an tumour suppressor gene (TSG) which plays a role in DNA repair, cell cycle checkpoint control and contributes to the maintenance of genomic stability (Soomro *et al.*, 2011; Wang *et al.*, 2009a). BRCA1 expression is also required for the differentiation of ER stem/progenitor cells into ER-luminal cells. The loss of double-strand DNA break repair seen in these tumours may contribute to the accumulation of genetically unstable breast cancer stem cells leading to carcinogenesis (Liu *et al.*, 2008). Experimental inhibition of BRCA1 resulted in accelerated growth in both normal and malignant breast cells, indicating that BRCA1 may normally serve as a negative regulator of mammary epithelial cell growth. This growth can be altered by mutations or alterations in gene expression (Thompson *et al.*, 1995). Genetic mutations in BRCA1 dramatically increase the risk for TNBCs which are likely to arise in BRCA1 mutation carriers and have similar gene expression profiles to BRCA1-deficient tumours (Haffty *et al.*, 2006). Tumour cells lacking functional BRCA1 have been found to be sensitive to PARP inhibitors (Farmer *et al.*, 2005). PARP enzymes are responsible for regulating cell

survival, cell death programmes and a number of other biological functions such as transcriptional regulation, telomere cohesion and mitotic spindle formation during cell division, intracellular trafficking and energy metabolism (Gibson & Kraus, 2012; Schreiber *et al.*, 2006). Olaparib (AZD2281), a novel, potent, orally active PARP inhibitor showed anti-tumour activity with few of the adverse side effects associated with conventional chemotherapy (Fong *et al.*, 2009). Another PARP inhibitor, 6-thioguanine (6-TG), has been shown to effectively treat BRCA1 mutant cancers and also to effectively kill PARP-inhibitor and platinum-based chemotherapy resistant cells (Issaeva *et al.*, 2010). Rodriguez et al. established that a defective DNA repair gene expression signature differentiates TNBCs which are sensitive to anthracyclines and resistant to taxanes, and should be considered as co-therapeutic agents along with PARP-inhibitors (Rodriguez *et al.*, 2010). However, it should be noted that BRCA1/2 status in TNBCs is not the sole determinant of PARP-inhibitor efficacy as different TNBC subtypes differ in sensitivity to different PARP-inhibitors (Lehmann *et al.*, 2011).

Although BRCA1 functions as a TSG, some studies have shown that BRCA1 dysfunction alone may not be sufficient for tumourigenesis (Schuyer & Berns, 1999). TP53, or p53, is another TSG found to be dysfunctional in almost half of all human cancers and in most, if not all, of BRCA1 breast cancer tumours (Holstege *et al.*, 2009; O'Toole *et al.*, 2013). TP53 cellular functions include regulation of cell cycle, apoptosis, senescence, DNA repair, and metabolism. Activity of p53 is ubiquitously lost in human tumours by mutations in the p53 gene or by loss of cell signalling up- or downstream of p53 (Bourdon, 2007). Greenblatt *et al.* (2001) found that somatic TP53 mutations are more common in breast cancers associated with BRCA1/2 mutations than in sporadic breast cancers. BRCA1-related tumours may be treated with drugs targeting both BRCA1 deficiency (PARP inhibitors) and p53-deficient cells. A negative regulator of p53; MDM4 may be an interesting target for drug development as its inactivation is crucial for p53 activation (Toledo & Wahl, 2006). Ibrahim *et al.* (2012) hypothesised that in BRCA-proficient TNBC, PI3K inhibition would result in homologous recombination impairment and subsequent sensitisation to PARP inhibitors. This provides a rationale to co-administer PI3K and PARP inhibitors in TNBC where targeted therapies are lacking. This combination of therapies is currently under clinical trial and raises interesting possibilities for targeted combination therapies in TNBCs.

Importantly, miRNAs have been documented as playing a role in breast cancer and TNBC. In particular, miR-155 is a putative oncogenic miRNA (oncomiR) which is located within the B cell integration cluster (BIC) and often upregulated in TNBC (Metzler *et al.*, 2004). Upregulated oncomiRs that function in tumour development mainly do so by repressing the expression of tumour suppressor- or tumour suppressor-like genes such as p53, MYC, and RAS (Esquela-Kerscher & Slack, 2006; Kent & Mendell, 2006). The R1699Q variation of Human BRCA1 does not interfere with DNA repair but was rather seen to abrogate the repression of miR-155 through HDAC2 association. The overexpression of miR-155 was shown to attenuate tumour growth *in vivo,* suggesting miR-155 as a possible target for BRCA1 deficient TNBC tumours (Chang *et al.,* 2011a). In the present study miR-155-5p was indeed upregulated in the TNBC cell lines. There was a great difference in expression of this miRNA between the two TNBC cell lines, suggesting that it may be useful in subclassifying TNBC types.

4.3.2. Targeting Cancer Stem Cells in TNBC

Breast cancer stem cells (CSCs) are increasingly thought to play a role in breast cancer growth and metastases (Takebe et al., 2011b). CSCs use a variety of signaling pathways to undergo self-renewal and differentiation, including Wnt, NOTCH, Hedgehog, and transforming growth factor β (TGF-β). These pathways are also involved in Epithelial-to-mesenchymal transition (EMT) (McGovern *et al.,* 2009; Peacock *et al.,* 2007; Reya & Clevers, 2005; Takebe *et al.,* 2011b). EMT guides the transformation of immobile epithelial-like cells into mobile, mesenchymal-like cells which have the ability to travel to distant anatomical sites in embryonic development. This transformation is also observed during tumour formation and may lead to metastasis. This process is however reversible through mesenchymal-to-epithelial (MET) transition, whereby migratory cells become anchored at distant sites and lose their migratory potential (Takebe *et al.,* 2011b). The so-called "Claudin-low" TNBC expresses low levels of Claudin 3 and E-cadherin and expressed EMT markers suggesting that these tumours may arise from immature progenitor or stem cells (Taube *et al.,* 2010). Their slow rate of growth and chemo-resistant characteristics suggest that CSCs may survive chemotherapy and re-initiate tumour growth later on (Frank *et*

al., 2010). This indicates that reoccurrence and drug resistance are both largely related to these pathways. To eradicate metastatic breast cancer it is necessary to either disrupt CSC niche interactions or eliminate CSCs (Ito *et al.*, 2012). EMT is linked with the acquisition of stem cell characteristics and the concept of CSCs conferring EMT and self-renewal properties provides the rationale for cancer cells to migrate and populate metastatic sites. Consequently, the development of CSC-targeted anti-tumour therapies may prevent metastasis in TNBC (Mani *et al.*, 2008).

4.3.2.1. Wnt Pathway Inhibition

The Wnt pathway is particularly important as it regulates cell proliferation. Wnt proteins bind to their transmembrane receptor Frizzled and act through the Disheveled cytoplasmic protein to suppress GSK-3β, inhibiting its ability to phosphorylate β-catenin, among other substrates. This results in a build-up of β-catenin in the cytoplasm and nucleus, allowing for its association with Tcf/Lef transcription factors, which drives the expression of pro-cell proliferation genes (Figure 21) (Weinberg, 2007).

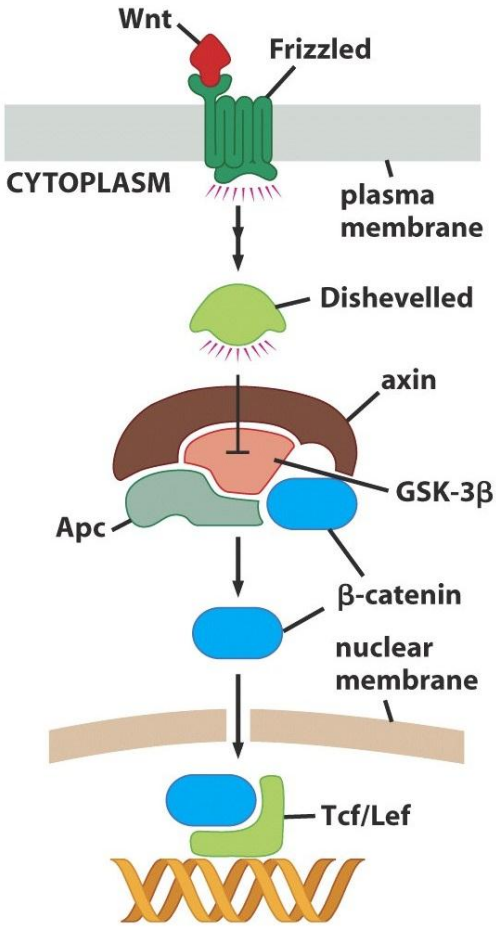


Figure 21: A summarised representation of the Wnt pathway. Phosphorylation of β-catenin leads to its degradation, however when the Wnt pathway is activated abnormally it leads to the buildup of β-catenin which subsequently leads to the overexpression of proliferation-promoting transcription factors. Adapted from Weinberg, (2007).

Activation of Wnt signaling has been associated with cancer, raising the possibility that the tightly regulated self-renewal mediated by Wnt signaling in stem and progenitor cells is subverted in cancer cells to allow malignant proliferation (Reya & Clevers, 2005). The relevance of Wnt signaling in cancer was re-enforced by the discovery that adenomatous polyposis coli (APC), a TS protein, was associated with β-catenin (Su *et al.,* 1993). It was found that β-catenin is downregulated by APC and upregulated by Wnt-1 (Munemitsu *et al.,* 1995; Polakis, 2012b). This suggests that β-catenin is a potential driver of human cancer. Mutations in the genes coding

for either β-catenin or APC resulted in the dysregulation of β-catenin, providing further support for the role of the Wnt pathway in cancer (Morin *et al.,* 1997).

β-catenin promotes tumourigenesis through its association with transcription factors which activate cell growth (Behrens *et al.,* 1996). It is an adherens junction protein responsible for regulating normal cell growth and behaviors, and also plays a role in contact inhibition signaling, which halts cell proliferation once an epithelial sheet is complete (Peifer, 1993). Notably, lack or reduction of β-catenin cell-membrane expression and/or nuclear accumulation were significantly associated with tumours of triple-negative and basal-like phenotype (Geyer *et al.,* 2011). Recent studies indicate that Wnt/β-catenin signaling is particularly activated in TNBC, such that the Wnt receptor frizzled-7 (FZD7) and the Wnt co-receptor LRP6 were found to be up-regulated (King *et al.,* 2012b). Another study found that Gugulipid, a natural compound derived from a species of tree, induced growth inhibition and apoptosis in human breast cancer due to downregulation of the β-catenin pathway (Jiang *et al.,* 2013). Autocrine Wnt signaling in TNBC occurs where Wnt mutations are absent and yet hyperactive signaling is apparent (Geyer *et al.,* 2011; Khramtsov *et al.,* 2010). Moreover, the overexpression of various Wnt pathway genes were observed in TNBC for example frizzled homolog 7 (FZD7), low density lipoprotein receptor-related protein 6 (LRP6), and transcription factor 7 (TF7). The Wnt pathway thus presents as a target for treatment of TNBC, as discussed below.

The knock down of FZD7 was proven to reduce expression of Wnt target genes and inhibited tumourigenesis in TNBC cells (Yang *et al.,* 2011a). Many breast tumours, including TNBC, show hypermethylation of secreted frizzled-related protein 1 (sFRP1) promoter region, causing lowered expression of this Wnt antagonist. Importantly, sFRP1 binds to and effectively competes with FZD receptors for Wnt ligands (Polakis, 2012a). Ectopic expression of sFRP1 in TNBC cells resulted in a significant reduction in tumour growth and blocked lung metastases (Matsuda *et al.,* 2009). The Wnt pathway co-receptor LRP6 is another possible target, particularly since salinomycin, a selective breast cancer stem cell killer, was recently demonstrated to be an inhibitor of Wnt/β-catenin signaling by inducing LRP6 degradation (King *et al.,* 2012b; Yue *et al.,* 2011). Subsequently, salinomycin effectively inhibited growth in a TNBC cell line and triggered apoptosis, and with the addition of chemotherapeutic drugs, was also able to potentiate the killing of TNBC cells (Al Dhaheri *et al.,* 2013). Another promising drug; niclosamide, is an

antihelminthic drug able to suppress LRP6 expression and phosphorylation, block Wnt3A-induced β-catenin accumulation, and inhibit Wnt signaling, as well as induce apoptosis in TNBC cells (Yue *et al.,* 2011). These results suggest that using sFRP1 to interfere with Wnt signaling in TNBC might be a valid therapeutic approach. Wend et al. identified HMGA2 as the most highly expressed gene along with WNT10B in Wnt10b-driven TNBC tumours. Treatment of TNBC cell lines with known Wnt pathway inhibitors results in downregulation of HMGA2 expression and cell proliferation markers. WNT10B has an epistatic effect on HMGA2, leading to decreased cell proliferation (Wend *et al.,* 2013).

4.3.2.1.1. Small Molecule Inhibitors of the Wnt Pathway

Two new classes of small molecules were identified for Wnt inhibition. The first inhibits *porcupine,* a membrane-bound acyltransferase essential for the production of Wnt proteins, and the second inhibits the destruction of axin proteins which suppress the Wnt pathway (Chen *et al.,* 2009a). The small molecule; ICG-001, downregulates β-catenin/T cell signaling by specifically binding to cyclic AMP (cAMP) response element-binding protein (Emami *et al.,* 2004). ICG-001 selectively induces apoptosis in transformed cells and reduces tumour growth. This is another molecule currently under investigation for treatment of TNBC.

4.3.2.1.2. Targeting Dishevelled

Dishevelled is a key protein in the Wnt signaling pathway which links extracellular signals and downstream signals. It may be manipulated to inhibit both canonical and non-cononical Wnt signaling pathways (Takebe *et al.,* 2011a). The interaction between Frizzled-7 receptor, Wnt receptor and the PDZ domain of Dishevelled can be disrupted by a small molecule; FJ9. This causes downregulation of canonical Wnt signaling, suppression of tumour growth and promotes apoptosis. FJ9 is one of the first non-peptide inhibitors to show therapeutic efficacy through PDZ protein-protein interaction disruption (Fujii *et al.,* 2007). Another small organic molecule capable of inhibiting Wnt signaling through the PDZ domain of disheveled is NSC668036, by inhibiting Wnt3A (Shan *et al.,* 2005). This is evidence for the rational design of high affinity inhibitors of the PDZ domain of disheveled to be tested in TNBC cell lines.

4.3.2.1.3. Targeting Wnt-1 and Wnt-2

Promoter methylation was identified to cause inhibition of the sFRP family in cancer; however, restoration of sFRP attenuates Wnt signaling even in the presence of downstream mutations. In order to elucidate the role of Wnt-1 survival factor in breast cancer, both a monoclonal antibody and siRNA were used to silence Wnt-1 signaling (He *et al.,* 2005). Both the monoclonal antibody and the RNAi induced apoptosis and caused downstream protein changes in Wnt-1 expressing cancer cells. The antibody also suppressed tumour growth. These findings hold promise as a novel therapeutic option in TNBC (He *et al.,* 2004). Wnt-2 may be an interesting target in TNBC as it too was found to induce apoptosis and downstream protein changes when inhibited by a monoclonal antibody and RNAi (You *et al.,* 2004)

4.3.2.1.4. MET and porcupine inhibitors

Proto-oncogene MET (MET) is a membrane receptor necessary for embryonic development and wound healing and is normally expressed in cells of epithelial origin. It is overexpressed in a significant percentage of human cancers and is amplified during the transition between primary tumours and metastasis (Giordano *et al.,* 1997). MET is primarily elevated in basal-like breast tumours and induces tumour signatures of Wnt and EMT (Ponzo *et al.,* 2009). Thus, MET inhibitors are another potential targeted therapeutic agent for TNBCs. Targeting the Wnt pathway with IGC-001 or the *porcupine* inhibitor; Wnt-C59 has great potential for TNBC. The potential for receptor-targeted inhibition with antibodies against Wnt ligands and/or LRP6 is an attractive approach currently under development (Wend *et al.,* 2013).

4.3.2.2. Notch Pathway Inhibition

Notch is a transmembrane protein which, once it binds its ligand, Jagged 1 or 2, it undergoes two proteolytic cleavages. The cleavage within its transmembrane domain releases a cytoplasmic protein fragment; the so-called Notch Intracellular Domain (NID), from its tethering to the plasma membrane, allowing it to migrate to the nucleus, where it functions as a transcription factor (Weinberg, 2007). Notch signaling plays an important role in regulating cell-to-cell communication during cell proliferation, embryogenesis, differentiation, and apoptosis and is also crucial for normal breast development and CSC self-renewal and survival (Artavanis-

Tsakonas *et al.*, 1999; Dontu *et al.*, 2004). Notch signalling is deregulated in human breast tumours and was found to be upregulated in TNBC-derived stem-like cells.

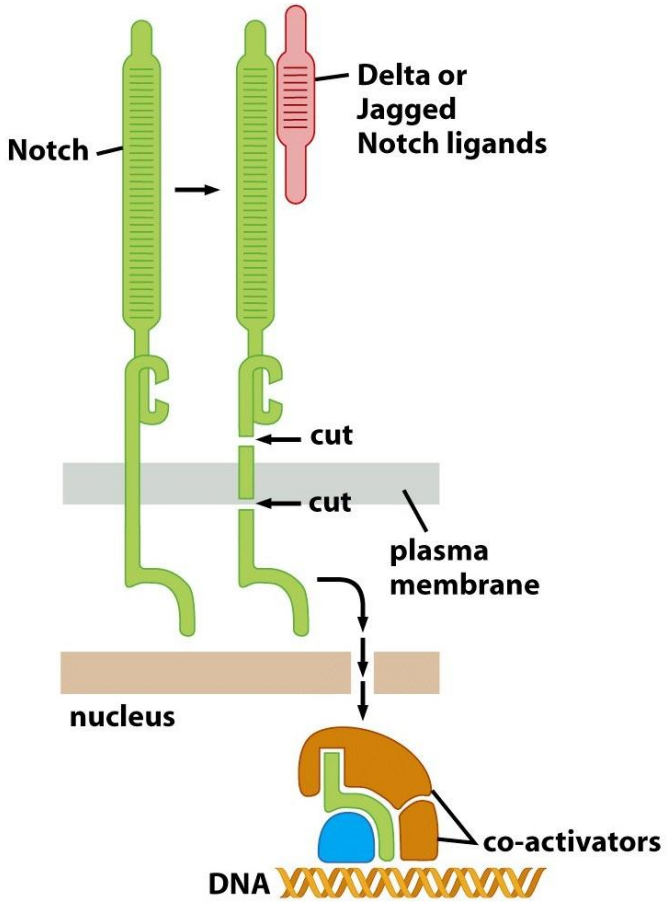


Figure 22: A summary of the Notch pathway. A proteolytic cleavage of Notch receptors occurs once a ligand binds; this ultimately leads to the activation of transcription. Adapted from Weinberg, (2007).

4.3.2.2.1. Gamma-secretase Inhibition

Notch and its ligands are overexpressed in breast cancer, and one method of effectively blocking Notch activity is preventing its cleavage at the cell surface with γ-secretase inhibitors (Olsauskas-Kuprys *et al.*, 2013). Three structurally distinct γ-secretase inhibitors (GSI) (LLNle, LY-411,575, or MRK003) and a Notch decoy protein (recombinant human Notch-1 Fc chimera) were used to determine that mammosphere formation was irreversibly eliminated and apoptosis was induced in cells treated with MRK003 (but not with the other two GSIs where the effect was transient). These findings suggest that Notch inhibition may have clinical benefits in targeting

TNBC SCs (Grudzien *et al.,* 2010). RO4929097, another GSI, was responsible for a mild response when treating TNBC cells. Sphere formation and tumour growth were not fully abrogated possibly indicating incomplete Notch inhibition (Azzam *et al.*, 2013). MK-0572 is a GSI under clinical investigation for treatment of advanced breast cancer both alone and in combination with chemotherapy (Al-Hussaini *et al.,* 2011). This might indicate that the use of a GSI in combination with conventional chemotherapy or other targeted therapies is a novel option for TNBC treatment. There is evidence to suggest that ionising radiation in combination with a GSI may be beneficial to TNBC treatment too (Chi, 2008).

4.3.2.2.2. ADAM-17 Protease Inhibition

ADAM-17 is a protease involved in Notch processing and in the activation of several ligands which promote EGFR signalling. This protease was found to be expressed at significantly higher levels in TNBCs as opposed to hormone-sensitive breast cancers. An ADAM-17 specific inhibitor, PF-5480090 was found to decrease the release of the EGFR ligand, TGFα, decrease levels of phosphorylated EGFR, and block cell proliferation in TNBC cells. Another finding was that pretreatment of cell lines with the ADAM-17 inhibitor enhanced response to several different chemotherapeutics (Seals & Courtneidge, 2003).

4.3.2.2.3. Monoclonal Antibodies

Mammalian membrane bound notch ligands consist of two structurally distinct families: Deltalike ligands (Dlls) 1, 3 and 4, and Jagged ligands 1 and 2 that interact with four transmembrane notch receptors (Takebe *et al*., 2011a). Dll4 is essential for embryonic vascular development and has a unique role on regulating endothelial cell proliferation and differentiation. A Dll4-selective antibody was used to neutralise Dll4 which rendered endothelial cells hyperproliferative and caused defective cell fate, in addition, inhibiting Dll4 blocked tumour growth (Ridgway *et al.,* 2006). Notch1 mAbs that bind specifically to the negative regulatory region of Notch1 were developed and tested in TNBC xenografts. Notch1 inhibition led to significant tumour growth inhibition, particularly in combination with docetaxel. These mAbs also caused a reduction in mammosphere formation and CD44+/CD24-/lo cell population, and resulted in longer time to reoccurrence. By directly targeting the CSC niche, these mAbs

may provide novel therapies to improve efficacy of chemotherapy and delay reoccurrence in TNBC patients (Qiu *et al.,* 2013).

Other Notch targeted therapies such as mastermind-like (MAML)–CSL–notch complex formation targeted agents and genetically engineered fusion proteins which inhibit Notch signalling are under clinical development but should be considered for TNBC targeted therapy as well (Takebe *et al.,* 2011a).

4.3.2.3. Hedgehog Pathway Inhibition

Hedgehog (Hh) proteins act as ligands for the Patched transmembrane receptor. Once an Hh protein binds to cell membrane associated Patched, it releases the downstream Smoothened protein (SMO) from inhibition, which is then able to emit downstream signals. The activated Smoothened protein prevents the cytoplasmic Gli protein from being cleaved, resulting in activation of gene transcription of cell proliferation factors (Figure 23) (Weinberg, 2007). The Hedgehog signaling pathway is known to regulate progenitor cell fate in normal development and homeostasis, as well as control tissue polarity, patterning maintenance, and stem cell maintenance in embryonic development (Ingham & McMahon, 2001; Peacock *et al.,* 2007). This pathway involves a signal transduction cascade that includes a member of the Hh family; sonic hedgehog (SHH), indian hedgehog (IHH), or desert hedgehog (DHH) (Tao *et al.,* 2011). Aberrant activation of this pathway has been associated with tumourigenesis in various human tissues, including brain, lung, mammary gland, and prostate (Berardi *et al.,* 2014; Harris *et al.,* 2012; Natarajan *et al.,* 2013; Tzelepi *et al.,* 2011; Yauch *et al.,* 2008).

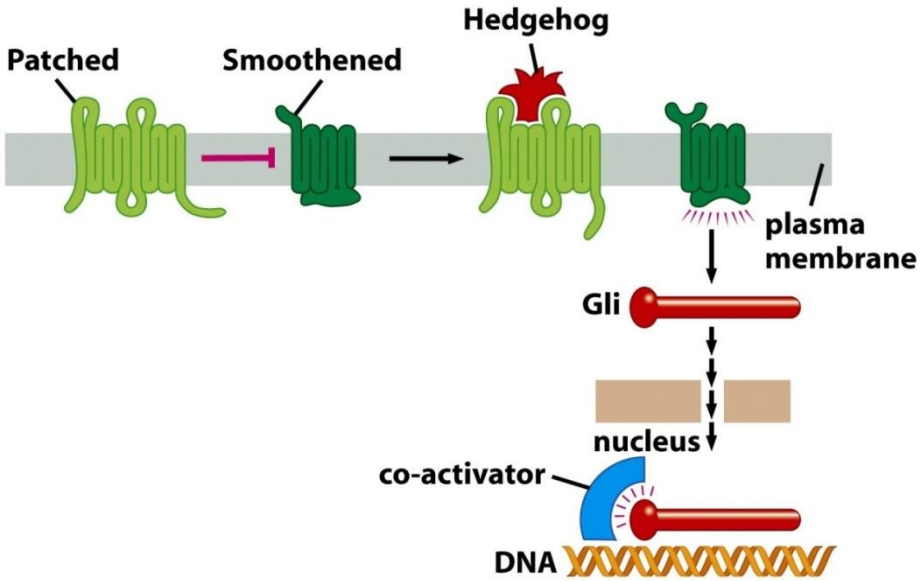


Figure 23: A summary of the Hedgehog pathway. The Hedgehog ligand binds to its receptor Patched, leading to the liberation of Smoothened which ultimately inhibits Gli and promotes cell proliferation. Adapted from Weinberg, (2007).

