

**Investigation into the role of the
long non-coding RNAs NEAT1
and MIAT in breast cancer**

Zainab Ahmed Mustafa Al-Mnaseer

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Abstract

Several long non-coding RNAs (lncRNAs) have been identified to play key, rate-limiting roles in malignancies, and the mechanisms involved are now being elucidated. This study addressed the roles of NEAT1 and MIAT lncRNAs, in breast cancer.

The short isoform NEAT1 _1 was found to be significantly up-regulated in advanced stages of breast cancer samples and in the ER/PR +ve and HER –ve molecular subtype, where its expression correlated positively with that of its neighbouring gene, MALAT1. NEAT1 transcripts in breast cancer cell lines were detected in both nuclear and cytoplasmic compartments. Silencing of cytoplasmic NEAT1 led to an increase in the expression of nuclear NEAT1, where such over-expression inhibited apoptosis and increased cell survival. Consistent with this, siRNA and ASO mediated knockdown of NEAT1 transcript levels decreased cell survival and migration and promoted cell death induced by different apoptotic stimuli including chemotherapeutic agents and UV-C irradiation. Reduced NEAT1 expression levels were also associated with changes in the expression of genes involved in the regulation of cell proliferation and survival. More importantly, it was found that NEAT1_1 regulates gene expression in cis and trans.

MIAT expression levels were significantly increased in triple negative breast cancer samples and its expression correlated with NEAT_1 expression. In breast cancer cell lines, MIAT expression was found to correlate with the expression of the transcription factor Oct4. MIAT down-regulation in breast cancer cells enhanced the basal apoptosis level and inhibited short and long-term survival. Induction of cell death by UV-C irradiation and chemotherapeutic drugs was also augmented in cells transfected with MIAT specific siRNA.

Taken together, the outcome of this study reveals important roles for NEAT1 and MIAT lncRNAs in breast cancer. Future work should explore the potential of these lncRNAs in the development of therapeutic drugs and as diagnostic and prognostic markers.

Abbreviations

ARF	ADP Ribosylation Factor 1
ART1	ADP-Ribosyltransferase 1
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BCAR4	Breast Cancer Anti-Estrogen Resistance 4
Bcl-2	B-cell lymphoma 2
Bcl-XI	B-cell lymphoma-extra large
CaSki	Cervical carcinoma cell
CCND1	Cyclin-D1
Cdkn1a	Cyclin dependent kinase inhibitor 1A
CDKN2B	Cyclin-dependent kinase 4 inhibitor B
CHK1	Checkpoint kinase 1
c-Myc	MYC proto-oncogene
DBHS family	Drosophila Melanogaster behaviour/human splicing proteins
DNA-PKcs	DNA-dependent protein kinase
E2F1	E2F transcription factor 1
E2F3	E2F Transcription Factor 3
eNOS	Endothelial NOS
ERBB2	Erb-b2 receptor tyrosine kinase 2
FAS	Fas cell surface death receptor
GADD45	Growth Arrest and DNA Damage
H3K27	Histone 3 lysine 27
HIF	Hypoxia-inducible factors
HOTAIR	HOX transcript antisense RNA
HOX	Homeobox genes
HULC	Highly Up-regulated in Liver Cancer (non-protein coding)
HuR	Human antigen R (RNA binding protein)
INK4	Family of cyclin-dependent kinase inhibitors
KCNQ1OT1	KCNQ1 overlapping transcript 1
KRAS	KRAS proto-oncogene, GTPase
LINK-A	long intergenic non-coding RNA for kinase activation
MDM2	Mouse double minute 2 homolog

MDMX	MIPS Digital Media eXtension
NFκB	Transcription factor
P15AS	P15 antisense RNA
PTENP1	Phosphatase And Tensin Homolog Pseudogene 1
RICTOR	Rapamycin-Insensitive Companion Of MTOR
RNA7SK	RNA, 7SK small nuclear
SFPQ	Splicing factor proline and glutamine rich
SPQF	Splicing factor proline/glutamine
XIST	X-inactive specific transcript

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Chapter 1

Introduction of Breast cancer

1.1 Anatomy and physiology of the breast

The breasts are bilateral prominent structures that lie in front of the chest above the pectoralis major and serratus muscles and are attached to the skin by a layer of connective tissues known as Cooper's ligaments, where neoplasm of the breast might affect them and causing a retraction of the overlying skin (Maceá and Fregnani, 2006). Each breast extends vertically from the collarbone (clavicle) to the sixth rib edge and horizontally from the middle breastbone (sternum) to the mid-axillary line below the armpit. The centre of each breast has a dark skin area called nipple surrounded by a circular pigmented dark skin area called the areola. Areola skin is characterised by appearance of little bumps that has related to modified sebaceous glands, which moisturise the nipples during breastfeeding (Darlington, 2015).