There was significant overexpression of the membrane protein, SMO and the transcription factor Gli1 in TNBC while Patched-1 (PTCH1) receptor expression was significantly decreased (Tao *et al.,* 2011). Both cell migration and invasion were decreased in TNBC cells when Gli1 was downregulated by RNAi, and it was also found that matrix metalloproteinase (MMP) 11 silencing in TNBC cells with overexpressed Gli1 abrogated the increased migration and invasion. Sustained suppression of Gli1 expression also decreased the growth of TNBC cells by inducing apoptosis and decreasing proliferation (Kwon *et al.,* 2011). Gant61 is a Gli inhibitor which has been used to decrease tumour growth, induce apoptosis, and inhibit CSC growth in various human cancers and would make an interesting study when applied to TNBC cell lines (Fu *et al.,* 2013; Mas & Ruiz i Altaba, 2010; Mazumdar *et al.,* 2011; Pan *et al.,* 2012; Wickström *et al.,* 2013). These findings suggest that research into the development of Gli inhibitors for TNBC treatment would possibly lead to a novel and effective targeted therapy option.

Yauch *et al.* (2008) found that Hh ligands fail to activate signaling in tumour epithelial cells, and rather support the idea of ligand-dependant activation of this pathway in the stromal microenviroment. Specific inhibition of Hh signaling using small molecule inhibitors, a

neutralising anti-Hh Ab, or genetic deletion of SMO resulted in growth inhibition of tumour cells. Another potential target of the hedgehog pathway is SMO which appears not to have been considered for TNBC treatment yet. There are several SMO inhibitors under investigation for other tumour types which may prove a useful tool in TNBC targeted therapy (Barginear *et al.*, 2009; Dijkgraaf *et al.*, 2011; Hyman *et al.*, 2009; Olive *et al.*, 2009; Rudin *et al.*, 2008; Stanton *et al.*, 2009; Taipale *et al.*, 2000).

4.3.2.4. TGFβ Pathway Inhibition

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine involved in regulation of cancer through mechanisms that function either within the tumour cell itself or through host-tumour cell interactions. Numerous physiological processes are involved, including cellular growth, differentiation, apoptosis, homeostasis, and EMT (Bierie & Moses, 2006; Taylor *et al.*, 2010). The activation of the TGF-β pathway leads to the dispatch of cytoplasmic Smad transcription factors to the nucleus, where they interact with other nuclear transcription factors to activate expression of many genes, to inhibit growth (Figure 24) (Weinberg, 2007).

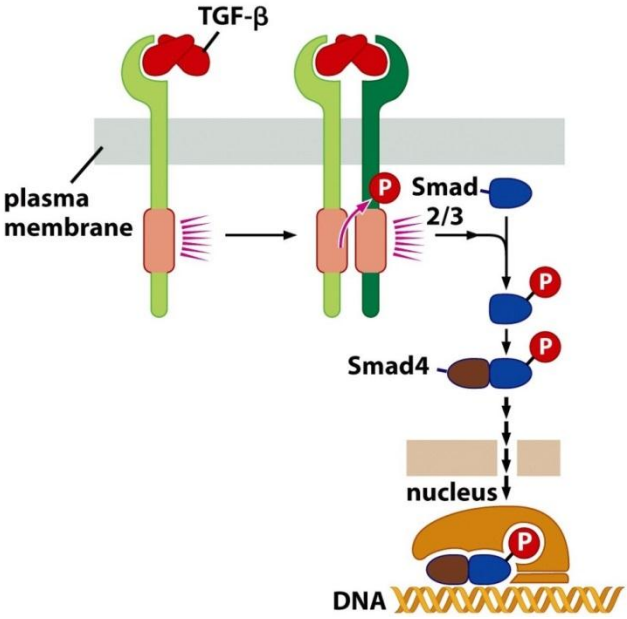


Figure 24: A summary of the TGF-β pathway. The binding of TGF-β to its membrane receptor causes the release of Smad transcription factors to the nucleus, resulting in growth inhibition. Adapted from Weinberg, (2007).

TGF-β ligands are often enriched in the TNBC tumour microenvironment and can be produced by tumour cells or by tumour-associated stromal and immune cells (Bhola *et al.,* 2013). The loss of TGF-β is associated with tumour initiation, progression and metastasis (Bierie *et al.,* 2008). Bhola *et al.* (2013) designed a study to evaluate the role of TGF-β in TNBC tumour metastasis before and after chemotherapy. LY2157299, a TGF-β type-1 receptor kinase inhibitor, a neutralising TGF-β type II receptor antibody, and SMAD4 siRNA all blocked paclitaxel-induced interleukin 8 (IL8) transcription and cancer stem cell expansion. They also found that treatment of TNBC xenografts with LY2157299 prevented re-establishment of tumours after paclitaxel treatment. This suggested that TGF-β signalling induced by chemotherapy enhances tumour reoccurrence through IL8-dependant expansion of cancer stem cells, and that TGF-β inhibitors prevent drug-resistance is cancer stem cells. This would support the use of TGF-β inhibitors in combination with chemotherapy in TNBC patients to reduce reoccurrence and risk of metastasis. Inflammatory-related genes are essential for growth in TNBC cells and the co-inhibition of IL8 and IL6 dramatically reduced colony formation and cell survival *in vitro*, and lowered tumour growth *in vivo* (Hartman *et al.,* 2013). Targeted therapies aimed at EMT induced by TGF-β during breast cancer progression may be a breakthrough in ameliorating metastasis in TNBC (Taylor *et al.,* 2010).

4.3.3. Growth Factor Signaling Pathways

4.3.3.1. Receptor Tyrosine kinase inhibition

Receptor tyrosine kinases (RTK) are a class of cell surface proteins which act as receptors for various ligands. RTKs play a role in the most fundamental cellular processes including cell cycle, cell migration, cell metabolism and survival, as well as cell proliferation and differentiation (Schlessinger, 2000). Included in this group of proteins and their receptors are epidermal growth factor (EGF), fibroblast growth factors (FGF's) and vascular endothelial growth factor (VEGF). These proteins are involved in cancer pathways activated by tyrosine kinase receptors (TRKs) namely, the PI3K/AKT and MAPK pathways. Overexpression or constitutive activation of these pathways plays an important role in pathogenesis and progression of breast cancer (Allen *et al.,* 2003).

Epidermal growth factor receptor (EGFR) and its ligands are cell signaling molecules involved in regulation of cell proliferation, differentiation, growth, motility, and survival, and in tissue development (Li *et al.,* 2014b; Wang *et al.,* 2004). Regulation of these cellular processes is mediated by clathrin-mediated endocytosis and non-clathrin-mediated endocytosis of EGFR. The former is the process whereby substances are transported into the cell through the plasma membrane with the aid of a clathrin coat, stimulated by ligand-binding to EGFR, subsequently leading to the recycling of EGFR or its degradation via ubiquitination (McMahon & Boucrot, 2011). The latter refers to the internalisation of EGFR in the presence of high growth factor concentration, and results in its degradation to lower EGFR abundance in the plasma membrane (Polo *et al.,* 2014). Degradation of internalised EGFR functions to decrease cell proliferation and tumourigenicity as reported in a study on non-small cell lung cancer (Li *et al.,* 2014b). TNBCs often show overexpression of EGFR and are dependent on the EGFR pathway for proliferation (Carey *et al.,* 2012; Nielsen *et al.,* 2004). Amend *et al.* (2006) found that 54% of TNBC tumours were positive for EGFR expression and were associated with more a aggressive clinical course and poor survival, independent of nodal status and tumour size. Within the RNAi pathway, EGFR interacts directly with AGO2, a critical component of the RISC, leading to inhibition of maturation of specific tumour suppressive miRNAs, such as miR-193a-5p, under hypoxic conditions (Shen *et al.,* 2013).

EGFR and Fibroblast growth factor receptor (FGFR) cell signalling pathways have been identified and compared. Both receptors stimulate a similar component of intracellular signalling pathways, however, where activated EGF receptors (EGFRs) function as the main platform for recruitment of signaling proteins, signaling through the FGF receptors (FGFRs) is mediated primarily by assembly of a multidocking protein complex. Moreover, FGFR signaling is subject to additional intracellular and extracellular control mechanisms that do not affect EGFR signaling (Schlessinger, 2004). There has been increasing interest in EGFR as a therapeutic target and several drugs, including therapeutic antibodies and in this regard small-molecule tyrosine kinase inhibitors have been developed (Baselga, 2002). Some of these are discussed below.

Gefitinib, a small molecule tyrosine kinase inhibitor of EGFR, has proven effective alone, and more so in combination with chemotherapeutic agents in the treatment of TNBC. Interestingly,

the combination of carboplatin and docetaxel appears to be antagonistic, but when gefitinib is added to the regimen it seems to create therapeutic synergy between the drugs, and improves efficacy by increasing apoptosis (Corkery *et al.,* 2009; Hoadley *et al.,* 2007). Cetuximab is a monoclonal antibody proven to inhibit growth in TNBC cell lines with EGFR overexpression (Baselga *et al.,* 1993). The addition of cetuximab to cisplatin showed a favourable overall response rate and prolonged progression-free patient survival in patients with TNBC (Baselga *et al.,* 2013). Another study showed that cetuximab had negligible activity in patients with metastatic TNBC, and when combined with the chemotherapeutic agent carboplatin, there was only a minor increase in effectiveness. This could suggest that cetuximab is ineffective (which is unlikely as it is effective in other cancer types) or that there are alternate mechanisms involved in TNBC that do not involve ligand-dependant EGFR-mediated activation (Carey *et al.,* 2012; Corkery *et al.,* 2009). Combination therapy of cetuximab or carboplatin, and taxanes showed good tolerability and high activity when used as first- and second-line therapy in patients with metastatic TNBC (Shamseddine & Farhat, 2011). Metformin (1,1-dimethylbiguanide hydrochloride) is a drug used to treat type II diabetes by inducing major shifts in glucose and fatty acid metabolism. TNBC appears to be particularly sensitive to metformin as it blocks cell cycle progression and selectively induces apoptosis via caspase activation. It also decreased the levels of cyclins and inactivates EGFR and downstream signalling in TNBC cells Two TNBC cell lines were identified with FGFR2 amplification and were highly sensitive to FGFR inhibitor PD173074 and to RNAi silencing of FGFR2 (Turner *et al.,* 2010). Another study suggested that the specific targeting of FGFR-IIIc activity and/or its transactivation by fibronectin may prevent the outgrowth of metastatic TNBCs that are resistant to EGFR-targeted therapies (Wendt *et al.,* 2012). It has been suggested that a combination of PARP inhibitors and metformin may be an interesting approach for treating TNBC in future (Liu *et al.,* 2009).

In TNBC there is higher intratumoural expression of vascular endothelial growth factor (VEGF), justifying research into this RTK as a treatment target to prevent angiogenesis. Bevacizumab is a monoclonal antibody directed against VEGF A which showed a 35% decrease in relapse but no effect on overall survival in TNBC patients (Linderholm *et al.,* 2009). Bevacizumab has been associated with several health risks due to toxicity however, and has thus been withdrawn for use in some countries (Choueiri *et al.,* 2011; Schutz *et al.,* 2011a; Schutz *et al.,* 2011b). Two

neoadjuvant trials have evaluated the addition of bevacizumab to anthracycline-taxane based chemotherapy. Two groups found that the addition of bevacizumab to neoadjuvant chemotherapy improved pathologic response rate of TNBC patients (Miles *et al.,* 2013; von Minckwitz *et al.,* 2012). However, another group found no significant improvement in pathologic response rate using this treatment strategy (Bear *et al.,* 2011). Bello *et al.* (2013) determined that a tyrosine kinase inhibitor; E-3810, showed superior antitumour effects in TNBC when administered concomitantly with paclitaxel. A novel small molecule, E-3810 inhibits vascular endothelial growth factor receptor-1, -2, and -3 and fibroblast growth factor receptor-1 tyrosine kinases. This activity was superior to combination chemotherapies and appeared be involved in proteolytic remodelling of the extracellular matrix (ECM). That, along with the cytotoxic effects of paclitaxel was proposed to contribute to the effective antitumour effects (Bello *et al.,* 2013).

Dasatinib is a small molecule kinase inhibitor of both the src and abl proteins (Finn *et al.,* 2007). Basal-like TNBCs have higher SRC family signalling, which suggests that they might be sensitive to src inhibitors like dasatinib (Boyd *et al.,* 2008). These patients do indeed appear to share a dasatinib-sensitive expression profile which is motivation to develop dasatinib as a novel targeted therapy for TNBC (Huang *et al.,* 2007). The use of cisplatin or carboplatin along with novel targeted therapeutics in TNBC seems promising. The optimal combinations of chemotherapy with tyrosine kinase inhibitors that will result in great synergistic interactions needs to be further explored. Although clinical efficacy for RTK inhibitors in breast cancer remains to be evaluated fully, targeted therapies along with cytotoxic agents may be appropriate for carefully selected subsets of patients, such as African women with triple-negative breast cancer. (Amend *et al.,* 2006).

4.3.3.2. The PI3K, AKT and mTOR Pathway

Phosphatidylinositol 3-kinase (PI3K) is activated through its association with Ras, resulting in the formation of phosphatidylinositol (3,4,5)-triphosphate (PIP_3). This, in turn, leads to the tethering of PH-containing molecules, such as Akt and Rho guanine nucleotide exchange proteins (Rho-GEFs) to the plasma membrane. Akt is also able to inactivate Bad; a protein

known to suppress apoptosis, GSK-3β; an antagonist of cell growth, and activates mTOR; a stimulant of cell growth and protein synthesis (Figure 25) (Weinberg, 2007).

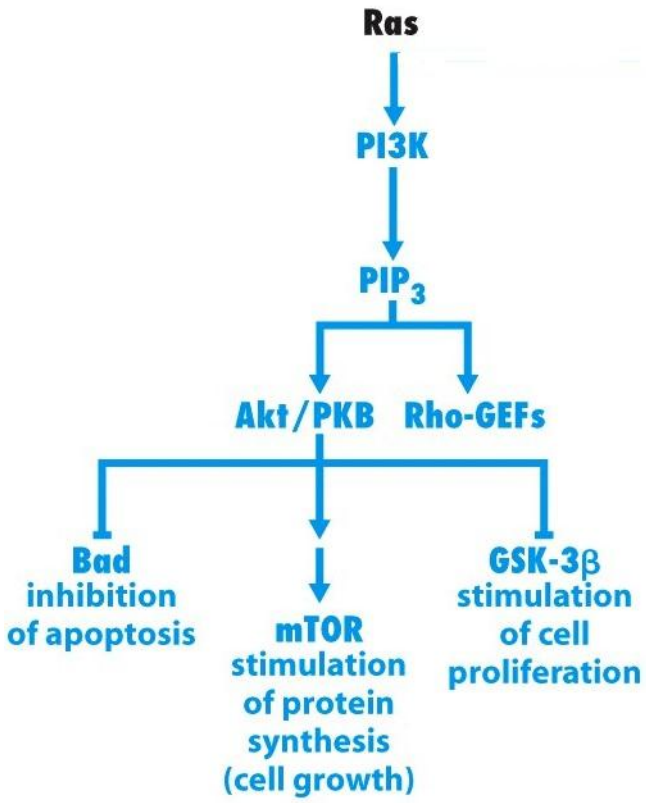


Figure 25: A summarised representation of the PI3K pathway. The aberrant activation of the PI3K pathway leads to excessive inhibition of apoptosis, and promotes cell proliferation and growth through the activation of mTOR and inactivation of Bad and GSK-3β. Adapted from Weinberg, (2007).

The PI3K pathway is frequently dysregulated in human tumours including breast cancers through akt1 and PIK3CA mutations and PTEN loss or mutation (Liu *et al.*, 2007; Saal *et al.*, 2005; Stemke-Hale *et al.*, 2008; Tokunaga *et al.*, 2007). The mammalian target of rapamycin; mTOR, is a drug target for the PI3K/AKT pathway, which is found in two structurally distinct protein complexes; mTOR1 and mTOR2 (Vilar *et al.*, 2011). mTOR1 is positively regulated by AKT, while mTOR2 activates AKT. An increase in AKT expression leads to mTOR1 activation (Rexer *et al.*, 2009). mTOR inhibition has generally proven problematic due to the fact that rapamycin and rapalouges exert an incomplete inhibition on mTORC1 and none on mTORC2. As mTORC2

is one of the major feedback modulators in this pathway, it would explain the limited activity of these drugs (Vilar *et al.,* 2011). These two complexes are regulated by different upstream proteins and several compensatory feedback loops. This allows diversification in signal response and has facilitated the development of novel mTOR inhibitors which inhibit catalytic activity of both mTOR complexes. Due to structural similarity in the catalytic domain of mTOR and the p101α subunit of PI3K, some of these inhibitors are able to inhibit PI3K as well (De *et al.,* 2013; Vilar *et al.,* 2011). These protein complexes are responsible for regulation of growth and proliferation, mRNA translation, cell survival, and cell cycle progression (Jacinto *et al.,* 2004; Thoreen *et al.,* 2012). The PI3K pathway is activated in basal-like TNBCs and up-regulated compared with HER2+ tumours as shown by a significantly increased activation of the downstream targets Akt and mTOR. There is evidence of decreased PTEN levels in these tumours as well. Both PI3K and mTOR inhibitors led to cell growth arrest, and apoptosis was observed specifically after PI3K inhibition (Marty *et al.,* 2008). Neoadjuvant chemotherapy with paclitaxel and everolimus (an mTOR inhibitor) in breast cancer patients with non-responsive tumours to epirubicin/cyclophosphamide, either with or without bevacizumab, did not improve the pathologic complete response, however long term data is not available yet (Huober *et al.,* 2013). NVP-BEZ235 is a dual mTOR/PI3K inhibitor which is able to specifically and effectively block PI3K pathway activation, inducing G_1 arrest (Maira *et al.,* 2008). According to the Lehmann *et al.* (2011) subtyping of TNBC, the mesenchymal-like cell lines preferentially responded to NVP-BEZ235, a dual PI3K/mTOR inhibitor. These results were seen in cell lines with PIK3CA mutations, as well as those without PIK3CA mutations or PTEN deficiencies. This suggests that the PI3K/mTOR pathway is important in the mesenchymal-like TNBC subtype, and other PI3K/mTOR inhibiting drugs should be researched.

4.3.3.3. The MAPK Pathway

Mitogen-activated protein kinase (MAPK) pathways generally consist of a cascade of phosphorylation of kinases, whereby the phosphorylation leads to the activation of the phosphorylated kinase. For example, Ras activates Raf kinase (a MAPKKK), which then proceeds to phosphorylate and activate MEK (a MAPKK). Active MEK is then able to phosphorylate and activate Erk1 and Ertk2 (MAPKs). Subsequently, Erk2 can then go on to

phosphorylate kinases in the cytoplasm to regulate translation as well as nuclear transcription factors (Figure 26) (Weinberg, 2007).

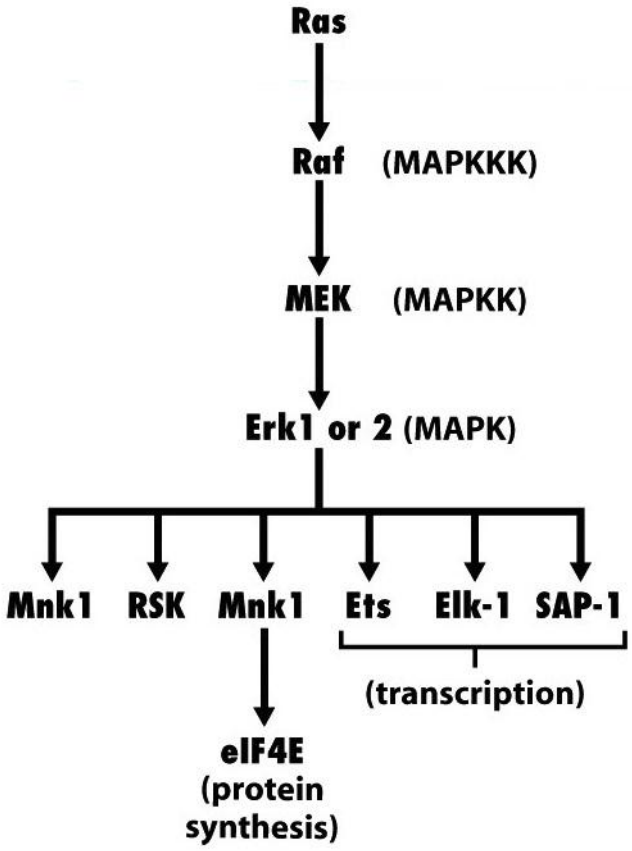


Figure 26: A summarised representation of the MAPK pathway. Activation of MAPK pathways by Ras, for example, leads to a cascade of phosphorylation events which ultimately upregulates transcription factor activity and protein synthesis. Adapted from Weinberg, (2007).

The MAPK pathway is important for cell proliferation, survival, and differentiation, and is frequently upregulated in many human cancers (Chung *et al.*, 2009). Targeting MEK, one of the downstream signal transducers in the pathway has proven to be effective in suppressing many important pathways involved in cell growth and apoptosis (McCubrey *et al.*, 2010b). MEK inhibitors differ from most other kinase inhibitors as they do not compete with ATP-binding (catalytic site) which confers high specificity (Delaney *et al.*, 2002). The advantage of MEK-targeted therapy is that a number of upstream signaling pathways can be blocked with MEK inhibition. Cl-1040 (PD184352) is an orally active, highly specific inhibitor of MEK1 and MEK2 which effectively blocks the phosphorylation of ERK and continued signal transduction

through the MAPK pathway in breast cancer (Allen *et al.,* 2003). As ERK5 deficiency leads to overexpression of VEGF, it may provide for another potential MAPK pathway target which could prevent metastasis and tumour progression (Sohn *et al.,* 2002). Other MEK inhibitors include Selumetinib and AZD6244. Selumetinib is an orally-active MEK1 inhibitor with significant tumour suppressor activity in preclinical models of breast cancer. The effects of selumetinib are enhanced significantly if the tumor has a mutation that activates the Raf/MEK/ERK signaling pathway, as occurs in TNBC (Rexer *et al.,* 2009). AZD6244 (ARRY-142886) is a potent, selective small molecule MEK1/2 inhibitor which is highly active in breast tumours (Yeh *et al.,* 2007). It has the ability to inhibit proliferation and to induce apoptosis and differentiation. When combined with cytotoxic chemotherapy, the efficacy of this inhibitor is enhanced (Davies *et al.,* 2007).

Basal-like TNBCs without PTEN loss respond effectively to single-agent MEK inhibition, while those with PTEN loss benefit from combined MEK/PI3K inhibition. This is due to the upregulation of the PI3K pathway resulting from PTEN loss (Hoeflich *et al.,* 2009; Rexer *et al.,* 2009). The MAPK and PI3K/AKT pathways are role players in drug resistance development in breast cancer, and thus, understanding the relationship between drug and target is crucial for the future of breast cancer treatment (McCubrey *et al.,* 2010a). Duncan et al. assessed kinome activity in response to MEK inhibition in TNBC cells and found that MEK inhibitor-induced RTK stimulation overcame MEK2 inhibition, but not MEK1 inhibition, reactivating ERK, and producing drug resistance. They suggested a kinase inhibitor combination to treat TNBC (Duncan *et al.,* 2012).

4.3.4. Other Potential Targets and TNBC

A RAS inhibitor, R115777, is known to target farnesyl transferase in order to prevent complete post-translational processing of RAS. Although only 5% of breast tumours have RAS mutations, there are several aberrant pathways which lead to the activation of RAS signaling. This is an interesting new target for treating breast cancer and its role, if any, in TNBC should be determined (Dy & Adjei, 2002). Manumycin A (Man A), a farnesyl transferase inhibitor, reduces cancer cell viability in TNBC through induction of non-apoptotic, non-autophagic cytoplasmic

vacuolation death. This compound also mediated the upregulation of p21, p27, and PTEN and downregulation of pAkt. These results support the use of Man A in TNBC treatment regimens (Singha *et al.*, 2013). Lehmann *et al*. (2011) based on their subclassification of TNBC, determined that the LAR subtype was likely to be driven by androgen receptor (AR) signalling. They found this subtype to be sensitive to treatment with AR antagonist bicalutamide and to treatment with 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG), a heat shock protein 90 (Hsp90) inhibitor, suggesting AR inhibition as a possible novel therapy option (Gucalp & Traina, 2010; Lehmann *et al.*, 2011). TNBC was found to be sensitive to a heat shock protein 90 (Hsp90) inhibitor; PU-H71, which showed potent and durable antitumour effects without toxicity or evidence of resistance. PU-H71 induces efficient and sustained downregulation and inactivation of multiple oncoproteins in complex with PU-H71-bound-Hsp90 in TNBC (Caldas-Lopes *et al.*, 2009).

Histone deacetylase (HDAC) inhibitors (HDI) induce endoplasmic reticulum stress and apoptosis, while promoting autophagy, which promotes cancer cell survival when apoptosis is compromised. Rao et al. found that co-treatment with an autophagy inhibitor and a pan-HDI resulted in accumulation of toxic polyubiquitylated proteins, exerted superior inhibitory effects on TNBC cell growth, and increased survival of TNBC xenografts (Rao *et al.*, 2012). Horiuchi *et al.* (2012) found that myelocytomatosis viral oncogene homolog (MYC) expression was elevated in TNBC along with altered expression of MYC regulatory proteins, resulting in increased activity in the MYC pathway. The MYC pathway plays important roles in cell growth and proliferation, metabolism, miRNA regulation, apoptosis, and survival (Dang, 1999; Eilers & Eisenman, 2008; Meyer & Penn, 2008). Cyclin-dependent kinase (CDK) inhibition was effective in TNBC tumours with elevated MYC expression. CDK inhibition increases BIM (a BCL-2 family member) protein expression which plays a role in apoptosis leading to tumour regression (Horiuchi *et al.*, 2012).

Another apoptosis promoting treatment option is to target TNBC cells with overexpression of the survival-promoting protein BCL-2. Treatment with ABT-737 alone was ineffective, however, when combined with docetaxel, showed a significant incease in apoptosis. This suggests that BH3 mimetics sensitise TNBC to chemotherapy, and should be investigated as a potential

combination therapy (Oakes *et al.,* 2012). TNBC cells with high Human antigen R (HuR) expression have alterations in cell cycle kinetics and faster growth rates; however HuR overexpression in mouse models significantly interfered with tumour growth. This appears to be due to an anti-angiogenic effect by increased expression of thromospondin1 (tSp1) and downregulation of VEGF. This suggests that HuR may be regulating genes involved in tumour angiogenesis and that an approach of modulating HuR levels may overcome limitations associated with monotherapies targeting angiogenesis (Gubin *et al.,* 2010).

Individual breast cancers have a variety of expressed fusion genes, some involving genes encoding microtubule-associated serine threonine kinase (MAST2). A TNBC cell line MDA-2B-468 is positive for endogenous ARID1A-MAST2 fusion; this led to the hypothesis that knocking down MAST2 in these cells could have beneficial antitumour effects. Multiple independent MAST2 or ARID1A-MAST2 fusion-specific siRNAs were used to knockdown the ARID1A-MAST2 fusion protein resulting in significant growth inhibition. shRNAs targeted at the MAST2 also showed efficient knockdown in ARID1A-MAST2 fusion transcript and protein, causing a dramatic reduction in growth and increased apoptosis with S-phase arrest (Robinson *et al.,* 2011). Perhaps targeting gene fusions in TNBC is an interesting and novel therapy option to be considered. The membrane bound CSPG4 is differentially expressed in breast cancer subtypes with a significantly higher frequency in TNBCs. There was also an association between level of CSPG4 expression and the frequency of CSCs in pleural effusions in TNBC. Targeting CSPG4 with mAb 225.28 inhibited the growth and metastasis, as well as regrowth of orthotopic primary tumours in human TNBC xenografts. These beneficial effects were associated with reduced proliferation, induction of apoptosis, reduction in vascular density in the tumour microenviroment, and inhibition of activation of signaling pathways regulating cell proliferation, migration, and survival. These findings support the use of CSPG4-specific mAbs and other molecules to target CSPG4 in the treatment of TNBC (Wang *et al.,* 2010).

Characterising associations between breast cancer risk factors and tumor subtypes to which they are related may allow improved risk assessment; and predicting the risk for specific tumor subtypes may lead to targeted early detection of breast cancer and implementation of prevention strategies. It would seem that combination therapy is the best current treatment option for

TNBCs as the targeted therapies available and being developed seem to be only somewhat effective alone. Targeted therapies need to be combined with chemotherapy, radiotherapy, or other targeted therapies in order to be effective (Chappell *et al.*, 2011). More future studies identifying differences in gene expression will help to target and enhance treatments more specifically. It is clear that novel approaches to solving these problems need to be researched and developed. A recent approach to profile miRNAs in TNBC can be used to determine a miRNA signature leading to improved diagnostic and treatment strategies.

Table 18: Targeted Therapies currently being developed for TNBC.

Pathway Targeted	Therapeutic Approach
DNA Repair: BRCA1	PARP Inhibitors
WNT/β-catenin signalling	Anti-WNT ligand mAbs soluble WNT receptor pan ligand inibitors Salinomycin Niclosamide
NOTCH	γ-secretase inhibitors anti-NOTCH mAbs ADAM inhibitors MAML inhibitors
Hedgehog	Anti-HH ligand mAbs small molecule inhibitors of SMO Gli antagonists
TGFβ Pathway	Receptor kinase inhibitors mAbs SMAD4 siRNA IL8 and IL6 inhibition

Growth factor Inhibition	**Anti-VEGF mAbs** **Anti-EGFR mAbs** **EGFR tyrosine kinase inhibitors** **Metformin** **FGFR inhibitors**
PI3K/mTOR signalling	mTOR inhibitors rapamycin analogues PI3K and AKT inhibitors dual PI3K and mTOR inhibitors
Apoptosis	BH3 mimetics BCL2 specific inhibitors TRAIL mAbs
Other	RAS inhibitors AR antagonists Hsp90 inhibitors Histone deacetylase inhibitors CDK inhibitors Fusion gene inhibition

4.4. Overexpressed miRNAs in TNBC

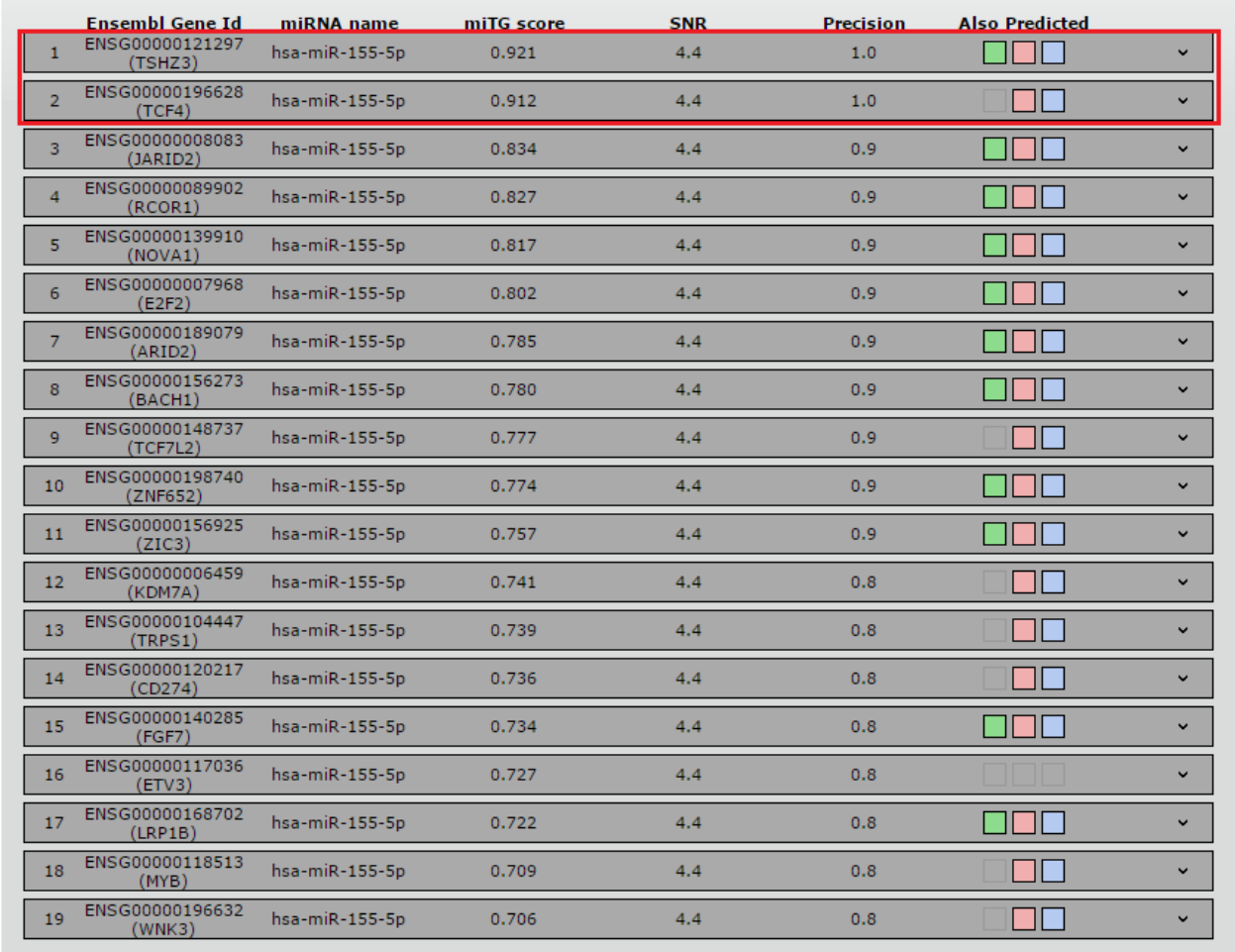
In this section the selected overexpressed miRNAs identified in the present study are discussed. Overexpression is of significance because it leads to the downregulation of expression of predicted protein targets through the miRNA binding at the 3' UTR of mRNA. This binding activates the RISC which is responsible for mRNA degradation and thus, downregulation of protein expression.

4.4.1. hsa-miR-155-5p

There are currently no publications specifically linking miR-155-5p to triple-negative breast cancer; however its overexpression has been implicated in several other diseases including BRCA1-deficient breast cancer. As identified in this study, miR-155-5p had 25 and 73 times higher expression on average when compared to normal breast cells and oestrogen-sensitive breast cancer cells in the TNBC cell lines MDA-MB-231 and MDA-MB-436, respectively. This may suggest that miR-155-5p expression might be useful in the subclassification of TNBC.

Chang *et al.* (2011b) reported that a mutation in BRCA1 led to increased expression of miR-155-5p in mouse embryonic stem cells, and that the knockdown of miR-155-5p in BRCA1-deficient breast cancer cells led to a reduction in tumour growth. This suggests that the two TNBC cell lines investigated in this study are likely to have BRCA1 mutations, which corresponds to previous studies (as mentioned under section 4.3.1.). In relation to p53 expression, Neilsen *et al.* (2013) found miR-155-5p expression to be considerably higher in breast tumours with p53 mutations. Moreover, the knockdown of endogenous p53 mutant R248Q in a breast cancer cell line resulted in significantly lower expression of miR-155-5p, indicating that mutant p53 drives invasion in breast tumours through the upregulation of miR-155-5p. In addition, the overexpression of miR-155 has been linked to tumour angiogenesis in TNBC, providing a basis for the aggressive nature of this breast cancer subtype (Kong *et al.*, 2014). Lastly, TGF-β induces miR-155 expression. When miR-155 was knocked down, it resulted in the suppression of TGF-β-induced EMT and tight junction dissolution, as well as migration and invasion in invasive breast cancer (Kong *et al.*, 2008). Together, these studies suggest that miR-155-5p is a potential therapeutic target for TNBCs with BRCA1 deficiency, p53 mutations, and those driven by TGF-β based EMT, and may be targeted to reduce the chance of metastasis.

In this study, the analysis of miR-155-5p, using the DIANA microT *in silico* prediction tool elucidated several validated targets with differing miTG scores (see Fig 27 below). The targets of significance; including Teashirt Zinc Finger Homeobox 3 (TSHZ3) (miTG score of 0.921) and Transcription factor 4 (TCF4) (miTG score of 0.912) are discussed below.



	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000121297 (TSHZ3)	hsa-miR-155-5p	0.921	4.4	1.0	
2	ENSG00000196628 (TCF4)	hsa-miR-155-5p	0.912	4.4	1.0	
3	ENSG00000008083 (JARID2)	hsa-miR-155-5p	0.834	4.4	0.9	
4	ENSG00000089902 (RCOR1)	hsa-miR-155-5p	0.827	4.4	0.9	
5	ENSG00000139910 (NOVA1)	hsa-miR-155-5p	0.817	4.4	0.9	
6	ENSG00000007968 (E2F2)	hsa-miR-155-5p	0.802	4.4	0.9	
7	ENSG00000189079 (ARID2)	hsa-miR-155-5p	0.785	4.4	0.9	
8	ENSG00000156273 (BACH1)	hsa-miR-155-5p	0.780	4.4	0.9	
9	ENSG00000148737 (TCF7L2)	hsa-miR-155-5p	0.777	4.4	0.9	
10	ENSG00000198740 (ZNF652)	hsa-miR-155-5p	0.774	4.4	0.9	
11	ENSG00000156925 (ZIC3)	hsa-miR-155-5p	0.757	4.4	0.9	
12	ENSG00000006459 (KDM7A)	hsa-miR-155-5p	0.741	4.4	0.8	
13	ENSG00000104447 (TRPS1)	hsa-miR-155-5p	0.739	4.4	0.8	
14	ENSG00000120217 (CD274)	hsa-miR-155-5p	0.736	4.4	0.8	
15	ENSG00000140285 (FGF7)	hsa-miR-155-5p	0.734	4.4	0.8	
16	ENSG00000117036 (ETV3)	hsa-miR-155-5p	0.727	4.4	0.8	
17	ENSG00000168702 (LRP1B)	hsa-miR-155-5p	0.722	4.4	0.8	
18	ENSG00000118513 (MYB)	hsa-miR-155-5p	0.709	4.4	0.8	
19	ENSG00000196632 (WNK3)	hsa-miR-155-5p	0.706	4.4	0.8	

Figure 27: **A screen shot representation of *in silico* DIANA microT predicted targets for miR-155-5p.**

4.4.1.1. Teashirt Zinc Finger Homeobox 3

The miR-155-5p predicted target, Teashirt Zinc Finger Homeobox 3 (TSHZ3) belongs to a gene family consisting of three teashirt zinc finger homeobox genes (TSHZ1, TSHZ2, and TSHZ3). Yamamoto *et al.* (2011) have speculated that this family of genes are novel tumour suppressor genes, having reported that the promoter region of TSHZ3 was hypermethylated in all breast cancer cell lines, including two TNBC cell lines (MDA-MB-231 and MDA-MB-468), thus causing its downregulation. According to our findings, another mechanism of TSHZ3 downregulation in breast cancers, particularly TNBCs, may be due to the overexpression of miR-155-5p which would lead to excessive post-transcriptional downregulation of the gene. The actual role of TSHZ genes/proteins in carcinogenesis is yet to be deciphered, however, they have

been implicated in the Wnt pathway as part of a gene-silencing complex in neuronal cells and can increase Wnt signaling in a β-catenin-dependent manner (Gallet *et al.,* 1999; Onai *et al.,* 2007). TSHZ genes were also identified as transcriptional repressors in Alzheimer's disease (Kajiwara *et al.,* 2009). This gene, in particular, has been related to grade III ER-negative breast tumours as an amplicon driver of the 19q12 locus which encompasses genes related to cancer cell survival. It was found that silencing TSHZ3, along with other 19q12 amplicon drivers via siRNA, selectively reduced cancer cell viability in cells containing amplification of this locus (Natrajan *et al.,* 2012).

Another study found that TSHZ3 transcripts have a lower expression in tumourigenic SCC-9 cells (an oral squamous cell line) and may play a role in the neoplastic transformation of oral keratinocytes. The mechanism behind this transformation was thought to be attributed to altered activity of the polycomb repressive complex-2 (PRC2), which is responsible for histone methylation for the maintenance of stem cell pluripotency and plasticity (Marcinkiewicz & Gudas, 2014; Shen *et al.,* 2008). While it seems that the downregulation of TSHZ3 may be a novel diagnostic marker in TNBC patients and it may be a possible target for therapy. Its role in cancer progression does however need to be more precisely determined.

4.4.1.2. Transcription Factor 4

Transcription factor TCF4 (also known as immunoglobulin transcription factor 2) is a broadly expressed basic helix-loop-helix (bHLH) protein that functions as a homo- or heterodimer. Missense, nonsense, frame-shift and splice-site mutations as well as translocations and large deletions encompassing TCF4 gene cause Pitt-Hopkins syndrome (PTHS), a rare developmental disorder characterized by severe motor and mental retardation, typical facial features and breathing anomalies (Sepp *et al.,* 2012). Aside from influencing the transcription of several genes, TCF4 is the main effector of the Wnt signaling pathway. It's activation of the Wnt pathway leads to the association of β-catenin with cytoplasmic B-Cell Cll/Lymphoma 9-Like protein (BCL9). BCL9 expression has been implicated in EMT in several carcinomas, and inhibition of BCL9 function induced β-catenin translocation from the nucleus to the cell membrane and an epithelial phenotype in kidney carcinoma cells (Brembeck *et al.,* 2004). Their

subsequent translocation to the nucleus, and their association with TCF4, resulting in activation of Wnt target genes involved in cell growth inhibition (Polakis, 2012b). Mutations in TCF4 seem to inactivate the protein in colorectal cancers leading to compromised ability to repress cell growth (Tang *et al.*, 2008). TCF4 also regulates a number of convergent signaling pathways involved in cell differentiation and survival. Knocking down TCF4 in neuroblastoma cells resulted in over-representation of genes involved in TGF-β signaling, EMT, and apoptosis (Forrest *et al.*, 2013).

In relation to TNBC, we would suggest that the overexpression of miR-155-5p may lead to the downregulation of TCF4, which in turn may too reduce its ability to repress cell growth and apoptosis, resulting in increased EMT in TNBC.

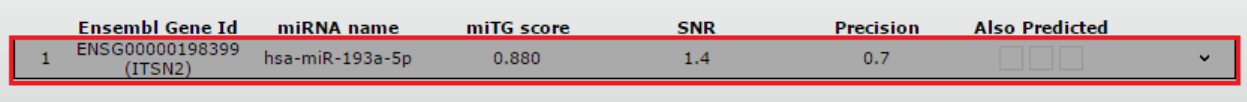
4.4.2. hsa-miR-193a-5p

Interestingly, the two TNBC cell lines analysed in this study both showed a marked increase in miR-193a-5p expression relative to 'normal' breast epithelial cells and ER+ breast cancer cells (about 2.5 times higher in either TNBC cell line on average). The predicted protein target of miR-193a-5p was Intersectin 2 (ITSN2) which is crucial for the initiation stage of clathrin-mediated endocytosis, caveolin-mediated endocytosis, actin cytoskeleton rearrangements, cell signaling, and survival (O'Bryan, 2010; Tsyba *et al.*, 2011).

4.4.2.1. Intersectin 2

The ITSN family consists of two scaffolding proteins (ITSN1 and ITSN2) which mediate protein-protein interactions (Wong *et al.*, 2012). ITSN2 was identified in a gene signature that predicts disease reoccurrence of breast cancer after adjuvant chemotherapy with several different drugs. A high level of ITSN2 was associated with longer disease-free survival (Specht *et al.*, 2009). This indicates that lower expression of ITSN2 may be a valuable prognostic marker in TNBC patients as the lower the expression, the shorter their time to relapse. The lowest levels of ITSN2 were indeed found in ER-negative, PR-negative breast tumours (Tsyba *et al.*, 2011). ITSN2 interacts with epidermal growth factor receptor kinase substrate 8 (Eps8) which is

overexpressed in several tumour types and has been targeted for vaccine development against breast cancer (He *et al.*, 2013b). Eps8 functions as part of the EGFR signaling pathway resulting the induction of Eps8 lysosomal degradation and downregulation of cell surface EGFR, indicating that ITSN2 may function as a tumour suppressor, the exact mechanism, however is undetermined (Ding *et al.*, 2012). With regards to the present study, this may suggest that ITSN2 plays a role in tumourigenesis in TNBCs, as the overexpression of miR-193a-5p would lower ITSN2 levels in cells, leading to the accumulation of Eps8. It is noteworthy that the DIANA microT *in silico* prediction analysis was the only source which identified ITSN2 as a target for miR-193a-5p (Figure 28). Target validation experiments should be performed in order to determine the importance of this potential interaction in TNBC tumourigenesis and progression.



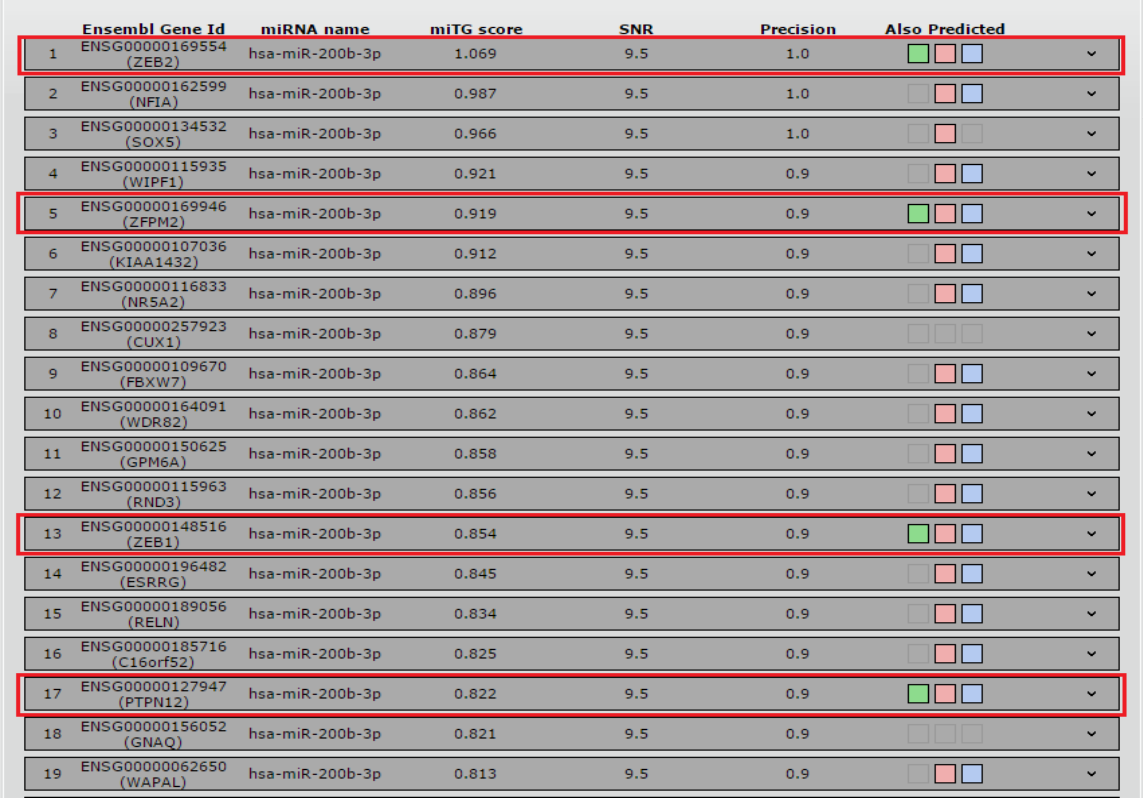
	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000198399 (ITSN2)	hsa-miR-193a-5p	0.880	1.4	0.7	

Figure 28: A screen shot representation of *in silico* DIANA microT predicted targets for miR-193a-5p. Note that ITSN2 is only predicted by DIANA microT and has not been experimentally validated.

4.4.3. hsa-miR-200b-3p and hsa-miR-200c-3p

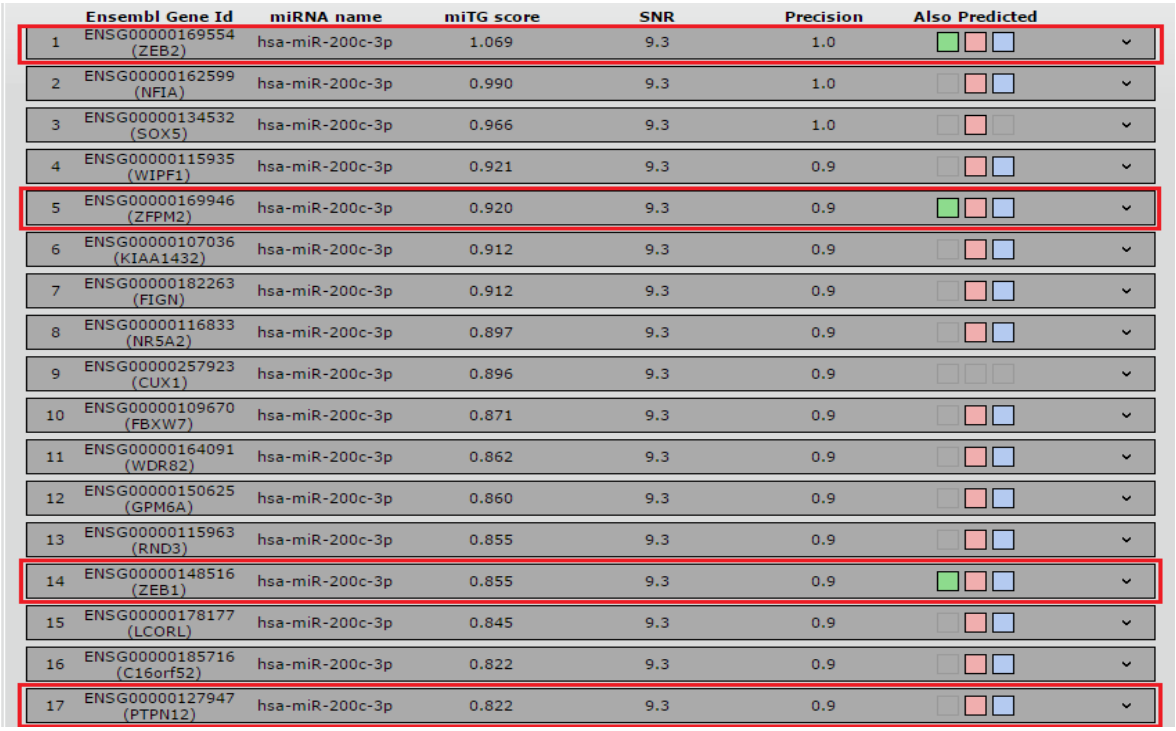
The miR200 family includes 5 miRNAs; miR-200a, -200b, -200c, -141, and -429, and are implicated in suppression of tumour cell motility and invasiveness in vitro (Howe *et al.*, 2012; Korpal *et al.*, 2008). In the present study, miR-200b-3p and miR-200c-3p were significantly overexpressed in TNBC cell lines when compared to hormone sensitive breast cancer and normal breast cell lines. There also appeared to be a marked difference in overexpression of these two miRNAs between the two TNBC cell lines. miR-200b-3p had a 2.65 fold increase, whereas miR-200c-3p had a 3.87 fold increase in expression in MDA-MB-231 and MDA-MB-436 cells respectively. miR-200c-3p had an average fold increase of 24 and 41 in the MDA-MB-231s and MDA-436s respectively. The differences in these fold increases may be useful in subclassification of TNBC types and may have prognostic value, however more insight is needed into the determining the relevance of these fold increases in this family of miRNAs, and their implications in disease progression.

The Enrichment of miR-200 in the serum of patients with metastatic cancers was observed and ectopic expression of miR-200 could confer metastatic capability on non-metastatic tumour cells (Le *et al.,* 2014). Contradictory to our findings, another group found that expression levels of miR-200b were not significantly different between different receptor statuses in breast tumours (Kolacinska *et al.,* 2014). Other opposing studies established that the down-regulation of miR-200 reduced E-cadherin expression to induce EMT, suggesting that more aggressive cancers are more likely to display decreased expression of miR-200 (Humphries *et al.,* 2014; Park *et al.,* 2008; Valastyan & Weinberg, 2009). High miR-200c expression was however correlated with a poorer prognosis in PR-negative breast cancer patients. It indicated a shorter relapse-free period, increased local and distant recurrence, and more frequent distant metastases and was also associated with high grade and low stage tumours (Tuomarila *et al.,* 2014). Taken together, this suggests that miR200c plays a role in invasive breast carcinoma and that PR status along with miR-200c expression may provide a novel prognostic marker in breast cancer. Interestingly, a recent study found opposing results to ours, reporting that miR-200c was underexpressed and miR-221 was overexpressed in TNBC in vitro and speculating that these two miRNAs had antagonistic roles in DICER1 (siRNA) regulation; with miR-221 repressing its activity. They suggested that a lack of DICER activity in TNBC was the reason for a large quantity of under-represented mature, functional miRNAs (Cochrane *et al.,* 2010). Potentially significant targets for these miRNAs are discussed below (Figures 29 and 30).



	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000169554 (ZEB2)	hsa-miR-200b-3p	1.069	9.5	1.0	
2	ENSG00000162599 (NFIA)	hsa-miR-200b-3p	0.987	9.5	1.0	
3	ENSG00000134532 (SOX5)	hsa-miR-200b-3p	0.966	9.5	1.0	
4	ENSG00000115935 (WIPF1)	hsa-miR-200b-3p	0.921	9.5	0.9	
5	ENSG00000169946 (ZFPM2)	hsa-miR-200b-3p	0.919	9.5	0.9	
6	ENSG00000107036 (KIAA1432)	hsa-miR-200b-3p	0.912	9.5	0.9	
7	ENSG00000116833 (NR5A2)	hsa-miR-200b-3p	0.896	9.5	0.9	
8	ENSG00000257923 (CUX1)	hsa-miR-200b-3p	0.879	9.5	0.9	
9	ENSG00000109670 (FBXW7)	hsa-miR-200b-3p	0.864	9.5	0.9	
10	ENSG00000164091 (WDR82)	hsa-miR-200b-3p	0.862	9.5	0.9	
11	ENSG00000150625 (GPM6A)	hsa-miR-200b-3p	0.858	9.5	0.9	
12	ENSG00000115963 (RND3)	hsa-miR-200b-3p	0.856	9.5	0.9	
13	ENSG00000148516 (ZEB1)	hsa-miR-200b-3p	0.854	9.5	0.9	
14	ENSG00000196482 (ESRRG)	hsa-miR-200b-3p	0.845	9.5	0.9	
15	ENSG00000189056 (RELN)	hsa-miR-200b-3p	0.834	9.5	0.9	
16	ENSG00000185716 (C16orf52)	hsa-miR-200b-3p	0.825	9.5	0.9	
17	ENSG00000127947 (PTPN12)	hsa-miR-200b-3p	0.822	9.5	0.9	
18	ENSG00000156052 (GNAQ)	hsa-miR-200b-3p	0.821	9.5	0.9	
19	ENSG00000062650 (WAPAL)	hsa-miR-200b-3p	0.813	9.5	0.9	

<u>Figure 29:</u> A screen shot representation of *in silico* DIANA microT predicted targets for miR-200b-3p.



	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000169554 (ZEB2)	hsa-miR-200c-3p	1.069	9.3	1.0	
2	ENSG00000162599 (NFIA)	hsa-miR-200c-3p	0.990	9.3	1.0	
3	ENSG00000134532 (SOX5)	hsa-miR-200c-3p	0.966	9.3	1.0	
4	ENSG00000115935 (WIPF1)	hsa-miR-200c-3p	0.921	9.3	0.9	
5	ENSG00000169946 (ZFPM2)	hsa-miR-200c-3p	0.920	9.3	0.9	
6	ENSG00000107036 (KIAA1432)	hsa-miR-200c-3p	0.912	9.3	0.9	
7	ENSG00000182263 (FIGN)	hsa-miR-200c-3p	0.912	9.3	0.9	
8	ENSG00000116833 (NR5A2)	hsa-miR-200c-3p	0.897	9.3	0.9	
9	ENSG00000257923 (CUX1)	hsa-miR-200c-3p	0.896	9.3	0.9	
10	ENSG00000109670 (FBXW7)	hsa-miR-200c-3p	0.871	9.3	0.9	
11	ENSG00000164091 (WDR82)	hsa-miR-200c-3p	0.862	9.3	0.9	
12	ENSG00000150625 (GPM6A)	hsa-miR-200c-3p	0.860	9.3	0.9	
13	ENSG00000115963 (RND3)	hsa-miR-200c-3p	0.855	9.3	0.9	
14	ENSG00000148516 (ZEB1)	hsa-miR-200c-3p	0.855	9.3	0.9	
15	ENSG00000178177 (LCORL)	hsa-miR-200c-3p	0.845	9.3	0.9	
16	ENSG00000185716 (C16orf52)	hsa-miR-200c-3p	0.822	9.3	0.9	
17	ENSG00000127947 (PTPN12)	hsa-miR-200c-3p	0.822	9.3	0.9	

<u>Figure 30:</u> A screen shot representation of *in silico* DIANA microT predicted targets for miR-200c-3p.

4.4.3.1. Zinc Finger E Box-Binding Homeobox 1 and Zinc Finger E Box-Binding Homeobox 2

The miR-200 family is involved in regulating tumour cell motility, and more specifically is responsible for the inhibition of EMT through the down-regulation of ZEB1 and ZEB2 which repress transcription of E-cadherin in the TGF-β pathway (Korpal *et al.,* 2008). Since E-cadherin is involved in cell-cell adhesion, a reduction in this protein leads to loss of epithelial integrity with tumour cells gaining invasive and metastatic properties. Low levels of E-cadherin have been noted in most TNBCs, however about a third of TNBCs are positive for E-cadherin. TNBCs negative for E-cadherin but positive for AR were associated with recurrence and metastasis, and a poorer prognosis (Tang *et al.,* 2012). A reduction in E-cadherin levels in TNBC has also been suggested as a prognosis marker as it too is associated with metastasis (Rakha *et al.,* 2007). Due to the seemingly contradictory increased expression of miR-200 reported here, it would be of interest to determine both ZEB1 and ZEB2 levels, as well as E-cadherin expression in these cell lines. It may be that another factor is involved in the repression of these EMT-regulating transcription factors, or alternatively some other factor may be driving the metastasis in some TNBCs; for example, mutations in P53 and RB1 resulted in EMT by reducing E-cadherin in some TNBCs (Jiang *et al.,* 2011). DNA methylation should also be considered as it plays an important role in miR-200b and -200c expression in breast cancer and may be involved in alternative expression of these miRNAs (Jacobsen *et al.,* 2013).

4.4.3.2. Zinc Finger Protein, Multitype 2

Zinc Finger Protein, Multitype 2 (ZFPM2) is a member of the friend of GATA (FOG) family of transcription factors implicated in the modulation of GATA proteins involved in cardiogenesis and haematopoiesis. Defects in the ZFPM2 gene have been associated with congenital heart disorders. A *de novo* deletion in BRCA1 in an early onset breast cancer patient was identified and speculated to be the cause for accumulation of genetic instabilities, including amplification of the ZFPM2 gene (Garcia-Casado *et al.,* 2011). ZFPM2 is also known as FOG2, plays a role in mammary gland involution. The deletion of FOG2 in breast cells led to a decrease in expression of genes responsible for cancerous transformation (Manuylov *et al.,* 2007). This suggests that FOG2 has a protective or tumour suppressor function in breast tissue which is interesting given

that this gene is expected to be downregulated due to the overexpression of miR-200 RNAs. The experimental validation of its mRNA transcript and protein levels would either confirm or refute its involvement in this breast cancer and its precise involvement in TNBC may be ascertained and possibly manipulated for a targeted therapy option. FOG2 also associates with GATA3 in some tissues which is interesting because GATA3 has been implicated in TNBC (Ali *et al.*, 2003). GATA3 is often upregulated, and has been suggested as a potential diagnostic marker in TNBC as it may add accuracy when used in conjunction with other diagnostic markers (Huo *et al.*, 2015). Higher levels of GATA3 have also been associated with a more favorable prognosis in TNBC patients as it inhibits metastasis through EMT (Dydensborg *et al.*, 2009; Usary *et al.*, 2004; Yan *et al.*, 2010; Yu *et al.*, 2013). MSL TNBC cell lines showed a general absence of GATA3, as mentioned previously, both TNBC cell lines in our study were classified as the MSL type (Yu *et al.*, 2013). It would be valid to investigate whether there is a relationship between FOG2 and GATA3 expression in TNBC, and if so, how they interact.

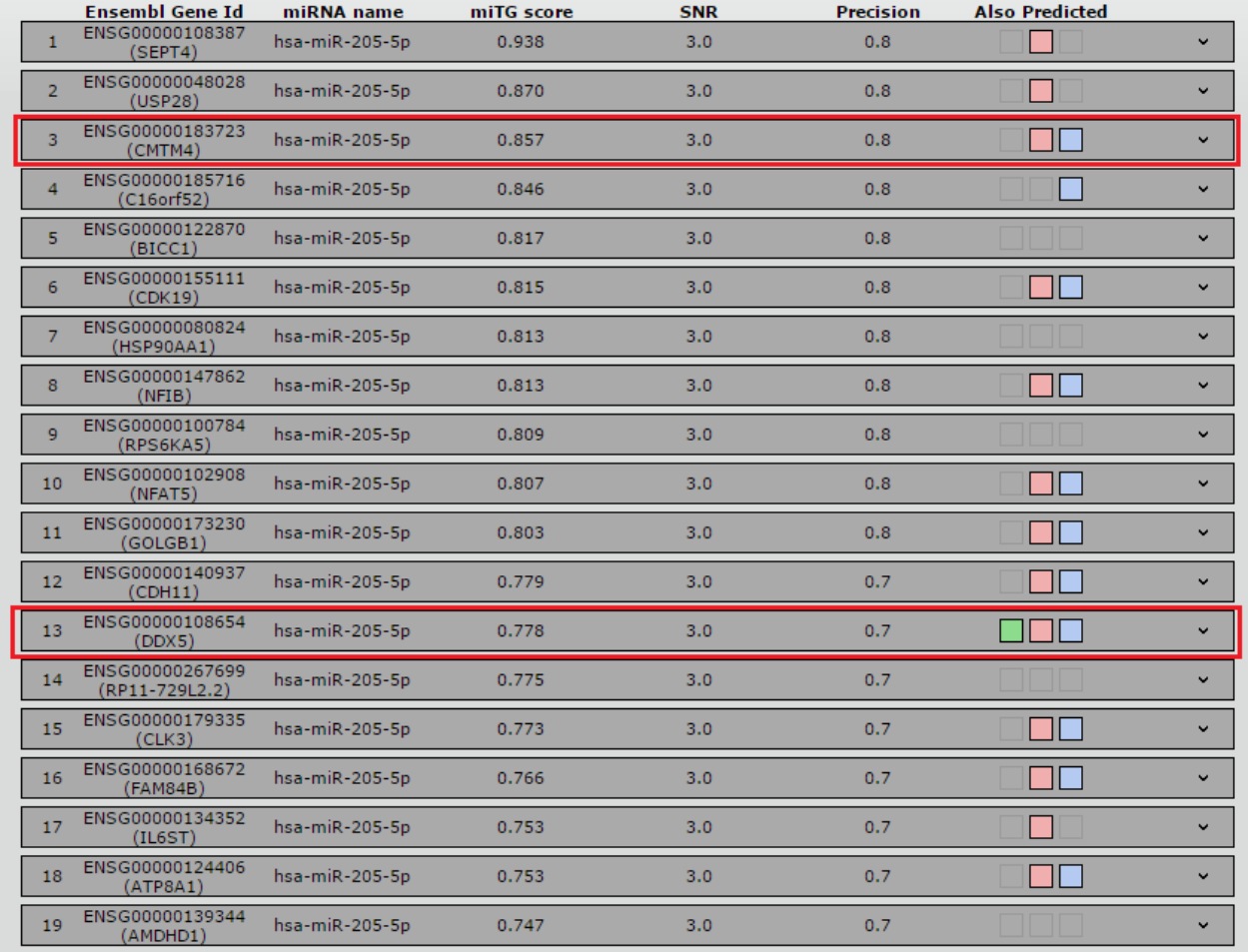
4.4.3.3. Protein-Tyrosine Phosphatase, Nonreceptor-Type, 12

Protein tyrosine phosphatases (PTPs), such as Protein-Tyrosine Phosphatase, Nonreceptor-Type, 12 (PTPN12), and protein tyrosine kinases (PTKs) are involved in the regulation of tyrosine phosphorylation-mediated signaling. Tyrosine-phosphorylated proteins can be specifically dephosphorylated through the action of PTPs, which therefore are likely to have an important role in the control of cellular growth and differentiation (Takekawa *et al.*, 1992). PTPN12 is often compromised and was identified as a tumour suppressor in TNBC as it potently suppresses mammary cell proliferation and transformation by interacting with, and inhibiting multiple oncogenic tyrosine kinases (Luo *et al.*, 2014; Sun e*t al.*, 2011). The overexpression of the miR-200s is seemingly a reason for the downregulation of PTPN12 in TNBC and by targeting those miRNAs it may be possible to upregulate PTPN12 and restore its function as a tumour suppressor. Sun *et al*. (2011) identified other mechanisms causal to the loss of function of PTPN12, including for example, loss of function mutations in its encoding gene and increased expression in miR-124, which is also responsible for regulating PTPN12 expression. It has been noted however that the majority of individuals with SNPs that inactivate PTPN12 tumour suppressor capacity never develop breast cancer, suggesting that the loss of PTPN12 alone is not

sufficient to drive tumour development. Mutations affecting other cancer pathways must be involved in conjunction with the loss of PTPN12 (Albeck & Brugge, 2011).

4.4.4. hsa-miR-205-5p

Yet another member of the miR-200 family; hsa-miR-205, has been implicated in mammary epithelial stem cell maintenance and differentiation (Avril-Sassen *et al.,* 2009; Greene *et al.,* 2010a). Based on a number of studies on the level of expression of this miRNA in different tumour types, it appears that high expression of miR-205 is associated with epithelial cancer types and has a better prognosis, while lower expression is apparent in more invasive cancers (Ayaz *et al.,* 2013; Chen *et al.,* 2014; Childs *et al.,* 2009; Lee *et al.,* 2015; Manikandan *et al.,* 2014; Schaefer *et al.,* 2010; Tang *et al.,* 2014; Wang *et al.,* 2008; Yu *et al.,* 2008). The role of miR-205 in breast cancer is likely dependent on the tumour subtype as well as the cell of origin and stage of progression (Greene *et al.,* 2010b). Enrichment of miR-205 was found in mammary epithelial stem cells and PTEN expression was found to be regulated by this miRNA, as well as ZEB1 and ZEB2 (Greene *et al.,* 2010a). This indicates that miR-205 may act as a tumour suppressor by negatively modulating EMT, cell survival, and proliferation. In HER2-positive breast tumours, miR-205-5p was differentially expressed, upregulation being antitumourigenic through its activation of the AKT/PI3K pathway (Iorio *et al.,* 2009; Li *et al.,* 2013). Ectopic expression of this miRNA significantly inhibited cell proliferation, invasion, and anchorage-dependent growth, as well as lung metastasis in different breast cancer subtypes, including TNBC. The mechanism for this antitumour effect was attributed to the direct targeting of VEGF-A and erbB3 by miR-205 (Wu *et al.,* 2009). Several targets were predicted *in silico* for miR-205-5p, two of which may be of some relevance (Figure 31).



	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000108387 (SEPT4)	hsa-miR-205-5p	0.938	3.0	0.8	
2	ENSG00000048028 (USP28)	hsa-miR-205-5p	0.870	3.0	0.8	
3	ENSG00000183723 (CMTM4)	hsa-miR-205-5p	0.857	3.0	0.8	
4	ENSG00000185716 (C16orf52)	hsa-miR-205-5p	0.846	3.0	0.8	
5	ENSG00000122870 (BICC1)	hsa-miR-205-5p	0.817	3.0	0.8	
6	ENSG00000155111 (CDK19)	hsa-miR-205-5p	0.815	3.0	0.8	
7	ENSG00000080824 (HSP90AA1)	hsa-miR-205-5p	0.813	3.0	0.8	
8	ENSG00000147862 (NFIB)	hsa-miR-205-5p	0.813	3.0	0.8	
9	ENSG00000100784 (RPS6KA5)	hsa-miR-205-5p	0.809	3.0	0.8	
10	ENSG00000102908 (NFAT5)	hsa-miR-205-5p	0.807	3.0	0.8	
11	ENSG00000173230 (GOLGB1)	hsa-miR-205-5p	0.803	3.0	0.8	
12	ENSG00000140937 (CDH11)	hsa-miR-205-5p	0.779	3.0	0.7	
13	ENSG00000108654 (DDX5)	hsa-miR-205-5p	0.778	3.0	0.7	
14	ENSG00000267699 (RP11-729L2.2)	hsa-miR-205-5p	0.775	3.0	0.7	
15	ENSG00000179335 (CLK3)	hsa-miR-205-5p	0.773	3.0	0.7	
16	ENSG00000168672 (FAM84B)	hsa-miR-205-5p	0.766	3.0	0.7	
17	ENSG00000134352 (IL6ST)	hsa-miR-205-5p	0.753	3.0	0.7	
18	ENSG00000124406 (ATP8A1)	hsa-miR-205-5p	0.753	3.0	0.7	
19	ENSG00000139344 (AMDHD1)	hsa-miR-205-5p	0.747	3.0	0.7	

Figure 31: A screen shot representation of *in silico* DIANA microT predicted targets for miR-205-5p.

Surprisingly, it was found here that miR-205-5p expression was almost 5 times higher in MDA-MB-231, and just over double in MDA-MB-436 TNBC cell lines, relative to normal breast cells and oestrogen-sensitive breast cancer cells. *In silico* target prediction identified a novel target; as being DEAD homeobox 5 (DDX5), commonly referred to as p68.

4.4.4.1. DEAD homeobox 5

DEAD box proteins are putative RNA helicases and are implicated in RNA metabolism, cell division, and EMT (Kittler *et al.*, 2004; Wang *et al.*, 2012; Yang *et al.*, 2006). The upregulation of DDX5 has previously been reported in the majority of malignant breast carcinomas, its expression strongly being correlated with that of Ki-67 and EGFR in TNBC. The knockdown of

DDX5 resulted in reorganisation of actin cytoskeleton, reduction of proliferation, upregulation of tumour suppressor PDCD4, and downregulation of miR-182, a modulator of cytoskeleton maintenance (Wang *et al.,* 2012). miR-182 is overexpressed in TNBC and promotes cell proliferation while potentially negatively regulating profilin 1 (PFN1) expression. Profilin 1 has been implicated in breast cancer previously as both a tumour suppressor and an oncogene and its downregulation has been identified in metastatic and invasive breast cancers (Choi *et al.,* 2014; Coumans *et al.,* 2014; Diamond *et al.,* 2015; Joy *et al.,* 2014; Rizwani *et al.,* 2014). Knockdown experiments of miR-182 led to decreased cell proliferation and increased apoptosis (Liu *et al.,* 2013a). Further evidence indicates the importance of DDX5 as it is linked to BRCA1 which promotes processing of pro-tumourigenesis pri-miRNAs through its direct association with Drosha and p68 (DDX5) of the Drosha microprocessing complex (Kawai & Amano, 2012). P53 also associates with p68 in the Drosha complex in order to promote the maturation of miRNAs with growth-suppressive properties (Suzuki *et al.,* 2009). A gradual loss of cytoplasmic Drosha expression was observed along tumour progression from DCIS, to invasive and metastatic breast cancer cell lines. This loss of Drosha was associated with BRCA1 and ER expression, and with a poorer prognosis in TNBC (Khoshnaw *et al.,* 2013). The loss of p68 function within the Drosha complex may be implicated in the invasiveness of TNBC. Most interestingly, another study identified TCF4 as a positive feedback loop regulator of DDX5 in the canonical Wnt pathway in breast cancer progression. As β-catenin/TCF4 mediated DDX5 regulation plays an important role in the upregulation of EMT marker proteins in TNBC (Guturi *et al.,* 2014). This suggests that DDX5 is a potential therapeutic target in TNBC to reduce EMT.

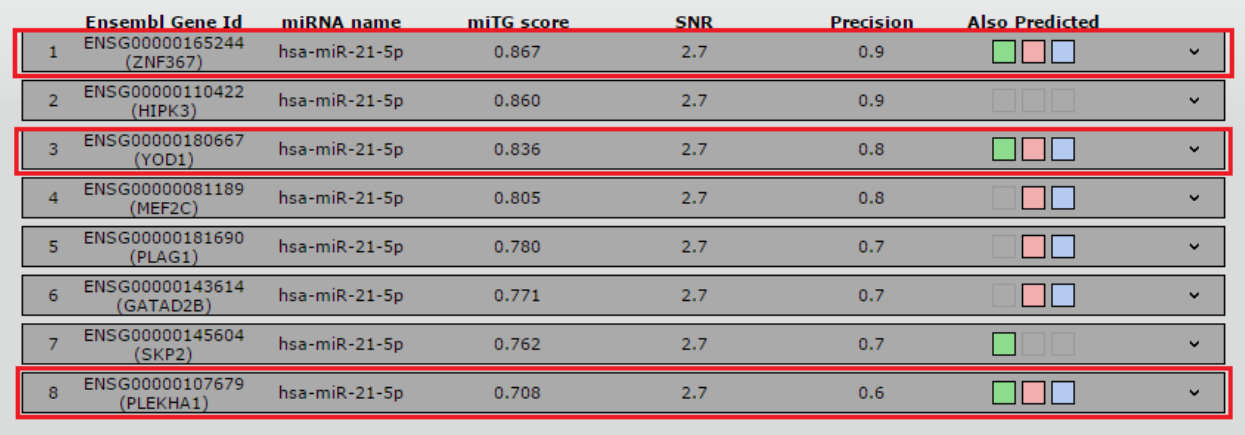
4.4.4.2. CKLF-like marvel transmembrane domain-containing 4

CKLF-like marvel transmembrane domain-containing 4 (CMTM4) expression is needed for normal cell division (Kittler *et al.,* 2004). Its downregulation due to overexpression of miR-205-5p may be implicated in abnormal cell division. Downregulation of other proteins from the same family have been implicated in several different tumour types but has not been identified or studied in TNBC as yet (Li *et al.,* 2014a). Wang *et al.* (2014a) did however find that CMTM4 was overexpressed in a TNBC cell line, and additionally that its knockdown promoted TNF-α-

induced apoptosis. The role of CMTM4 in breast cancer and particularly TNBC, tumourigenesis requires further investigation as a novel therapeutic target.

4.4.5. hsa-miR-21-5p

miR-21-5p overexpression was very prominent in the TNBC cell lines. There was also a large difference in expression of this miRNA between the two TNBC cell lines, indicating that it may be useful in the subclassification of TNBCs. The average expression of miR-21-5p in the MDA-MB-231 was about 76 times higher than normal or oestrogen-sensitive cell lines, while expression in the MDA-MB-436 cell line was on average, about 165 times higher. Overexpression of miR-21 has been reported in the majority of cancers to date and targets several components of the P53, TGF-β, and mitochondrial apoptosis tumor-suppressive pathways (Papagiannakopoulos *et al.,* 2008). RAS is an oncogene in the MAPK signaling pathway which regulates transcription of proteins involved in cell growth and differentiation. Overexpression of RAS results in overactivity of the MAPK pathway, which leads to excessive growth and proliferation. The RAS pathway is often dysregulated in TNBC. A zinc finger transcription factor; Krüppel-like factor 4 (KLF4) promotes the expression of miR-21 and miR-206, which in turn promotes RAS-ERK signaling in TNBC (Sharma *et al.,* 2014). A study found that overexpression of miR-21 in MCF-7 breast cancer cells resulted in increased soft agar colony formation, suggesting an increased tumourigenicity. The mechanism was speculated to be inhibition of apoptosis as miR-21 binds the 3' UTR of the apoptotic gene programmed cell death 4 (PDCD4), thus downregulating the Pdcd4 protein (Lu *et al.,* 2008). Overexpression of miR-21 promotes tumour cell growth by downregulating krev interaction trapped 1(KRIT1) expression (Orso *et al.,* 2013). KRIT1 is of importance as it is associated with EMT, angiogenesis, and cell-cell adhesion in different studies (Gunel *et al,.* 2002; Maddaluno *et al.,* 2013; Marchuk *et al.,* 2003). In this study, three predicted targets were elucidated using DIANA microT, these being ZNF367, YOD1 and PLEKHA1 (Figure 32). Since each of these was predicted by three different bioinformatic tools, this indicates a raised significance and each is discussed below.



	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000165244 (ZNF367)	hsa-miR-21-5p	0.867	2.7	0.9	
2	ENSG00000110422 (HIPK3)	hsa-miR-21-5p	0.860	2.7	0.9	
3	ENSG00000180667 (YOD1)	hsa-miR-21-5p	0.836	2.7	0.8	
4	ENSG00000081189 (MEF2C)	hsa-miR-21-5p	0.805	2.7	0.8	
5	ENSG00000181690 (PLAG1)	hsa-miR-21-5p	0.780	2.7	0.7	
6	ENSG00000143614 (GATAD2B)	hsa-miR-21-5p	0.771	2.7	0.7	
7	ENSG00000145604 (SKP2)	hsa-miR-21-5p	0.762	2.7	0.7	
8	ENSG00000107679 (PLEKHA1)	hsa-miR-21-5p	0.708	2.7	0.6	

Figure 32: A screenshot representation of *in silico* DIANA microT predicted targets for miR-21-5p.

4.4.5.1. Zinc finger protein 367

Zinc finger protein 367 (ZNF367), also known as ZFF29 and CDC14B, is a member of the zinc finger protein family. It is normally expressed in embryonic or foetal erythroid tissue and is absent in other normal adult tissues (Asano *et al.,* 2004; Gilligan *et al.,* 2002). ZNF367 is overexpressed in a variety of endocrine cancers causing cell invasion, migration, and adhesion. Its overexpression was related to a loss of miR-195 expression (Jain *et al.,* 2014). miR-21-5p may be another miRNA involved in the modulation of ZNF367 expression. Overexpression of miR-21-5p would be expected to decrease the levels of ZNF367 in TNBC due to increased DICER activity, which is contradictory to previous studies. It may be that there is another mechanism involved, or that ZNF367 functions differently in epidermal tissue as opposed to endocrine tissues. Additional research would be necessary to determine the relationship between miR-21-5p and ZNF367, and to elucidate their potential role in TNBC progression.

4.4.5.2. Ovarian Tumour Deubiquitinating Enzyme 1

YOD1 is a deubiquitinating enzyme (DUB) of the ovarian tumour family whose function is yet to be fully determined in mammalian physiology. YOD1 has been identified as a component in the P97 dislocation complex which is involved in the ubiquitin proteasome system (UPS). UPS is the major pathway for the destruction of misfolded proteins which takes place in the cytosol.

Dislocation is the process whereby misfolded proteins are translocated into the cytosol for destruction by UPS (Ernst *et al.,* 2009). Aberrant proteostasis can trigger apoptosis and it has been proposed that targeting UPS and other cellular stress response systems may be a target in cancer treatment (Liu & Ye, 2011). It has been speculated that the P97 complex is involved in controlling proliferation and its activity is altered in cancer cells. There is also the possibility that this complex plays a role in maintaining cancer viability through the clearing of potentially toxic misfolded proteins. This suggests that the P67 complex may be a potential target for cancer therapy (Haines, 2010). Multiple DUBs have been classified as tumour suppressors or oncogenes based on their regulatory roles of proteins involved in tumour development (Fraile *et al.,* 2012). YOD1 was identified as having a potential carcinogenic role in bladder cancer (Catto *et al.,* 2009). The specific role of YOD1 has not been fully elucidated yet, but it may be involved in tumourigenesis of TNBC. Future studies should be performed to assess the potential benefits of targeting this DUB for TNBC patients.

4.4.5.3. Pleckstrin Homology Domain-Containing Protein

Pleckstrin Homology Domain-Containing Protein (PLEKHA1), also known as TAPP1, has not been studied in much detail regarding cancer, and especially breast cancer pathology. It has however been shown to interact with multiple PDZ domain protein (MPDZ) which is implicated in protein-protein interactions, and was identified as an oncogene prognostic marker in breast cancer cell lines (Kimber *et al.,* 2002; Tsai *et al.,* 2011). PLEKHA1 was one of five mesenchymal-enriched inclusion events found in basal-like TNBC cell lines, indicating that it may be involved in the EMT and the increased cellular invasiveness of the aggressive phenotype of TNBC (Shapiro *et al.,* 2011). As the cellular functions of PLEKHA1 are largely unknown; it would be of interest to study this protein and its role in cancer biology, particularly in the context of TNBC.

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4.4.6. hsa-miR-296-5p

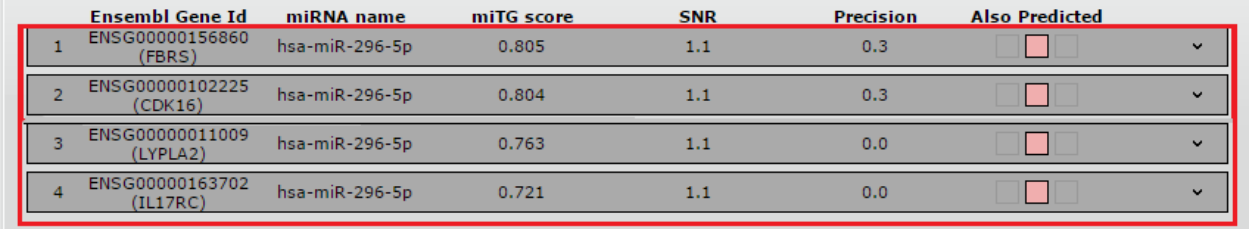
miR-296-5p is a central regulator of signaling pathways affecting development, stem cell differentiation and cancer progression. Further, it is a nodal miRNA deregulated in diverse human cancers. In the present study, there was significant overexpression of miR-296-5p recorded in both of the TNBC cell lines, with an average fold increase of 10, in comparison to the two control cell lines. This difference in expression suggests that this miRNA may be a useful diagnostic marker in TNBC.

There was significant overexpression of miR-296-5p seen in the two TNBC cell lines. There was an average fold increase of 10 in comparison to the two control cell lines. This suggests that this miRNA may be a useful diagnostic marker in TNBC. miR-296-5p was underexpressed in primary breast tumours which was correlated to the shortest disease-free survival and earlier metastases. This came as a result of overexpression of its target SCRIBBLE1 (SCRIB) which is a cytoplasmic multimodular scaffold protein targeted to epithelial adherens junctions and neuronal presynaptic compartments and plays a role in breast carcinogenesis (Savi *et al.,* 2014). miR-296-5p was also implicated in chemotherapy resistance by inducing quiescence in breast tumour cells (van Jaarsveld *et al.,* 2014). Another study found that low expression of miR-296-5p was linked to overexpression of PIN1 in prostate cancer. PIN1 overexpression plays a role in cell proliferation and anchorage-dependent growth in prostate cancer cell lines (Lee *et al.,* 2014a). The above studies have identified miR-296-5p as a tumour suppressor which is contradictory to our findings. However, in TNBC, miR-296-5p overexpression may downregulate tumour suppressor proteins through different pathways.

None of the predicted gene targets for miR-296-5p were sufficiently validated by other prediction algorithms, nor did they meet the selection criteria for significance (Figure 33). The top three targets have not been linked to triple negative breast cancer and a search for publications was negative. Nevertheless, one potential target, fibrosin is discussed below. There is a possibility that none of these predicted targets are real based on the DIANA microT results, as all of them have low precision and low miTG scores. There may be other unidentified targets of this miRNA which are implicated in TNBC tumourigenesis.

4.4.6.1. Fibrosin

Fibrosin (FBRS), also known as FBS1, is a lymphokine secreted by activated lymphocytes that induces fibroblast proliferation (Prakash & Robbins, 1998). Fibroblasts are a component in human connective tissue and are implicated in EMT, angiogenesis, metastasis, and cell survival (Balakrishnan *et al.*, 2013; Keller *et al.*, 2012; Pavlides *et al.*, 2012). Further, it is implicated in the development of fibrosis; when expressed, it stimulates proliferation of myofibroblasts and increases production of alpha smooth muscle actin fibers (α SMA). These specialised cells are involved in wound contraction and in retractile phenomena observed during fibrotic disease (Prakash *et al.*, 2007). Although there is currently no data linking fibrosin to any kind of breast cancer, it would be interesting to assess its validity as a target for miR-296-5p (Bastid *et al.*, 2014; Cochaud *et al.*, 2013).



	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000156860 (FBRS)	hsa-miR-296-5p	0.805	1.1	0.3	
2	ENSG00000102225 (CDK16)	hsa-miR-296-5p	0.804	1.1	0.3	
3	ENSG00000011009 (LYPLA2)	hsa-miR-296-5p	0.763	1.1	0.0	
4	ENSG00000163702 (IL17RC)	hsa-miR-296-5p	0.721	1.1	0.0	

Figure 33: A screenshot representation of *in silico* DIANA microT predicted targets for miR-296-5p.

4.4.7. hsa-miR-342-3p

The overexpression of miR-342-3p in the two TNBC cell lines was quite different. The MDA-MB-231 cell line showed overexpression of about 5 fold higher than the two control cell lines, while the MDA-MB-436s was expressed approximately 33 times higher than in the control cell lines. The marked difference in expression may represent a novel diagnostic marker in TNBC. In addition, it may potentially aid with the subclassification of TNBCs.

The literature reports on the overexpression of miR-342 wherein it upregulated BRCA1 expression through its target inhibitor of DNA binding 4 (ID4) in TNBC cell line MDA-MB-231 (Crippa *et al.*, 2014). ID4 is part of a family of proteins known to inhibit binding to DNA and

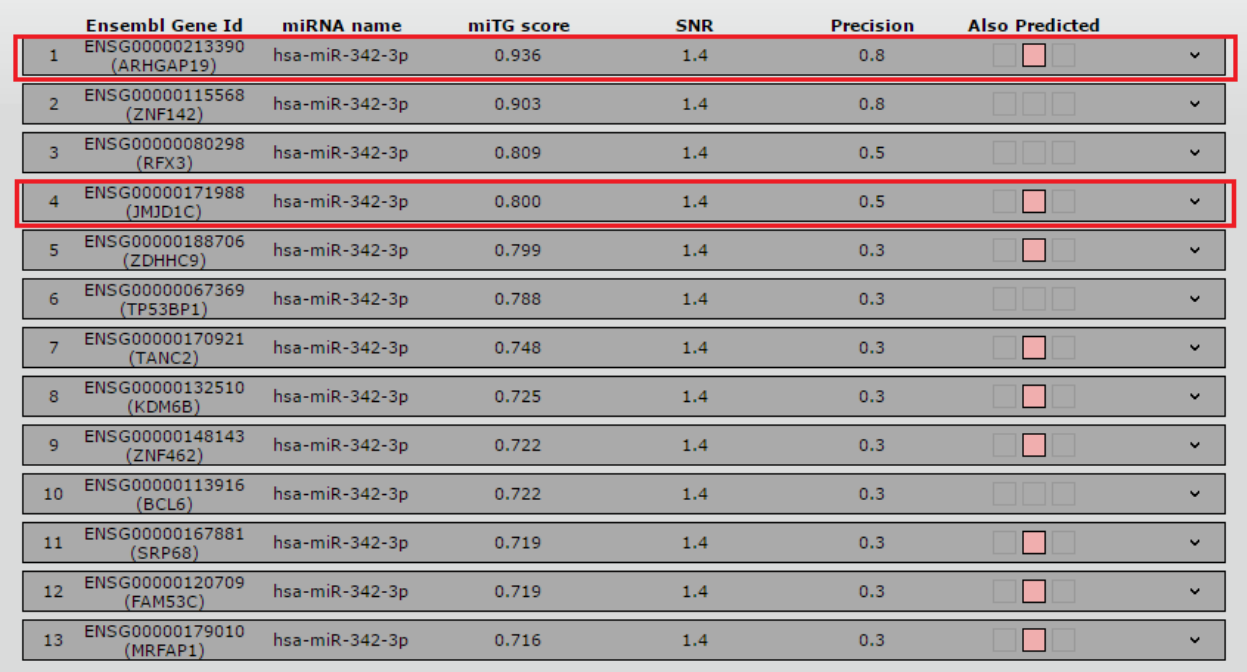
transcriptional transactivation by heterodimerization with basic helix-loop-helix proteins. Interestingly, downregulation of miR-342 in oestrogen-sensitive MCF-7 breast cancer cells was associated with the development of tamoxifen resistance (Cittelly *et al.,* 2010). Further, a positive correlation has been demonstrated between miR-342 expression and ER mRNA levels in MCF-7 breast cancer cells. Ectopic expression of miR-342 in MCF-7 cells also sensitised them to tamoxifen (He *et al.,* 2013a). This might suggest that miR-342 has a role in ER status in breast cancer, and its expression may be manipulated in TNBC to allow for treatment by hormone therapies. miR-342-3p expression was altered in postpartum breast tumours which occur in younger women are more aggressive (Munoz-Rodriguez *et al.,* 2014). Decreased expression of miR-342 was observed in TNBC cells, contradictory to our findings (Lowery *et al.,* 2009). Decreased expression was also observed in cancer-associated fibroblasts suggesting that this miRNA may be involved in cancer cell proliferation, invasion, and metastasis (Zhao *et al.,* 2012).

4.4.7.1. Rho GTPase Activating Protein 19

Rho GTPase Activating Protein 19 (ARHGAP19) was the only target of miR-342-3p with an miTG score greater than 0.9 (Figure 34), suggesting its significance as a valid target It is part of the Rho-activating family of proteins which is responsible for negative regulation of Rho GTPases, which are intrinsic to cell migration, proliferation, and differentiation, actin remodeling, and G1 cell cycle progression (Lv *et al.,* 2007). ARHGAP19 has yet to be linked specifically to TNBC, however other members of this protein family and proteins involved in the Rho pathway, have been implicated in TNBC tumourigenesis (Feng *et al.,* 2014; Okada *et al.,* 2015; Zeng *et al.,* 2012).

4.4.7.2. Jumonji domain-containing 1C

Jumonji domain containing 1C (JMJD1C) for example, was downregulated in breast cancer tissue suggesting a tumour suppressor role in breast cancer (Wolf *et al.,* 2007). The role of this miRNA remains to be elucidated in TNBC tumourigenicity.



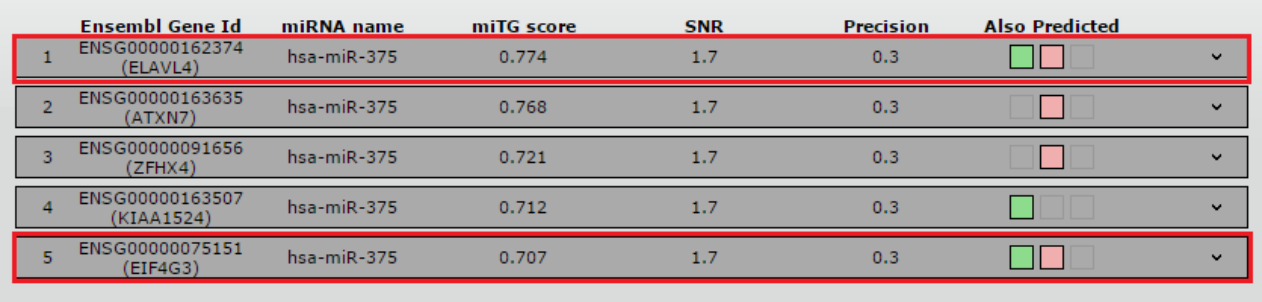
	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000213390 (ARHGAP19)	hsa-miR-342-3p	0.936	1.4	0.8	
2	ENSG00000115568 (ZNF142)	hsa-miR-342-3p	0.903	1.4	0.8	
3	ENSG00000080298 (RFX3)	hsa-miR-342-3p	0.809	1.4	0.5	
4	ENSG00000171988 (JMJD1C)	hsa-miR-342-3p	0.800	1.4	0.5	
5	ENSG00000188706 (ZDHHC9)	hsa-miR-342-3p	0.799	1.4	0.3	
6	ENSG00000067369 (TP53BP1)	hsa-miR-342-3p	0.788	1.4	0.3	
7	ENSG00000170921 (TANC2)	hsa-miR-342-3p	0.748	1.4	0.3	
8	ENSG00000132510 (KDM6B)	hsa-miR-342-3p	0.725	1.4	0.3	
9	ENSG00000148143 (ZNF462)	hsa-miR-342-3p	0.722	1.4	0.3	
10	ENSG00000113916 (BCL6)	hsa-miR-342-3p	0.722	1.4	0.3	
11	ENSG00000167881 (SRP68)	hsa-miR-342-3p	0.719	1.4	0.3	
12	ENSG00000120709 (FAM53C)	hsa-miR-342-3p	0.719	1.4	0.3	
13	ENSG00000179010 (MRFAP1)	hsa-miR-342-3p	0.716	1.4	0.3	

Figure 34: A screen shot representation of *in silico* DIANA microT predicted targets for miR-332-3p.

4.4.8. hsa-miR-375

miR-375 has been identified as both a tumour suppressor and oncogenic miRNA in breast cancer based on its targets (Shafi *et al.,* 2014). With regards to this study, we report here that miR-375 was overexpressed in both TNBC cell lines, relative to the two control cell lines; MDA-MB-231 had about 3 fold higher expression, while the MDA-MB-436s had about 5 times higher expression on average.

In both breast and head and neck cancers, low expression of miR-375 resulted in an increase in expression of its target metadherin (MTDH) (Hui *et al.,* 2011; Shafi *et al.,* 2014). Ectopic expression of miR-375 in head and neck cancer cells led to decreased cell viability, cell survival, metastasis, and *in vivo* tumour formation (Hui *et al.,* 2011). Though miR-375 was downregulated in oral squamous epithelial carcinomas, its targets were however not analysed (Towle *et al.,* 2014). The targets of this particular miRNA do require verification and their roles in cancer progression examined, particularly in TNBC. Of the 5 targets predicted *in silico* for miR-375 all lacked significant miTG or precision scores, and only 2 were validated in two other ways (Figure 35).



	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000162374 (ELAVL4)	hsa-miR-375	0.774	1.7	0.3	
2	ENSG00000163635 (ATXN7)	hsa-miR-375	0.768	1.7	0.3	
3	ENSG00000091656 (ZFHX4)	hsa-miR-375	0.721	1.7	0.3	
4	ENSG00000163507 (KIAA1524)	hsa-miR-375	0.712	1.7	0.3	
5	ENSG00000075151 (EIF4G3)	hsa-miR-375	0.707	1.7	0.3	

Figure 35: A screenshot representation of *in silico* DIANA microT predicted targets for miR-375.

4.4.8.1. ELAV Like Neuron-Specific RNA Binding Protein 4

ELAV Like Neuron-Specific RNA Binding Protein 4 (ELAVL4), also known as HUD, has been implicated in the development of T-cell leukaemia through its involvement in the Notch signaling pathway (Bellavia *et al.,* 2007). HUD overexpression has also been implicated in the progression of neuroblastoma through inhibition of RNA decay mediated by MYCN. This suggests that HUD may contribute to the malignant phenotype of neuroblastoma (Manohar *et al.,* 2002). This target is yet to be linked to any type of breast cancer.

4.4.8.2. Eukaryotic translation initiation factor 4-gamma 3

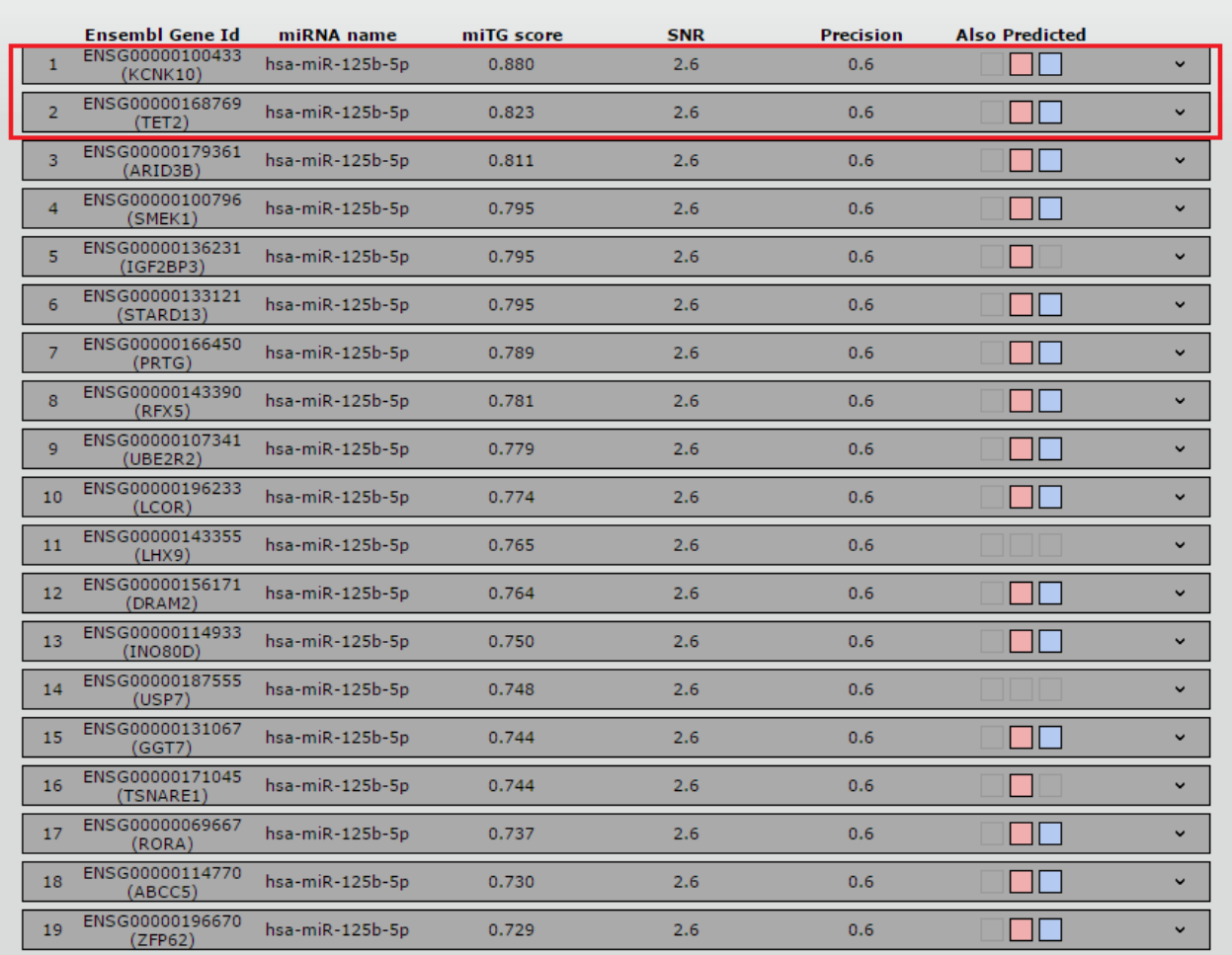
Eukaryotic translation initiation factor 4-gamma 3 (eIF4G3) is a protein synthesis initiation factor in eukaryotes associated with the promotion of cancer. Upregulation of this target has been identified in breast cancer, and regulates both cap-dependant and cap-independent protein synthesis initiation, resulting in cancer cell survival, proliferation, and tumour progression (de la Parra *et al.,* 2012; Wagner *et al.,* 2014). Another study postulated that a decrease in the germline copy number of eIF4G3 may lead to reduction or failure in translation of some transcripts which may confer malignant potential to breast cells (Suehiro *et al.,* 2013). Overexpression of miR-375 may lead to downregulation of certain gene expression modulated by eIF4G3 which may result in the same malignant potential in TNBC cells.

4.5. Underexpressed miRNAs in TNBC

In the present study very few targets predicted for the underexpressed miRNAs determined as significantly different to the control lines meet the criteria for significance. It is clear that the targets for these miRNAs need to be properly validated in order to make any conclusions regarding their specific roles in TNBC biology. Possible links to breast cancer are discussed below.

4.5.1. hsa-miR-125b-5p

miR-125-5p is part of the miR-125 family which play crucial roles in many cellular processes, including differentiation, proliferation, and apoptosis, and are often downregulated in disease states (Bi *et al.,* 2012; Bousquet *et al.,* 2012; Ge *et al.,* 2011; Sun *et al.,* 2013). In the present study, miR-125b-5p showed an average decrease in expression in the two TNBC cell lines of about 9 fold, when compared to the control cell lines. Downregulation of this miRNA has not been associated with TNBC in any way thus far. It has however been implicated in several other types of cancer including prostate, bladder, lymphomas, oesophagel, and colorectal (Canturk *et al.,* 2014; Goto *et al.,* 2015; Liao *et al.,* 2014; Manfè *et al.,* 2013; Wang *et al.,* 2015). When miR-125b was overexpressed in the HER2-overexpressing breast cancer cell line SKBR3, this resulted in the suppression of both ERBB2 and ERBB3 at the transcript and the protein level. This subsequently led to impaired anchorage-dependent growth and a reduction in migration and invasion capacity by reducing phosphorylation of ERK1/2 and AKT (Scott *et al.,* 2007). As indicated by the analysis here, there is potential of this miRNA as a promising biomarker and therapeutic marker in TNBC. Two potential targets of miR-125b-5p which are of interest are potassium channel, subfamily K, member 10 (KCNK10) and TET oncogene family, member 2 (TET2) (see fig 36).



	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000100433 (KCNK10)	hsa-miR-125b-5p	0.880	2.6	0.6	
2	ENSG00000168769 (TET2)	hsa-miR-125b-5p	0.823	2.6	0.6	
3	ENSG00000179361 (ARID3B)	hsa-miR-125b-5p	0.811	2.6	0.6	
4	ENSG00000100796 (SMEK1)	hsa-miR-125b-5p	0.795	2.6	0.6	
5	ENSG00000136231 (IGF2BP3)	hsa-miR-125b-5p	0.795	2.6	0.6	
6	ENSG00000133121 (STARD13)	hsa-miR-125b-5p	0.795	2.6	0.6	
7	ENSG00000166450 (PRTG)	hsa-miR-125b-5p	0.789	2.6	0.6	
8	ENSG00000143390 (RFX5)	hsa-miR-125b-5p	0.781	2.6	0.6	
9	ENSG00000107341 (UBE2R2)	hsa-miR-125b-5p	0.779	2.6	0.6	
10	ENSG00000196233 (LCOR)	hsa-miR-125b-5p	0.774	2.6	0.6	
11	ENSG00000143355 (LHX9)	hsa-miR-125b-5p	0.765	2.6	0.6	
12	ENSG00000156171 (DRAM2)	hsa-miR-125b-5p	0.764	2.6	0.6	
13	ENSG00000114933 (INO80D)	hsa-miR-125b-5p	0.750	2.6	0.6	
14	ENSG00000187555 (USP7)	hsa-miR-125b-5p	0.748	2.6	0.6	
15	ENSG00000131067 (GGT7)	hsa-miR-125b-5p	0.744	2.6	0.6	
16	ENSG00000171045 (TSNARE1)	hsa-miR-125b-5p	0.744	2.6	0.6	
17	ENSG00000069667 (RORA)	hsa-miR-125b-5p	0.737	2.6	0.6	
18	ENSG00000114770 (ABCC5)	hsa-miR-125b-5p	0.730	2.6	0.6	
19	ENSG00000196670 (ZFP62)	hsa-miR-125b-5p	0.729	2.6	0.6	

<u>Figure 36:</u> A screenshot representation of *in silico* DIANA microT predicted targets for miR-125-5p.

4.5.1.1. Potassium Channel, Subfamily K, Member 10

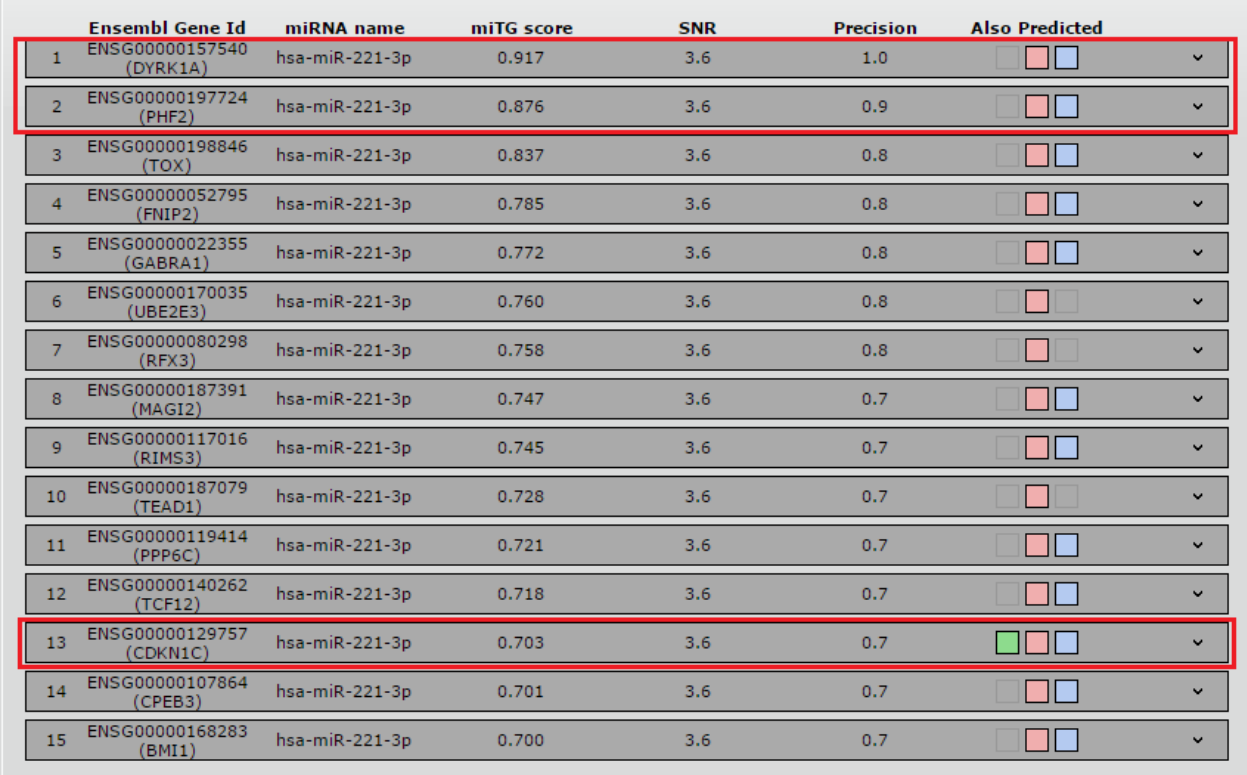
KCNK10, also known as TREK2 is a member of potassium channel proteins which have recently been identified as key players in cell behaviors linked to cancer, including regulation of cell proliferation, migration, apoptosis, and angiogenesis (Comes *et al.*, 2014; Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011; Lang *et al.*, 2005; Schwab *et al.*, 2012). Currently, its role in hormone insensitive breast cancer is unknown. KCNK10 was reported as being underexpressed in hormone-sensitive breast carcinomas compared to normal breast tissue which is contradictory to our findings (Williams *et al.*, 2013). In contrast, TREK2 overexpression was evident in ovarian cancers (Innamaa, 2013). KCNK10 needs to be validated as a true target for miR-125b-5p and its role in TNBC tumourigenicity determined.

4.5.1.2. TET oncogene family, member 2

TET2 is part of the family of methylcytosine dioxygenases which is known to silence the anti-metastatic miR-200 family through demethylation of its promoter region (Song *et al.,* 2013). This suggests that overexpression of TET2 may play a role in EMT in breast carcinoma. TET2 has been implicated in resistance to breast cancer therapy and mutations in the TET2 gene has been observed in many types of cancer (Bhat-Nakshatri *et al.,* 2013; Katz *et al.,* 2014). More insight into the role of TET2 in TNBC pathology is needed to potentially develop a targeted therapy aimed at repressing its overexpression.

4.5.2. hsa-miR-221-3p

The TNBC cell lines investigated here showed marked downregulation of miR-221-3p expression, relative to the two control cell lines. The MDA-MB-231's had almost 10 times less expression than the control lines, while the MDA-MB-436's had approximately 5 times less expression. Due to this great difference in expression between the two TNBC cell lines, there may well be potential for this miRNA to be used as a subclassification marker in TNBC. Currently evidence indicates that miR-221 and miR-222 play a role in oestrogen expressing cell lines, since both were found to interact directly with the 3' UTR of the ER gene. Further, the ectopic expression of these miRNAs in ER-positive cell lines resulted in a reduction in ER protein expression, but not mRNA. Conversely, the knockdown of these two miRNAs partially restored ER protein expression. In relation to the TNBC cell line MDA-MB-468, knockdown of miR-221 and -222 resulted in increased tamoxifen sensitivity, indicating that these two miRNAs play an important role in the regulation of ER expression. It was suggested that they and could be targeted in TNBC patients in conjunction with tamoxifen treatment (Zhao *et al.,* 2008). There are no other links between miR-221-3p and TNBC thus far. Our data suggest three targets of interest for miR-221-3p, these being DRYK1A, PHF2, and CDKN1C (Figure 37). Each of these targets are discussed further below.



	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000157540 (DYRK1A)	hsa-miR-221-3p	0.917	3.6	1.0	
2	ENSG00000197724 (PHF2)	hsa-miR-221-3p	0.876	3.6	0.9	
3	ENSG00000198846 (TOX)	hsa-miR-221-3p	0.837	3.6	0.8	
4	ENSG00000052795 (FNIP2)	hsa-miR-221-3p	0.785	3.6	0.8	
5	ENSG00000022355 (GABRA1)	hsa-miR-221-3p	0.772	3.6	0.8	
6	ENSG00000170035 (UBE2E3)	hsa-miR-221-3p	0.760	3.6	0.8	
7	ENSG00000080298 (RFX3)	hsa-miR-221-3p	0.758	3.6	0.8	
8	ENSG00000187391 (MAGI2)	hsa-miR-221-3p	0.747	3.6	0.7	
9	ENSG00000117016 (RIMS3)	hsa-miR-221-3p	0.745	3.6	0.7	
10	ENSG00000187079 (TEAD1)	hsa-miR-221-3p	0.728	3.6	0.7	
11	ENSG00000119414 (PPP6C)	hsa-miR-221-3p	0.721	3.6	0.7	
12	ENSG00000140262 (TCF12)	hsa-miR-221-3p	0.718	3.6	0.7	
13	ENSG00000129757 (CDKN1C)	hsa-miR-221-3p	0.703	3.6	0.7	
14	ENSG00000107864 (CPEB3)	hsa-miR-221-3p	0.701	3.6	0.7	
15	ENSG00000168283 (BMI1)	hsa-miR-221-3p	0.700	3.6	0.7	

Figure 37: A screenshot representation of *in silico* DIANA microT predicted targets for miR-221-3p.

4.5.2.1. Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1a

The dual-specificity tyrosine phosphorylation-regulated kinase 1a (DYRK1A) has to-date not been especially associated with TNBC. It does however regulate the MAP kinase and ERK signaling pathways in neuronal differentiation, these being central pathways involved in tumourigenesis. Overexpression of DYRK1A is known to potentiate nerve growth factor-mediated neuronal differentiation through the upregulation of the MAPK signaling pathway and also prolongs ERK activation through its interaction with Ras, Braf, and MEK1. This suggests that DYRK1A is required for modulation of neuronal differentiation (Kelly & Rahmani, 2005). It is proposed here that overexpression of this target may be involved in the MAPK pathway activation, so contributing to TNBC tumourigenicity. DYRK1A has also been shown to negatively regulate intrinsic apoptosis in the developing retina through its association with caspase-9 (Laguna *et al.*, 2008). Thus, it is feasible that DYRK1A overexpression could have a functional role in the inhibition of apoptosis in TNBC. It has indeed been found that DYRK1A

inhibits the activity of forkhead O transcription factors (FOXOs) which have been implicated in breast cancer tumourigenesis and modulate apoptosis along with other cellular processes (Yang & Hung, 2009). Experimental verification of this target will clarify whether it truly does play a role in TNBC progression.

4.5.2.2. Phd finger protein 2

Phd finger protein 2 (PHF2) belongs to a diverse group of transcriptional regulators affecting eukaryotic gene expression by influencing chromatin structure (Hasenpusch-Theil *et al.*, 1999). Downregulation of miR-221-3p would be expected to result in overexpression of its target genes, however one study found that PHF2 had tumour suppressor properties and its deletion or other inhibition of expression resulted in breast cancer tumourigenesis in younger women (Sinha *et al.*, 2008). PHF2 deletions present in colon cancer were also associated with P53 function, thus having a tumour suppressive role (Lee *et al.*, 2014b). It may be that PHF2 has both tumour suppressor and oncogenic roles depending on the tissue it acts on. Currently, its potential role in TNBC is unknown.

4.5.2.3. Cyclin-dependent kinase inhibitor 1C

The cyclin-dependent kinase inhibitor 1C (CDKN1C) gene encodes p57 (KIP2), a potent tight-binding inhibitor of several G1 cyclin/Cdk complexes and a negative regulator of cell proliferation (Lee *et al.*, 1995). Decreased expression of CDKC1 leads to accelerated G1-S transition, which has been suggested to support breast cancer (Yoo & Hennighausen, 2012). CDKN1C has been identified as a tumour suppressor gene in TNBC which potentially contradicts our findings (Barrdahl *et al.*, 2015; Vasilatos *et al.*, 2013). There may be another regulatory mechanism behind its oncogenic role or it may not be a real target for miR-221-3p.

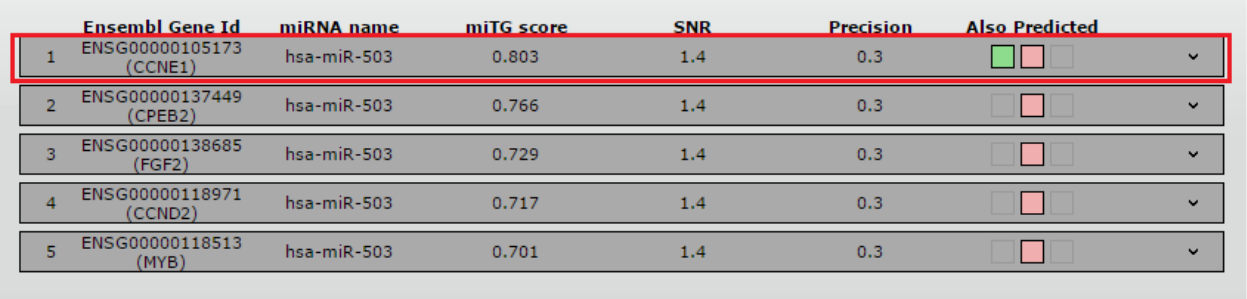
4.5.3. hsa-miR-503

As reported here, miR-503 expression was almost tripled in the two TNBC cell lines compared to the hormone sensitive cancer cells and the normal breast cells. Since mir-424-503 is a polycistronic microRNA cluster that promotes differentiation and G1 arrest of the cell cycle, it follows that its downregulation may result in excessive cell proliferation (Forrest *et al.,* 2009). Downregulation of this cluster may thus result in excessive cell proliferation. One study found that BCL2 and IGF1R were targets for miR-503 involved in the TGF-β pathway during involution of mammary tissue after lactation. The downregulation of BCL2 and IGF1R results in apoptosis and tissue remodelling (Llobet-Navas *et al.,* 2014). Abbarent expression of miR-503 could play a role in breast cancer development during milkstasis. Conversely, miR-503 downregulation was associated with resistence to cisplatin in gastric cancer cells due to the upregulation of IGF1R and BCL2. Ectopic expression of miR-503 resulted in inhibition of proliferation and sensitised the cancer cells to cisplatin-induced apoptosis (Wang *et al.,* 2014b). miR-503 has been inversely related to metastasis and targets protooncogene DDHD2, suggesting that it may have a tumour supressor role in breast cancer biology (Gong *et al.,* 2014; Polioudakis *et al.,* 2015).

4.5.3.1. Cyclin E1

A potentially important target, cyclin E1 (CCNE1) a cell cycle regulatory gene has been experimentally validated as a target for miR-503 (Figure 38) (Forrest *et al.,* 2009; Nakashima *et al.,* 2010). Cyclin E1 (CCNE1) forms a complex with cyclin-dependent kinase 2 (CDK2) to regulate cell cycle progression from G1 to S phase (synthesis phase where DNA replication takes place), and has kinase-independent activities, such as DNA replication (Caldon & Musgrove, 2010). Overexpression of cyclin-E accelerates entry into S phase and promotes polyploidy, independent of CDK2, disrupted DNA replication, and genomic instability (Akli & Keyomarsi, 2003; Matsumoto & Maller, 2004). This results in a shorter G1 and a longer S phase, and may reneder cells dependant on homologous recombination DNA repair (Zariwala *et al.,* 1998). With regards to breast cancer, cyclin-E1? was overexpressed in grade III ER-negative breast cancers, and when inhibited, the cancer cells showed reduced viability (Natrajan *et al.,* 2012). Other

studies have also report that increased expression of cyclin-E leads to hyperplasia and breast carcinoma, and further, has been related to a poorer prognosis (Bortner & Rosenberg, 1997; Porter *et al.*, 1997). However, as amplification of CCNE1 is not associated with dysfunction of the BRCA1 pathway , these tumours are insensitive to PARP inhibitors (Etemadmoghadam *et al.*, 2013). CCNE1 is downregulated by miR-15a which has been found to be downregulated in TNBC, this may suggest that miR-15a works with miR-503 to regulate cyclin-E expression and cell cycle progression (Luo *et al.*, 2013). Thus, cyclin-E inhibition presents as a good target for novel therapy of TNBC, possibly together with with chemotherapy.



	Ensembl Gene Id	miRNA name	miTG score	SNR	Precision	Also Predicted
1	ENSG00000105173 (CCNE1)	hsa-miR-503	0.803	1.4	0.3	
2	ENSG00000137449 (CPEB2)	hsa-miR-503	0.766	1.4	0.3	
3	ENSG00000138685 (FGF2)	hsa-miR-503	0.729	1.4	0.3	
4	ENSG00000118971 (CCND2)	hsa-miR-503	0.717	1.4	0.3	
5	ENSG00000118513 (MYB)	hsa-miR-503	0.701	1.4	0.3	

Figure 38: A screenshot representation of *in silico* DIANA microT predicted targets for miR-503.

Further studies regarding these particular gene targets may be beneficial to development of novel targeted therapies for TNBC, especially used in conjunction with chemotherapy and radiation. Combination therapy where several miRNAs and/or targets are targeted simultaneously is likely to be the best strategy for treating TNBCs as a whole. Once further classification of TNBC is possible, it should be possible to develop even more indiviualised treatment strategies for patients.

4.6. Subclassification of TNBC

Triple-negative breast cancer is a highly diverse disease which has been difficult to effectively sub-divide into significant groups due to a lack of clear classification criteria. This presents a major challenge to the development of targeted therapy for TNBCs. Lehmann *et al.* (2011) managed to subclassify TNBC into 6 groups based on gene expression profiling and gene cluster analysis. According to their analysis, the six TNBC subtypes differed in their aberrantly activated or dysregulated pathways. The two basal-like (BL1 and BL2) subtypes had higher expression of cell cycle and DNA repair genes, while the mesenchymal (M) and mesenchymal stem-like (MSL) subtypes were enriched for EMT and growth factor pathways. These findings are consistent with our data; the two TNBC cell lines used in our study were both categorised as MSL TNBC, and they seem to have a miRNA profile reflecting upregulation in EMT and growth factor signaling pathways (Table 15). Although this subclassification system helps to select a particular pathway to target in TNBC therapy, it is not particularly specific as to which element in the pathway should be targeted. Another limitation with gene expression analysis is that aberrant gene expression may be caused by other factors in tumour biology and targeting a specific gene may not always be an effective treatment option.

In relation to our findings, we suggested that miRNA expression profiles be used to subclassify TNBCs, as our two TNBC cell lines showed a significant difference of 25% in miRNA expression, either greater than two-fold increase or decrease in expression (Figure 19). This result is very significant based on the subclassification of both the TNBC cell lines assessed here as MSL, by Lehmann *et al*. (2011), indicating that there are large variations within their subtypes. This implies that TNBC could very likely be subclassified more precisely, into more groups which may be able to be targeted with the same therapies. While some of the miRNAs discussed above may serve as important markers in the subclassification of TNBC, the entire list of differentially expressed miRNAs is included in appendix A (Table A17). Another study reported in the literature adopted miRNA, mRNA, and DNA analysis in order to subclassify breast cancer, reporting that the different subtypes showed clear differences in miRNA expression patterns (de Rinaldis *et al.*, 2013). This provides good evidence that miRNA profiles are a potential novel system for reliable subclassification of TNBC. The best way to subclassify

TNBCs is probably to implement a system where both miRNA expression profiles and receptor status are used in combination.

5. Future Prospects

Both TNBC cell lines investigated in this study were derived from Caucasian women, however it would be interesting to profile miRNAs from TNBC cell lines derived from African women to compare whether different cellular pathways are affected, and how this might affect the aggressive potential of TNBC in these different populations. A candidate cell line would be MDA-2B-468 which has ductal carcinoma histology and falls within the basal-like 1 subtype based on gene expression analysis. This cell line is intrinsically subtyped as basal and basal subtyped as basal A. It has mutations in *PTEN*, *RB1*, *SMAD4*, and *TP53* which is notably different to the mutations in the two Caucasian cell lines (Lehmann *et al.,* 2011).

Several other miRNAs showed significant over- or under-expression in TNBC cell lines compared to the "normal" and hormone-sensitive cell lines. Further research should be done to determine the implications of such cellular changes in order to determine roles of other significant miRNAs in TNBC tumour development and progression. Further investigation should be done to validate the findings of this study in the laboratory by performing knockdown or knockout experiments in order to determine actual protein targets of aberrantly expressed miRNAs in TNBC cell lines. Once those relationships have been established it would be possible to try silencing particular miRNAs as a potential targeted therapy. The production of small molecule inhibitors of particular targets may be worth investigating as well. miRNA levels in blood plasma of cancer patients seems to have a unique profile dependant on cancer type (Chen *et al.,* 2008). Liu *et al*. (2008), found that miR-155 was overexpressed in the serum of breast cancer patients and was directly correlated with clinical stage and Ki-67 inversely correlated with P53 status (Liu *et al.,* 2013b). It would be of great interest to profile serum of TNBC patients to establish a profile which could add prognostic value as well as an easy and efficient diagnostic tool.

There appears to be a significant difference between different TNBC cell lines in terms of miRNA expression profiles (25% difference in this study). These differences could be useful in subclassification of TNBC types which could lead to more personalised treatment for patients as well as a more accurate prognosis. Currently TNBC is only classified according to receptor status which does not even begin to describe how complex the differences between different TNBCs are. It is worth profiling all TNBC cell lines in order to determine the difference in miRNA expression which could elucidate more defined parameters for subclassification and better treatment.

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Appendix A: Results

Table A1: MiRNAs common to both TNBC cell lines (MDA-2B-231 and MDA-2B-436), with at least a two-fold increase in average expression, in comparison to normal breast cells (MCF-10A) and hormone sensitive breast cancer cells (MCF-7) respectively.

miRNA	Expression Level				*Average Fold Increase*	
	MCF-10A	MCF-7	MDA-2B-231	MDA-2B-436	*MDA-2B-231*	*MDA-2B-436*
hsa-miR-155-5p	1.89	1.85	46.76	136.91	*24.9*	*72.93*
hsa-miR-193a-5p	38.16	32.26	88.3	82.88	*2.53*	*2.37*
hsa-miR-200b-3p	23.03	28.69	67.58	98.69	*2.65*	*3.87*
hsa-miR-200c-3p	28.61	29.2	691.3	1184.39	*23.92*	*40.98*
hsa-miR-205-5p	39.99	37.05	185.24	87.09	*4.81*	*2.27*
hsa-miR-21-5p	1.89	1.85	142.66	309.64	*76*	*164.94*
hsa-miR-296-5p	13.23	22.92	149.37	183.95	*8.9*	*10.96*
hsa-miR-342-3p	8.58	19.75	140.54	519.06	*4.66*	*33.03*
hsa-miR-375	25.4	34.23	78.94	143.56	*2.71*	*4.92*

Table A2: MiRNAs common to both TNBC cell lines (MDA-2B-231 and MDA-2B-436), with at least a two-fold decrease in average expression, in comparison to normal breast cells (MCF-10A) and hormone sensitive breast cancer cells (MCF-7) respectively.

miRNA	Expression Level				*Average Fold Decrease*	
	MCF-10A	MCF-7	MDA-2B-231	MDA-2B-436	*MDA-2B-231*	*MDA-2B-436*
hsa-miR-125b-5p	10569.48	8685.89	1110.36	1113.87	*8.67*	*8.64*
hsa-miR-221-3p	737.69	786.36	77.86	167.21	*9.78*	*4.56*
hsa-miR-503	125.20	138.11	50.80	45.98	*2.59*	*2.86*

Table A3: **miRNAs underexpressed in TNBC cell line MDA-2B-231 relative to hormone-sensitive breast cancer and normal breast cells.**

miRNA	MCF-10A	MCF-7	MDA-2B-231
hsa-let-7i-5p	826.8525		397.26
hsa-miR-125b-5p	10569.4875	8685.895	1110.3625
hsa-miR-181a-5p	426.2525		178.965
hsa-miR-221-3p	737.6925	786.36	77.8675
hsa-miR-4443	182.32		44.1625
hsa-miR-503	125.2	138.1125	50.8025
hsa-miR-518a-3p	29.9575		14.33
hsa-miR-100-5p		450.215	48.9325
hsa-miR-556-5p		16.1	7.825
hsa-miR-591		24.9925	12.1975
hsa-miR-593-3p		31.6	13.88
hsa-miR-92b-3p		41.6925	16.755

Table A4: **miRNAs underexpressed in TNBC cell line MDA-2B-436 relative to hormone-sensitive breast cancer and normal breast cells.**

miRNA	MCF-10A	MCF-7	MDA-2B-436
hsa-miR-1	31.97	40.39	15.02
hsa-miR-100-5p	721.21	450.215	73.6975
hsa-miR-10a-5p	75.63	67.4025	23.1475
hsa-miR-1224-5p	38.815	41.38	17.9425
hsa-miR-1234	70.52	70.885	21.02
hsa-miR-1236	47.02	58.02	20.2875
hsa-miR-1238	48.8875	49.4525	10.77
hsa-miR-1245b-3p	49.105	58.835	18.37
hsa-miR-1246	1.8975	1.8575	0.5475
hsa-miR-1248	38.1375	36.8775	15.56
hsa-miR-1250	26.2925	31.2425	12.72
hsa-miR-1253	1.8975	1.8575	0.5475
hsa-miR-1255b-5p	46.0025	44.8425	17.675
hsa-miR-1257	48.4525	66.5325	14.28
hsa-miR-125b-5p	10569.49	8685.895	1113.878
hsa-miR-1262	31.79	32.72	14.875
hsa-miR-1267	74.1275	85.4975	19.015
hsa-miR-1270	54.98	70.5425	19.305
hsa-miR-1273c	39.3175	38.5275	13.7825

hsa-miR-1273e	24.7575	24.53	10.8625
hsa-miR-1273f	22.8425	35.16	7.7525
hsa-miR-127-3p	34.1875	36.815	16.3725
hsa-miR-1277-3p	45.2475	57.995	21.645
hsa-miR-1283	1.8975	1.8575	0.5475
hsa-miR-1285-3p	54.3525	54.3625	11.975
hsa-miR-1286	86.9325	85.9275	18.02
hsa-miR-1288	48.5725	51.9375	10.7375
hsa-miR-1291	45.645	45.915	16.0275
hsa-miR-1293	21.155	24.16	9.655
hsa-miR-1323	42.4025	42.4875	20.0375
hsa-miR-139-3p	38.1775	42.57	16.645
hsa-miR-142-3p	58.95	60.525	15.18
hsa-miR-142-5p	37.9075	27.3425	12.2625
hsa-miR-143-3p	1.8975	1.8575	0.5475
hsa-miR-150-5p	20.44	20.33	9.13
hsa-miR-1825	27.27	31.395	12.265
hsa-miR-1827	117.835	128.2825	18.3475
hsa-miR-185-5p	101.505	102.4525	38.635
hsa-miR-188-5p	221.2625	278.21	71.09
hsa-miR-1908	37.955	49.7625	17.7275
hsa-miR-1912	39.4625	39.225	12.575
hsa-miR-1913	17.6475	23.9575	7.255
hsa-miR-1915-3p	27.91	37.8675	10.155
hsa-miR-192-5p	41.4425	40.44	11.97
hsa-miR-1972	22.8625	23.6175	11.3425
hsa-miR-199a-5p	43.5925	59.8475	19.3525
hsa-miR-2054	22.4225	23.2625	11.0325
hsa-miR-206	41.34	41.08	20.4575
hsa-miR-2113	43.475	50.5775	12.355
hsa-miR-216b	58.1575	57.375	26.0175
hsa-miR-219-2-3p	19.0875	18.48	8.4325
hsa-miR-221-3p	737.6925	786.36	167.2125
hsa-miR-2277-3p	42.1425	45.785	10.365
hsa-miR-2682-5p	88.9	108.0975	24.3025
hsa-miR-297	122.2625	137.1125	56.495
hsa-miR-302b-3p	48.765	50.1325	18.6325
hsa-miR-302d-3p	178.33	223.455	47.075
hsa-miR-302f	30.1	32.64	14.855
hsa-miR-3127-5p	20.93	23.825	10.3325
hsa-miR-3136-5p	36.525	38.025	15.305
hsa-miR-3151	21.825	23.73	8.655
hsa-miR-3164	25.5575	34.105	8.895

hsa-miR-3168	44.895	42.765	17.9175
hsa-miR-3175	33.99	32.97	13.3975
hsa-miR-3182	50.6575	65.1225	11.585
hsa-miR-3184-5p	38.2925	39.145	13.555
hsa-miR-3187-3p	39.755	48.035	13.8
hsa-miR-331-3p	191.0775	203.775	79.4775
hsa-miR-337-3p	52.265	58	24.5525
hsa-miR-337-5p	29.13	31.9775	9.8875
hsa-miR-338-3p	43.21	51.24	14.2825
hsa-miR-33a-5p	28.25	30.3025	13.3
hsa-miR-3614-5p	42.535	72.0225	12.385
hsa-miR-367-3p	35.295	37.6575	11.9475
hsa-miR-378c	29.345	36.0575	10.27
hsa-miR-378e	226.42	302.5875	63.4975
hsa-miR-378h	33.4725	34.4675	15.8825
hsa-miR-423-5p	73.77	85.135	31.765
hsa-miR-4286	69.68	76.29	30.1175
hsa-miR-4425	24.695	28.8625	8.3125
hsa-miR-4431	28.5025	31.2775	12.2625
hsa-miR-4443	182.32	58.1475	13.32
hsa-miR-4455	67.5925	90.065	14.38
hsa-miR-4458	34.145	42.7275	8.4625
hsa-miR-4508	20.5675	28.225	3.3275
hsa-miR-450a-5p	39.1975	38.43	16.08
hsa-miR-450b-3p	37.175	36.9575	11.1275
hsa-miR-487a	31.205	45.7125	11.4125
hsa-miR-487b	26.1675	29.955	9.705
hsa-miR-492	26.02	27.6225	9.515
hsa-miR-503	125.2	138.1125	45.98
hsa-miR-504	41.8775	47.705	18.9325
hsa-miR-507	29.65	27.2025	12.5325
hsa-miR-509-3-5p	24.9775	29.7575	9.085
hsa-miR-512-3p	31.27	39.335	15.325
hsa-miR-513b	19.075	24.9675	8.235
hsa-miR-514b-5p	96.8225	118.1	30.0925
hsa-miR-516a-3p	36.4725	44.8125	17.955
hsa-miR-519d	66.34	68.4675	26.7875
hsa-miR-520c-3p	33.695	27.6175	11.145
hsa-miR-520d-5p+hsa-miR-518a-5p+hsa-miR-527	43.515	61.075	17.1725
hsa-miR-523-3p	27.21	31.8275	12.9125
hsa-miR-542-3p	61.7375	66.06	18.5775
hsa-miR-548a-5p	67.3075	76.2275	31.5475

hsa-miR-548an	34.615	41.15	12.305
hsa-miR-548c-5p	63.7175	83.1175	25.8725
hsa-miR-548g-3p	41.76	42.12	20.5975
hsa-miR-548j	15.6025	22.905	6.365
hsa-miR-548k	38.0925	38.0075	18.19
hsa-miR-548m	31.6	45.85	13.205
hsa-miR-548n	39.76	49.5225	18.725
hsa-miR-548p	43.5175	48.5025	19.205
hsa-miR-548y	30.0475	35.6625	14.965
hsa-miR-549	1.8975	1.8575	0.5475
hsa-miR-550a-5p	39.785	40.2425	14.715
hsa-miR-556-3p	21.93	23.0775	5.69
hsa-miR-557	23.9075	24.23	10.5325
hsa-miR-561-3p	14.5425	19.7325	6.8925
hsa-miR-563	33.075	36.9425	10.9175
hsa-miR-566	38.24	33.96	14.71
hsa-miR-571	22.5525	38.955	9.04
hsa-miR-576-3p	28.62	25.575	11.8875
hsa-miR-578	44.1175	47.6825	16.22
hsa-miR-582-5p	20.515	21.92	8.8625
hsa-miR-583	35.3	42.6225	12.895
hsa-miR-585	23.93	30.455	11.2775
hsa-miR-587	62.24	59.42	17.585
hsa-miR-589-5p	16.77	20.75	7.185
hsa-miR-592	36.82	43.295	15.505
hsa-miR-599	26.8275	22.545	7.0075
hsa-miR-601	30.375	29.69	13.865
hsa-miR-606	38.8725	33.335	11.58
hsa-miR-612	104.55	151.705	38.915
hsa-miR-614	50.65	53.285	14.7
hsa-miR-615-5p	28.73	29.5075	10.28
hsa-miR-631	266.1325	310.985	76.2725
hsa-miR-633	21.2225	28.445	9.0925
hsa-miR-646	28.36	34.635	12.5275
hsa-miR-648	28.57	30.995	8.485
hsa-miR-650	26.7875	36.3625	9.9725
hsa-miR-655	90.9	103.1075	23.1625
hsa-miR-657	26.34	32.2	10.5275
hsa-miR-658	28.255	27.9125	13.04
hsa-miR-663b	24.2975	21.6925	9.17
hsa-miR-671-5p	26.8425	25.385	12.3825
hsa-miR-708-5p	20.4375	22.8025	9.66
hsa-miR-761	77.9825	93.6475	21.9675

hsa-miR-762	33.825	32.1925	15.2075
hsa-miR-765	19.1625	29.0675	8.3225
hsa-miR-766-3p	34.3725	39.08	15.5425
hsa-miR-767-5p	30.195	29.49	12.9075
hsa-miR-769-5p	60.71	71.3025	19.9725
hsa-miR-770-5p	36.4075	47.865	13.04
hsa-miR-802	33.8825	43.7275	15.4175
hsa-miR-876-5p	38.05	44.5525	11.0725
hsa-miR-885-5p	25.855	25.6375	11.1075
hsa-miR-888-5p	129.1775	150.2625	35.4475
hsa-miR-889	33.7725	20.485	8.205
hsa-miR-890	34.39	36.685	14.69
hsa-miR-891a	50.1875	65.74	21.9625
hsa-miR-892a	26.6075	32.3325	11.2375
hsa-miR-922	39.0025	40.925	13.875
hsa-miR-933	29.5125	25.36	12.31
hsa-miR-938	42.425	39.6625	12.38
hsa-miR-943	38.985	40.68	11.6175
hsa-miR-944	46.1025	57.535	19.9175
hsa-miR-9-5p	54.5625	53.82	25.95

Table A5: ***In silico*** **Target Prediction results for hsa-miR-155-5p as predicted by DIANA-microT online software.**

Target	ENSMBL Codes	miT Score	Also Predicted
Teashirt Zinc Finger Homeobox 3 (TSHZ3)	ENSG00000121297	1.00	3
Transcription Factor 4 (TCF4)	ENSG00000196628	0.912	2

Table A6: ***In silico*** **Target Prediction results for hsa-miR-193a-5p as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
Intersectin 2 (ITSN2)	ENSG00000198399	1.00	0

Table A7: ***In silico*** **Target Prediction results for hsa-miR-200b-3p as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
Zinc Finger E Box-Binding Homeobox 2 (ZEB2)	ENSG00000169554	1.00	3
Zinc Finger Protein, Multitype 2 (ZFPM2)	ENSG00000169946	0.999	3
Zinc Finger E Box-Binding Homeobox 1 (ZEB1)	ENSG00000148516	1.00	3
Protein-Tyrosine Phosphatase, Nonreceptor-Type, 12 (PTPN12)	ENSG00000127947	1.00	2

Table A8: ***In silico*** **Target Prediction results for hsa-miR-200c-3p as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
Zinc Finger E Box-Binding Homeobox 2 (ZEB2)	ENSG00000169554	1.00	3
Zinc Finger Protein, Multitype 2 (ZFPM2)	ENSG00000169946	0.999	3
Zinc Finger E Box-Binding Homeobox 1 (ZEB1)	ENSG00000148516	1.00	3

Table A9: ***In silico*** **Target Prediction results for hsa-miR-205-5p as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
DEAD Homeobox 5 (DDX5)	ENSG00000108654	0.993	3
CKLF-like Marvel Transmembrane Domain-containing 4 (CMTM4)	ENSG00000183723	0.857	2

Table A10: ***In silico*** **Target Prediction results for hsa-miR-21-5p as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
Zinc Finger Protein 367 (ZNF367)	ENSG00000165244	1.00	3
Ovarian Tumour Deubiquitinating Enzyme 1 (YOD1)	ENSG00000180667	0.999	3
Pleckstrin Homology Domain-Containing Protein, Family A, Member 1 (PLEKHA1)	ENSG00000107679	0.990	3

Table A11: ***In silico*** **Target Prediction results for hsa-miR-296-5p as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
Fibrosin 1 (FBRS)	ENSG00000156860	0.996	1
Cyclin-Dependent Kinase 16 (CDK16)	ENSG00000102225	0.804	1
Lysophospholipase II (LYPLA2)	ENSG00000011009	0.763	1

Table A12: ***In silico*** **Target Prediction results for hsa-miR-342-3p as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
Rho GTPase Activating Protein 19 (ARHGAP19)	ENSG00000213390	0.936	1
Jumonji Domain Containing 1C (JMJD1C)	ENSG00000171988	0.8	1

Table A13: ***In silico*** **Target Prediction results for hsa-miR-375 as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
ELAV Like Neuron-Specific RNA Binding Protein 4 (ELAVL4)	ENSG00000162374	1.000	2
Eukaryotic Translation Initiation Factor 4 Gamma, 3 (eIF4G3)	ENSG00000075151	0.992	2

Table A14: ***In silico*** **Target Prediction results for hsa-miR-125b-5p as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
Potassium Channel, Subfamily K, Member 10 (KCNK10)	ENSG00000100433	0.880	2
TET Oncogene Family, Member 2 (TET2)	ENSG00000168769	0.823	2

Table A15: ***In silico*** **Target Prediction results for hsa-miR-221-3p as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
Dual-Specificity Tyrosine Phosphorylation-Regulated Kinase 1a (DYRK1A)	ENSG00000157540	0.917	2
Phd Finger Protein 2 (PHF2)	ENSG00000197724	0.876	2
Thymocyte Selection-Associated High Mobility Group Box (TOX)	ENSG00000198846	0.837	2

Table A16: ***In silico*** **Target Prediction results for hsa-miR-503 as predicted by DIANA-microT online software.**

Target	ENSEMBL Code	miT Score	Also Predicted
Cyclin E1 (CCNE1)	ENSG00000105173	0.998	2

Table A17: Difference in miRNA expression between two TNBC cell lines. A total of 14 miRNAs had increased expression by at least two-fold in the MDA-2B-436 cells, relative to the MDA-2B-231 cells (Shaded in blue). A total of 186 miRNAs had decreases expression by at least half in the MDA-2B-436 cells, relative to the MDA-2B-231 cells (Shaded in red).

miRNA	MDA-2B-231	MDA-2B-436	Fold Change
hsa-let-7f-5p	194	571	3
hsa-miR-106a-5p+hsa-miR-17-5p	137	338	2
hsa-miR-106b-5p	81	184	2
hsa-miR-1181	10	23	2
hsa-miR-155-5p	47	137	3
hsa-miR-15a-5p	177	387	2
hsa-miR-21-5p	143	310	2
hsa-miR-221-3p	78	167	2
hsa-miR-342-3p	141	519	4
hsa-miR-34a-5p	29	59	2
hsa-miR-497-5p	9	22	2
hsa-miR-610	15	34	2
hsa-miR-628-3p	8	18	2
hsa-miR-98	97	213	2
hsa-miR-1179	34	15	2
hsa-miR-1184	23	11	2
hsa-miR-1185-5p	30	14	2
hsa-miR-1193	36	17	2
hsa-miR-1208	52	19	3
hsa-miR-1234	64	21	3
hsa-miR-1236	42	20	2
hsa-miR-1237	39	11	4

hsa-miR-1238	40	11	4
hsa-miR-1245b-3p	54	18	3
hsa-miR-1246	2	1	3
hsa-miR-1247-5p	57	27	2
hsa-miR-1248	37	16	2
hsa-miR-1251	30	14	2
hsa-miR-1253	2	1	3
hsa-miR-1255b-5p	37	18	2
hsa-miR-1257	48	14	3
hsa-miR-1262	31	15	2
hsa-miR-1263	20	8	3
hsa-miR-1267	63	19	3
hsa-miR-1269b	25	12	2
hsa-miR-1270	58	19	3
hsa-miR-1271-5p	28	11	2
hsa-miR-1273c	40	14	3
hsa-miR-1273e	23	11	2
hsa-miR-1273f	25	8	3
hsa-miR-1273g-5p	23	9	2
hsa-miR-127-3p	33	16	2
hsa-miR-127-5p	20	9	2
hsa-miR-1277-3p	50	22	2
hsa-miR-1278	36	16	2
hsa-miR-1280	23	8	3
hsa-miR-1282	27	12	2
hsa-miR-1283	2	1	3
hsa-miR-1285-3p	44	12	4
hsa-miR-1286	76	18	4
hsa-miR-1288	45	11	4
hsa-miR-1291	40	16	2
hsa-miR-1293	20	10	2
hsa-miR-1302	22	10	2
hsa-miR-139-3p	36	17	2
hsa-miR-140-5p	24	12	2
hsa-miR-142-3p	46	15	3
hsa-miR-142-5p	27	12	2
hsa-miR-143-3p	2	1	3
hsa-miR-1468	22	10	2
hsa-miR-146b-3p	21	10	2
hsa-miR-152	44	19	2
hsa-miR-153	27	13	2

hsa-miR-154-5p	18	9	2
hsa-miR-1825	29	12	2
hsa-miR-1827	96	18	5
hsa-miR-185-5p	81	39	2
hsa-miR-188-5p	221	71	3
hsa-miR-1908	36	18	2
hsa-miR-1912	33	13	3
hsa-miR-1913	19	7	3
hsa-miR-1915-3p	31	10	3
hsa-miR-192-5p	33	12	3
hsa-miR-199a-3p+hsa-miR-199b-3p	28	14	2
hsa-miR-199a-5p	43	19	2
hsa-miR-205-5p	185	87	2
hsa-miR-206	42	20	2
hsa-miR-2113	45	12	4
hsa-miR-2116-5p	58	25	2
hsa-miR-218-5p	21	10	2
hsa-miR-219-2-3p	23	8	3
hsa-miR-2276	41	16	3
hsa-miR-2277-3p	33	10	3
hsa-miR-23c	40	17	2
hsa-miR-2682-5p	101	24	4
hsa-miR-297	118	56	2
hsa-miR-302b-3p	41	19	2
hsa-miR-302d-3p	168	47	4
hsa-miR-3136-5p	40	15	3
hsa-miR-3164	24	9	3
hsa-miR-3175	27	13	2
hsa-miR-3180-5p	30	13	2
hsa-miR-3182	41	12	4
hsa-miR-3184-5p	37	14	3
hsa-miR-3187-3p	36	14	3
hsa-miR-320d	27	13	2
hsa-miR-323a-5p	22	11	2
hsa-miR-328	31	13	2
hsa-miR-337-5p	25	10	2
hsa-miR-338-3p	37	14	3
hsa-miR-339-3p	31	15	2
hsa-miR-340-5p	17	6	3
hsa-miR-361-3p	127	48	3
hsa-miR-3614-5p	54	12	4

hsa-miR-363-3p	2	1	3
hsa-miR-371a-3p	143	37	4
hsa-miR-372	25	12	2
hsa-miR-378c	34	10	3
hsa-miR-378d	26	11	2
hsa-miR-378e	250	63	4
hsa-miR-378h	33	16	2
hsa-miR-3934	50	21	2
hsa-miR-4286	73	30	2
hsa-miR-4425	30	8	4
hsa-miR-4435	23	11	2
hsa-miR-4443	44	13	3
hsa-miR-4455	58	14	4
hsa-miR-4458	34	8	4
hsa-miR-4485	15	7	2
hsa-miR-4508	18	3	5
hsa-miR-450b-3p	34	11	3
hsa-miR-4516	28	13	2
hsa-miR-452-5p	21	9	2
hsa-miR-4531	43	17	2
hsa-miR-487a	31	11	3
hsa-miR-487b	25	10	3
hsa-miR-507	26	13	2
hsa-miR-509-3-5p	26	9	3
hsa-miR-512-3p	38	15	2
hsa-miR-512-5p	62	21	3
hsa-miR-514b-5p	87	30	3
hsa-miR-516b-5p	28	10	3
hsa-miR-519d	67	27	3
hsa-miR-520b	65	25	3
hsa-miR-520c-3p	23	11	2
hsa-miR-520d-5p+hsa-miR-518a-5p+ hsamiR-527	50	17	3
hsa-miR-524-3p	28	13	2
hsa-miR-542-3p	49	19	3
hsa-miR-548a-5p	66	32	2
hsa-miR-548ac	21	11	2
hsa-miR-548ae	26	12	2
hsa-miR-548an	26	12	2
hsa-miR-548c-3p	33	15	2
hsa-miR-548c-5p	62	26	2

hsa-miR-548e	25	11	2
hsa-miR-548j	15	6	2
hsa-miR-548k	45	18	2
hsa-miR-548m	39	13	3
hsa-miR-548p	40	19	2
hsa-miR-548s	20	9	2
hsa-miR-548v	25	12	2
hsa-miR-548y	37	15	2
hsa-miR-549	2	1	3
hsa-miR-550a-5p	36	15	2
hsa-miR-556-3p	12	6	2
hsa-miR-561-3p	20	7	3
hsa-miR-563	30	11	3
hsa-miR-566	33	15	2
hsa-miR-571	22	9	2
hsa-miR-576-3p	25	12	2
hsa-miR-577	29	14	2
hsa-miR-578	38	16	2
hsa-miR-583	33	13	3
hsa-miR-587	55	18	3
hsa-miR-589-5p	18	7	3
hsa-miR-592	37	16	2
hsa-miR-599	19	7	3
hsa-miR-600	30	12	2
hsa-miR-606	30	12	3
hsa-miR-612	102	39	3
hsa-miR-614	46	15	3
hsa-miR-615-5p	21	10	2
hsa-miR-622	24	11	2
hsa-miR-627	43	21	2
hsa-miR-631	261	76	3
hsa-miR-633	29	9	3
hsa-miR-648	23	8	3
hsa-miR-650	31	10	3
hsa-miR-653	25	13	2
hsa-miR-655	66	23	3
hsa-miR-658	27	13	2
hsa-miR-663b	20	9	2
hsa-miR-708-5p	20	10	2
hsa-miR-761	83	22	4
hsa-miR-767-3p	33	16	2

hsa-miR-769-5p	61	20	3
hsa-miR-770-5p	34	13	3
hsa-miR-874	23	7	3
hsa-miR-876-5p	25	11	2
hsa-miR-885-5p	24	11	2
hsa-miR-888-5p	116	35	3
hsa-miR-889	18	8	2
hsa-miR-890	31	15	2
hsa-miR-891a	52	22	2
hsa-miR-892a	24	11	2
hsa-miR-922	39	14	3
hsa-miR-938	33	12	3
hsa-miR-943	41	12	4
hsa-miR-944	48	20	2
hsa-miR-9-5p	52	26	2

Appendix B: Information Sheets

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical))

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

Ref: W-CJ-13-3

30/01/2013

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Dr C Penny, Ms N Crawford (student no 307250).**

Project title: **miRNA profiling of triple negative breast cancer; identification of potential diagnostic markers and drug targets.**

Reason: This is a wholly laboratory study using commercial cell lines including MCF-7, MCF10A, MDA-2B-231, MDA-2B-436 and MDA-2B-468. No humans are involved.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2013 -01- 30
JOHANNESBURG

Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

ATCC

Product Sheet

MCF7 (ATCC® HTB-22™)

Please read this FIRST

Storage Temp.
liquid nitrogen vapor phase

Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: 0.01 mg/ml human recombinant insulin; fetal bovine serum to a final concentration of 10%.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: MCF7 (ATCC® HTB-22™)

American Type Culture Collection
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www.atcc.org

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Description

Organism: *Homo sapiens*, human
Tissue:
mammary gland/breast; derived from metastatic site: pleural effusion
Disease: adenocarcinoma
Cell Type: epithelial
Age: 69 years adult
Gender: female
Morphology: epithelial
Growth Properties: adherent
Isoenzymes:
AK-1, 1
ES-D, 1-2
G6PD, B
GLO-I, 1-2
PGM1, 1-2
PGM3, 1
DNA Profile:
Amelogenin: X
CSF1PO: 10
D13S317: 11
D16S539: 11,12
D5S818: 11,12
D7S820: 8,9
THO1: 6
TPOX: 9,12
vWA: 14,15
Cytogenetic Analysis: modal number = 82; range = 66 to 87.
The stemline chromosome numbers ranged from hypertriploidy to hypotetraploidy, with the 2S component occurring at 1%. There were 29 to 34 marker chromosomes per S metaphase; 24 to 28 markers occurred in at least 30% of cells, and generally one large submetacentric (M1) and 3 large subtelocentric (M2, M3, and M4) markers were recognizable in over 80% of metaphases. No DM were detected. Chromosome 20 was nullisomic and X was disomic.

SAFETY PRECAUTION

ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

Unpacking & Storage Instructions

1. Check all containers for leakage or breakage.
2. Remove the frozen cells from the dry ice packaging and immediately place the cells at a temperature below -130°C, preferably in liquid nitrogen vapor, until ready for use.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C. Storage at -70°C will result in loss of viability.

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
3. It is recommended that the cryoprotective agent be removed immediately. Centrifuge the cell suspension at approximately 125 x g for 5 to 10 minutes. Discard the supernatant and resuspend the cell pellet in an appropriate amount of fresh growth medium.
4. Transfer the cell pellet to an appropriate size vessel. It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).

ATCC

Product Sheet

MCF7 (ATCC® HTB-22™)

Please read this FIRST

Storage Temp.
liquid nitrogen vapor phase

Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: 0.01 mg/ml human recombinant insulin; fetal bovine serum to a final concentration of 10%.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: MCF7 (ATCC® HTB-22™)

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5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).
2. **If the cells are still attached,** aseptically remove all but 5 to 10 mL of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.
3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 x g for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 mL of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Volumes used in this protocol are for 75 cm^2 flasks; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

Note: if floating cells are present, it is recommended that they be transferred at the first two (2) subcultures as described below. It is not necessary to transfer floating cells for subsequent subcultures.

1. Remove culture medium to a centrifuge tube.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.
3. Add 2.0 to 3.0 mL of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting.
5. Transfer the cell suspension to the centrifuge tube with the medium and cells from step 1, and centrifuge at approximately 125 xg for 5 to 10 minutes. Discard the supernatant.
6. Resuspend the cell pellet in fresh growth medium. Add appropriate aliquots of the cell suspension to new culture vessels.
7. Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:6 is recommended
Medium Renewal: 2 to 3 times per week

Cryopreservation Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC® Catalog No. 4-X.

Comments

The MCF7 line retains several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes. The cells express the WNT7B oncogene.
ref
Growth of MCF7 cells is inhibited by tumor necrosis factor alpha (TNF alpha).
Secretion of IGFBP's can be modulated by treatment with anti-estrogens.

References

References and other information relating to this product are available online at www.atcc.org.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication of the *Biosafety in Microbiological and Biomedical Laboratories* from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes

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Product Sheet

MCF7 (ATCC® HTB-22™)

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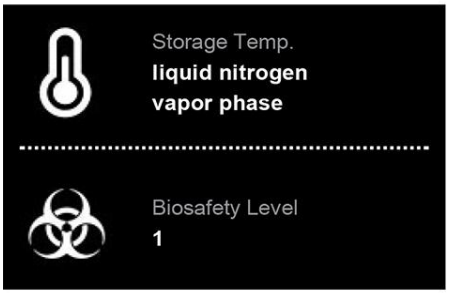

Intended Use

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Complete Growth Medium

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: 0.01 mg/ml human recombinant insulin; fetal bovine serum to a final concentration of 10%.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: MCF7 (ATCC® HTB-22™)

for Health.

ATCC Warranty

The viability of ATCC® products is warranted for 30 days from the date of shipment, and is valid only if the product is stored and cultured according to the information included on this product information sheet. ATCC lists the media formulation that has been found to be effective for this strain. While other, unspecified media may also produce satisfactory results, a change in media or the absence of an additive from the ATCC recommended media may affect recovery, growth and/or function of this strain. If an alternative medium formulation is used, the ATCC warranty for viability is no longer valid.

Disclaimers

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This product is sent with the condition that you are responsible for its safe storage, handling, and use. ATCC is not liable for any damages or injuries arising from receipt and/or use of this product. While reasonable effort is made to insure authenticity and reliability of strains on deposit, ATCC is not liable for damages arising from the misidentification or misrepresentation of cultures.
Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org

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ATCC

Product Sheet

MCF 10A (ATCC® CRL-10317™)

Please read this FIRST

Storage Temp.
liquid nitrogen vapor phase

Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line (MEBM) along with the additives can be obtained from Lonza/Clonetics Corporation as a kit: MEGM, Kit Catalog No. CC-3150. ATCC does not use the GA-1000 (gentamycin-amphotericin B mix) provided with kit. To make the complete growth medium, you will need to add the following components to the kit (sold separately):

- 100 ng/ml cholera toxin

Note: Do not filter complete medium

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: MCF 10A (ATCC® CRL-10317™)

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Description

Organism: *Homo sapiens*, human
Tissue: mammary gland/breast
Disease: fibrocystic disease
Cell Type: epithelial
Age: 36 years
Gender: female
Morphology: epithelial
Growth Properties: adherent
Isoenzymes:
AK-1, 1
ref
ES-D, 1
ref
G6PD, B
ref
GLO-I, 1-2
ref
PGM1, 1-2
ref
PGM3, 1
ref
DNA Profile:
Amelogenin: X
CSF1PO: 10,12
D13S317: 8,9
D16S539: 11,12
D5S818: 10,13
D7S820: 10,11
THO1: 8,9.3
TPOX: 9,11
vWA: 15,17

SAFETY PRECAUTION

ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

Unpacking & Storage Instructions

1. Check all containers for leakage or breakage.
2. Remove the frozen cells from the dry ice packaging and immediately place the cells at a temperature below -130°C, preferably in liquid nitrogen vapor, until ready for use.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C. Storage at -70°C will result in loss of viability.

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete culture medium and spin at approximately 125 x g for 5 to 10 minutes.
4. Resuspend the cell pellet with the recommended complete medium (see the specific batch information for the culture recommended dilution ratio) and dispense into a 25 cm^2 or a 75 cm^2 culture flask. It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).

ATCC

Product Sheet

MCF 10A (ATCC® CRL-10317™)

Please read this FIRST

Storage Temp.
liquid nitrogen vapor phase

Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line (MEBM) along with the additives can be obtained from Lonza/Clonetics Corporation as a kit: MEGM, Kit Catalog No. CC-3150. ATCC does not use the GA-1000 (gentamycin-amphotericin B mix) provided with kit. To make the complete growth medium, you will need to add the following components to the kit (sold separately):

- 100 ng/ml cholera toxin

Note: Do not filter complete medium

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: MCF 10A (ATCC® CRL-10317™)

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5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).
2. **If the cells are still attached,** aseptically remove all but 5 to 10 mL of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.
3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 x g for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Remove medium and rinse monolayer with PBS (ATCC 30-2200). Add 3.0 mL 0.05% trypsin, 0.53 mM EDTA and incubate at 37°C for 15 minutes. To neutralize trypsin, add 3 mL solution of 0.1% soybean trypsin inhibitor. Centrifuge cell suspension at 125 x g for 5 to 10 minutes. Resuspend cell pellet in complete culture medium. Add appropriate aliquots of cell suspension to new culture vessels.
Subcultivation Ratio: A subcultivation ratio of 1:3 to 1:4 is recommended
Medium Renewal: Every 2 to 3 days

Cryopreservation Medium

Complete growth medium described above supplemented with 7.5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Comments

The MCF 10A cell line is a non-tumorigenic epithelial cell line.
The cells are positive for epithelial sialomucins, cytokeratins and milk fat globule antigen.
They exhibit three dimensional growth in collagen, and form domes in confluent cultures.
Thus far, the cells have shown no signs of terminal differentiation or senescence.
The line is responsive to insulin, glucocorticoids, cholera enterotoxin, and epidermal growth factor (EGF).
By electron microscopy the cells display characteristics of luminal ductal cells but not of myoepithelial cells.
They also express breast specific antigens as detected by positive reaction with MFA-Breast and MC-5 monoclonal antibodies.
The calcium content of the medium exerts a strong effect on the morphology of the cells.

References

References and other information relating to this product are available online at www.atcc.org.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication of the *Biosafety in Microbiological and Biomedical Laboratories* from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes for Health.

ATCC Warranty

The viability of ATCC® products is warranted for 30 days from the date of shipment, and is valid only if the product is stored and cultured according to the information included on this product information sheet. ATCC lists the media formulation that has been found to be effective for this strain. While other, unspecified media may also produce satisfactory results, a change in media or the absence of an additive from the ATCC recommended media may affect recovery, growth and/or function of this strain. If an alternative medium

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Product Sheet

MCF 10A (ATCC® CRL-10317™)

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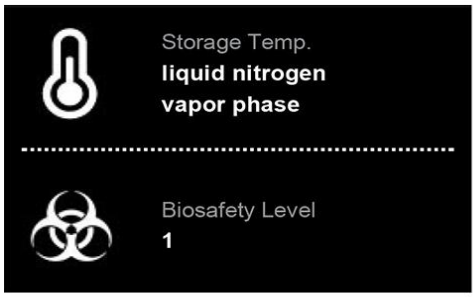

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line (MEBM) along with the additives can be obtained from Lonza/Clonetics Corporation as a kit: MEGM, Kit Catalog No. CC-3150. ATCC does not use the GA-1000 (gentamycin-amphotericin B mix) provided with kit. To make the complete growth medium, you will need to add the following components to the kit (sold separately):

- 100 ng/ml cholera toxin

Note: Do not filter complete medium

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: MCF 10A (ATCC® CRL-10317™)

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This product is sent with the condition that you are responsible for its safe storage, handling, and use. ATCC is not liable for any damages or injuries arising from receipt and/or use of this product. While reasonable effort is made to insure authenticity and reliability of strains on deposit, ATCC is not liable for damages arising from the misidentification or misrepresentation of cultures.
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ATCC

Product Sheet

MDA-MB-231 (ATCC® HTB-26™)

Please read this FIRST

Storage Temp.
liquid nitrogen vapor phase

Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Leibovitz's L-15 Medium, Catalog No. 30-2008. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: MDA-MB-231 (ATCC® HTB-26™)

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Description

Organism: *Homo sapiens*, human
Tissue: mammary gland/breast; derived from metastatic site: pleural effusion
Disease: adenocarcinoma
Cell Type: epithelial
Age: 51 years adult
Gender: female
Morphology: epithelial
Growth Properties: adherent
Isoenzymes:
AK-1, 1
ES-D, 1
G6PD, B
GLO-I, 2
Me-2, 1-2
PGM1, 1-2
PGM3, 1
DNA Profile:
Amelogenin: X
CSF1PO: 12,13
D13S317: 13
D16S539: 12
D5S818: 12
D7S820: 8,9
THO1: 7,9.3
TPOX: 8,9
vWA: 15,18
Cytogenetic Analysis: The cell line is aneuploid female (modal number = 64, range = 52 to 68), with chromosome counts in the near-triploid range. Normal chromosomes N8 and N15 were absent. Eleven stable rearranged marker chromosomes are noted as well as unassignable chromosomes in addition to the majority of autosomes that are trisomic. Many of the marker chromosomes are identical to those shown in the karyotype reported by K.L. Satya-Prakash, et al.

SAFETY PRECAUTION

ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

Unpacking & Storage Instructions

1. Check all containers for leakage or breakage.
2. Remove the frozen cells from the dry ice packaging and immediately place the cells at a temperature below -130°C, preferably in liquid nitrogen vapor, until ready for use.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C. Storage at -70°C will result in loss of viability.

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
3. Transfer the vial contents to a centrifuge tube containing 9.0 mL complete culture medium and spin at approximately 125 x g for 5 to 7 minutes.
4. Resuspend cell pellet with the recommended complete medium (see the specific batch information for the culture recommended dilution ratio) and dispense into a new culture flask.
5. Incubate the culture at 37°C in a suitable incubator. **(without CO_2)**

ATCC

Product Sheet

MDA-MB-231 (ATCC® HTB-26™)

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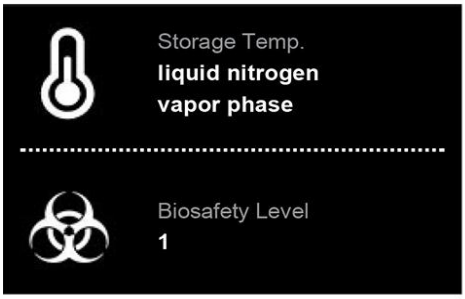

Intended Use

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Complete Growth Medium

The base medium for this cell line is ATCC-formulated Leibovitz's L-15 Medium, Catalog No. 30-2008. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Citation of Strain

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Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).
2. **If the cells are still attached,** aseptically remove all but 5 to 10 mL of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a free gas exchange with atmospheric air until they are ready to be subcultured.
3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 x g for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 mL of this medium and add to 25 cm^2 flask. Incubate at 37° in a free gas exchange with atmospheric air until cells are ready to be subcultured.

Subculturing Procedure

Volumes are given for a 75 cm^2 flask. Increase or decrease the amount of dissociation medium needed proportionally for culture vessels of other sizes.

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 mL of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.
6. Incubate cultures at 37°C without CO_2.

Subcultivation Ratio: A subcultivation ratio of 1:2 to 1:4 is recommended
Medium Renewal: 2 to 3 times per week

Cryopreservation Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

References

References and other information relating to this product are available online at www.atcc.org.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication of the *Biosafety in Microbiological and Biomedical Laboratories* from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes for Health.

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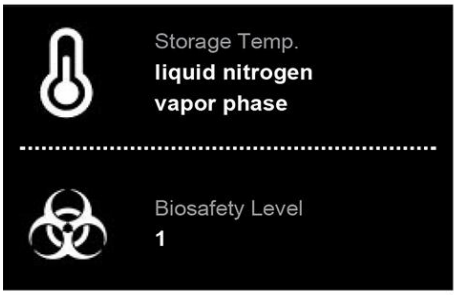
MDA-MB-231 (ATCC® HTB-26™)

Please read this FIRST



Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Leibovitz's L-15 Medium, Catalog No. 30-2008. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: MDA-MB-231 (ATCC® HTB-26™)

patents are provided for informational purposes only. ATCC does not warrant that such information has been confirmed to be accurate.

This product is sent with the condition that you are responsible for its safe storage, handling, and use. ATCC is not liable for any damages or injuries arising from receipt and/or use of this product. While reasonable effort is made to insure authenticity and reliability of strains on deposit, ATCC is not liable for damages arising from the misidentification or misrepresentation of cultures.

Please see the enclosed Material Transfer Agreement (MTA) for further details regarding the use of this product. The MTA is also available on our Web site at www.atcc.org

Additional information on this culture is available on the ATCC web site at www.atcc.org.

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800.638.6597 or 703.365.2700
Fax: 703.365.2750
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Or contact your local distributor

ATCC

Product Sheet

MDA-MB-436 (ATCC® HTB-130™)

Please read this FIRST

Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

Leibovitz's L-15 medium with 10 mcg/ml insulin, 16 mcg/ml glutathione, 90%; fetal bovine serum, 10%

Citation of Strain

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Description

Organism: *Homo sapiens*, human
Tissue:
mammary gland/breast; derived from metastatic site: pleural effusion
Disease: adenocarcinoma
Age: 43 years
Gender: female
Morphology: pleomorphic with multinucleated component cells
Growth Properties: adherent
Isoenzymes:
AK-1, 1
ES-D, 1
G6PD, B
GLO-I, 2
Me-2, 1
PGM1, 1
PGM3, 1
DNA Profile:
Amelogenin: X
CSF1PO: 12
D13S317: 10
D16S539: 9,11
D5S818: 13
D7S820: 10
THO1: 9.3
TPOX: 8
vWA: 14,20
Cytogenetic Analysis: modal number = 45

SAFETY PRECAUTION

ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

Unpacking & Storage Instructions

1. Check all containers for leakage or breakage.
2. Remove the frozen cells from the dry ice packaging and immediately place the cells at a temperature below -130°C, preferably in liquid nitrogen vapor, until ready for use.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C. Storage at -70°C will result in loss of viability.

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
3. Transfer the vial contents to a centrifuge tube containing 9.0 mL complete culture medium and spin at approximately 125 x g for 5 to 7 minutes. Discard supernatant.
4. Resuspend the cell pellet with the recommended complete medium and dispense into a 25 cm^2 culture flask. It is important to avoid excessive alkalinity of the medium during recovery of the cells.
5. Incubate the culture at 37°C in a suitable incubator in a free gas exchange with atmospheric air

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination.

ATCC

Product Sheet

MDA-MB-436 (ATCC® HTB-130™)

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Biosafety Level
1

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Complete Growth Medium

Leibovitz's L-15 medium with 10 mcg/ml insulin, 16 mcg/ml glutathione, 90%; fetal bovine serum, 10%

Citation of Strain

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Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 mL of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a **free gas exchange** until they are ready to be subcultured.
3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 x g for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 mL of this medium and add to 25 cm^2 flask. Incubate at 37°C until cells are ready to be subcultured; in a **free gas exchange** with atmospheric air.

Subculturing Procedure

Subcultures are prepared by scraping. Remove spent medium, add 3 to 5 ml of fresh medium, dislodge cells from the floor of the flask, aspirate and dispense into new flasks. Subculture every 6 to 8 days.
Subcultivation Ratio: A subcultivation ratio of 1:2 is recommended
Medium Renewal: 2 to 3 times per week

Cryopreservation Medium

Complete culture medium described above supplemented with 5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC® Catalog No. 4-X.

Comments

The line is pleomorphic and most cells react intensely with anti tubulin antibody as demonstrated by indirect immunofluorescence staining.

References

References and other information relating to this product are available online at www.atcc.org.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the current publication of the *Biosafety in Microbiological and Biomedical Laboratories* from the U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes for Health.

ATCC Warranty

The viability of ATCC® products is warranted for 30 days from the date of shipment, and is valid only if the product is stored and cultured according to the information included on this product information sheet. ATCC lists the media formulation that has been found to be effective for this strain. While other, unspecified media may also produce satisfactory results, a change in media or the absence of an additive from the ATCC recommended media may affect recovery, growth and/or function of this strain. If an alternative medium formulation is used, the ATCC warranty for viability is no longer valid.

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Appendix C: Recipes

1 X TBE (1 Liter):

- 108g Tris base
- 55g Boric acid
- 40 mL 0.5M EDTA (pH 8.0)
- Autoclaved for 60 minutes

2% Agarose Gel (100 mL):

- 2 g Agarose (Seakem, cat #: 50002)
- 1 X TBE Buffer up to 100 mL

Cell culture media:

Cell line	Medium Recipe for 50mL
MCF-7/ MDA-MB-231	• Lonza BioWhittaker DMEM-F12 (cat #: BE12-719F) • 100µL Penstrep (Biowhittaker, cat #: 17-602E) • 5mL FBS (Sigma, cat #: F1051)
MDA-MB-436	• Lonza BioWhittaker Leibovitz L15 Medium (cat #: BE12-700F) • 100µL penstrep • 5mL FBS • 50µL Sigma insulin solution, human (cat #: 192748-5ML)

MCF-10A	• **Lonza Clonetics MEBM (cat #: CC-3151)** • **100µL Penstrep** • **5mL FBS** • **Lonza Clonetics MEGM SingleQuots (cat #: CC-4136) (50µL insulin, 50µL, EGF, 200µL BPE, 50µL Hydrocortisol**

Cryogenic Medium:

- 20% FBS
- 10% DMSO (Sigma, cat #: D-5879)
- Culture medium

PBS: (Sigma-Aldrich Phosphate Buffered Saline, cat #: P4417-100TAB)

- 1 PBS tablet dissolved in 200 mL distilled water
- Autoclaved and stored at 4°C

Appendix D: Nanostring Raw Data

Table D1: An example of the raw Nanostring data as received from Nanostring Technologies. Samples were sent without cell line identification as we blinded them. The sample identifications are mentioned in table 11.

Type			Frozen	Frozen	Frozen	Frozen	Frozen	Frozen	Frozen	Frozen	RNAStable	RNAStable	RNAStable	RNAStable	RNAStable	RNAStable	RNAStable	RNAStable
Sample Date			2014/01/07	2014/01/07	2014/01/07	2014/01/07	2014/01/07	2014/01/07	2014/01/16	2014/01/16	2014/01/07	2014/01/07	2014/01/07	2014/01/07	2014/01/07	2014/01/07	2014/01/16	2014/01/16
File Version			1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
Lane ID			1	2	3	4	5	6	1	2	7	8	9	10	11	12	3	4
FOV Count			280	280	280	280	280	280	280	280	280	280	280	280	280	280	280	280
FOV Counted			279	280	280	280	280	280	279	280	280	280	280	280	280	280	279	280
Scanner ID			KB0006	KB0006	KB0006	KB0006	KB0006	KB0006	KB0006	KB0006	KB0006	KB0006	KB0006	KB0006	KB0006	KB0006	KB0006	KB0006
Stage Position			2	2	2	2	2	2	1	1	2	2	2	2	2	2	1	1
Binding Density			0.3	0.29	0.43	0.35	0.38	0.38	0.78	0.62	0.37	0.36	0.39	0.33	0.34	0.25	0.61	0.51
Top 100 Normalization Factor:			*2.72*	*1.97*	*1.97*	*2.23*	*1.53*	*1.05*	*1.6*	*1.3*	*1.45*	*1.78*	*1.78*	*2.5*	*0.27*	*0.41*	*0.58*	*0.93*
Positive	POS_A	nmiR00813.1	32868	24910	42279	29343	44326	37008	41843	32981	29622	33562	43760	33056	35631	19767	40547	37573
Positive	POS_B	nmiR00809.1	2560	1918	3699	2593	3872	3217	8188	5618	2733	2906	3762	3050	2905	1709	6925	6047
Positive	POS_C	nmiR00811.1	1440	1132	1962	1420	2089	1439	2412	1503	1479	1455	1824	1626	1680	852	1754	1415
Positive	POS_D	nmiR00822.1	1086	698	1198	1049	1326	1232	801	504	980	984	1205	1072	1084	694	726	614
Positive	POS_E	nmiR00801.1	97	74	148	94	160	116	225	173	103	126	163	155	133	75	216	195
Positive	POS_F	nmiR00825.1	60	51	95	62	91	81	95	76	67	76	91	70	88	40	78	65
Negative	NEG_A	nmiR00810.1	51	45	46	30	60	52	26	23	35	47	64	45	48	22	22	26
Negative	NEG_B	nmiR00828.1	64	30	62	48	57	44	32	16	41	43	51	42	47	26	19	19
Negative	NEG_C	nmiR00803.1	42	40	55	54	64	51	29	24	50	40	67	50	45	28	16	23
Negative	NEG_D	nmiR00823.1	72	44	91	78	99	92	59	38	82	83	108	89	76	56	50	27
Negative	NEG_E	nmiR00827.1	30	26	50	32	31	43	15	22	32	46	49	22	33	24	11	12
Negative	NEG_F	nmiR00805.1	9	10	18	10	16	17	20	24	15	22	21	14	17	10	18	23
Housekeepi	ACTB	NM_001101.2	2687	1809	4117	3389	1779	2374	1158	1422	8883	2473	3006	3264	2839	4097	3771	4344
Housekeepi	B2M	NM_004048.2	3556	3090	2663	3614	299	401	318	378	6850	1780	1680	2607	4070	8005	10330	10533
Housekeepi	GAPDH	NM_002046.3	6755	6121	11138	5900	3919	3383	2188	3403	13872	3106	3744	5439	6777	11598	15581	18990
Housekeepi	RPL19	NM_000981.3	4335	3399	7291	2930	1715	1420	950	1709	8296	2634	3016	3219	2544	4102	5435	5895
Housekeepi	RPLP0	NM_001002.3	10619	8890	13905	12856	5802	4098	2069	3148	17711	4764	5283	6357	9032	14971	9921	11424
Endogenous	hsa-let-7a-5p	MIMAT0000062	3678	6758	4180	4688	2235	3048	4950	4934	3538	5588	4820	4388	3915	4669	9240	8138
Endogenous	hsa-let-7b-5p	MIMAT0000063	1190	2008	1920	1430	855	555	668	950	1412	616	504	631	1140	1383	1967	1967
Endogenous	hsa-let-7c	MIMAT0000064	128	146	207	183	158	106	141	187	128	90	64	66	75	77	199	189
Endogenous	hsa-let-7d-5p	MIMAT0000065	365	508	402	450	500	629	500	562	374	509	385	397	808	944	926	857
Endogenous	hsa-let-7e-5p	MIMAT0000066	231	290	359	330	459	369	437	524	400	327	275	290	202	232	337	349
Endogenous	hsa-let-7f-5p	MIMAT0000067	120	252	101	120	140	187	625	761	86	223	128	153	218	232	406	490
Endogenous	hsa-let-7g-5p	MIMAT0000414	2317	5193	1778	2217	1030	1851	1666	2174	816	2870	2037	1215	1402	1808	2360	2171
Endogenous	hsa-let-7i-5p	MIMAT0000415	487	1512	552	675	405	586	816	865	515	793	581	519	278	320	555	450
Endogenous	hsa-miR-1	MIMAT0000416	46	26	45	49	23	18	8	15	35	21	33	34	36	32	12	25
Endogenous	hsa-miR-100-5p	MIMAT0000098	354	841	357	312	20	6	14	17	531	1158	511	621	95	75	139	124
Endogenous	hsa-miR-101-3p	MIMAT0000099	30	14	22	20	21	15	12	15	19	12	17	12	18	35	16	21
Endogenous	hsa-miR-103a-3p	MIMAT0000101	16	6	12	13	9	12	10	10	24	12	10	7	7	12	13	15
Endogenous	hsa-miR-105-5p	MIMAT0000102	27	34	16	33	29	6	15	14	19	18	16	20	25	40	22	20
Endogenous	hsa-miR-106a-5p+l	MIMAT0000103	90	191	108	107	48	54	112	173	90	152	150	84	244	202	551	514
Endogenous	hsa-miR-106b-5p	MIMAT0000680	109	130	69	47	91	141	297	266	45	39	60	39	39	52	89	84
Endogenous	hsa-miR-107	MIMAT0000104	136	171	166	114	158	212	172	193	165	242	224	194	120	157	172	136
Endogenous	hsa-miR-10a-5p	MIMAT0000253	87	110	91	69	58	25	17	21	54	51	49	60	62	35	26	29
Endogenous	hsa-miR-10b-5p	MIMAT0000254	41	14	51	45	34	11	11	12	37	22	32	32	23	42	17	18
Endogenous	hsa-miR-1178	MIMAT0005823	35	26	22	20	21	14	10	16	38	30	26	20	14	22	16	24
Endogenous	hsa-miR-1179	MIMAT0005824	76	30	45	56	32	17	12	12	53	36	38	46	59	30	13	22
Endogenous	hsa-miR-1180	MIMAT0005825	57	69	142	102	68	65	58	67	146	64	62	57	57	37	82	76
Endogenous	hsa-miR-1181	MIMAT0005826	19	14	30	13	6	9	14	16	18	8	13	11	5	17	24	37
Endogenous	hsa-miR-1182	MIMAT0005827	27	8	20	11	15	15	4	7	29	8	22	20	18	12	9	12
Endogenous	hsa-miR-1183	MIMAT0005828	103	97	138	147	95	75	49	52	136	106	126	137	148	105	80	99
Endogenous	hsa-miR-1184	MIMAT0005829	27	18	28	31	20	19	10	7	24	14	19	12	32	22	12	14