Structurally, Breast tissues consist of glandular tissues, which are formed by 15-20 lobes that are separated by adipose and connective tissues (Cooper's ligaments). These connective tissues provide support to the breast (Ellis and Mahadevan, 2013). Each lobe made up of multiple smaller lobules, in which milk-secreting glands (alveoli) are found. Milk produced in the alveoli passes into a series of small ducts or tubules that drain toward the apex of the nipple (Ellis and Mahadevan, 2013). At birth, the mammary glands are not developed and appear as a slightly elevated region in the chest. The female breast begins to develop with the onset of puberty by forming the lobules and their ducts, and increasing fat deposition leading to an increase in breast size. The size and pigmentation of the areola and nipple also increase (Ellis and Mahadevan, 2013). The development of the breast during puberty is under the influence of oestrogen and progesterone hormones. Estrogen affects the growth of the ducts while progesterone stimulates the growth of lobules

(Ellis and Mahadevan, 2013). Notably, mammary gland tissue development starts at puberty and is completed during pregnancy and lactation. During menopause, atrophy in the glandular tissue occurs beside to a decrease in breast consistency as a result to the reduction in collagen amounts (Ellis and Mahadevan, 2013).

In addition, the breast contains nerves, blood vessels and lymphatics, which drain the fluids to the axillary and internal thoracic lymph nodes. Approximately, 75% of breast lymphatic fluids drain to the axillary lymph nodes while the remaining 25% passes to the internal thoracic lymph nodes (Darlington, 2015; Neville and Neifert, 1983; Ellis and Mahadevan, 2013).

1.2 Aetiology and histopathology of breast cancer

Breast cancer is a heterogeneous group of diseases that results from a multistep process of accumulating genetic alterations of several proto-oncogenes and tumour-suppressor genes as well as other genetic changes including chromosomal rearrangements and copy number amplifications (Osborne et al, 2004). Breast cancer comes in several clinical and histological forms and represents the most common worldwide disease and the leading cause of death among women in less developed countries (Cancer, I.A.F.R.O. 2013). According to British cancer statistics (2014), the incidence of breast cancers in United Kingdom represents 15% of all new cases of cancer (UK, C. R. 2014). Higher incidence of breast cancer is reported in women with early onset of menarche and late menopause, low number of pregnancies in addition to their first full term pregnancy after age of 40 and short duration of breastfeeding (van den Brandt and Goldbohm, 2002; UK, C. R. 2014). Breast cancer risk factors include frequent exposing to ionizing radiation at a

younger age, excessive alcohol consumption, high body mass index and the use of exogenous hormones like hormonal replacement therapy (UK, C. R. 2014).

According to Histopathological examination, breast cancer can either be in situ (localized in their site of origin), or invasive carcinoma (invade the underlying tissue) (Pinder, 2010). Carcinoma in situ is an epithelial hypertrophy of the ducts and lobules without invading the basement membrane. About 20-25% of all breast cancers are ductal carcinoma in situ (DCIS), while 1-2 % represents lobular carcinoma in situ (Pinder, 2010; Tuzlali, 2016; Cutuli et al, 2015).

Although, the invasive breast cancers (ductal and lobular carcinoma) differ in their morphological characteristics as well as in their metastatic ability, both of them develop from the duct-lobular junction and represent the most common breast cancer types (Turashvili et al, 2007). In contrast to invasive ductal carcinoma, lobular carcinoma represents 15% of all invasive breast cancers and metastasizes markedly to the gastrointestinal system, gynaecologic organs, and peritoneum (Turashvili et al, 2007; Borst and Ingold, 1993; Winn et al, 2016; Gatzka and Carey, 2016).

1.3 Classification of breast cancer

Breast cancer is a heterogeneous disease that comes with distinct histopathological, biological and molecular subtypes leading to disparate response to therapeutics and clinical outcomes. Such classification is clinically important as it allows informed decision regarding treatment, management and determine the prognosis (Viale, 2012; Rakha & Green, 2016; Brenton et al, 2005). Histopathological classification is considered as a worldwide reliable method of subdividing breast cancer in two types, ductal and lobular carcinoma. Although, the basic importance of this

classification is to give an idea about the morphological characteristics of breast cancer, but it has a minimum clinical application particularly in choosing the suitable method of management. The histopathological identification of breast carcinoma together with the tumour size and grade represent the basic outline of a pathological report (Viale, 2012). The biological classification of breast cancer is based on their molecular signature i.e. expression of protein biomarkers and gene expression profiles. Such classification provides information for treatment decision and choosing appropriate therapeutic strategies such as systematic hormonal therapy and chemotherapy (Viale, 2012; Rakha & Green, 2016). The classification used conventionally for patient prognosis and management is based on the expression of the classical immunohistochemistry markers namely oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Viale, 2012; Rakha & Green, 2016). However, high-throughput technologies like gene expression profiling and microarray analysis led to the identification of further at least five molecular breast cancer subtypes: luminal, normal breast-like, HER2, and basal-like (Brenton et al, 2005; Vidal et al, 2016). The luminal subtype is further sub-categorised into type A and B. Both subtypes are ER positive and represent 65-70% of breast cancers. However, the characteristic profile of luminal type A includes low ratio of Ki-67 ($\leq 14\%$), a p53 mutation rate of 13% and an excessive expression of ER related genes such as GATA binding protein 3 (GATA-3), X-box binding protein 1 (XBP-1), forkhead box A1 (FOXA1) and Alcohol dehydrogenase 1B (ADH1B) (Zhang et al, 2014; Sørli et al, 2001). Whereas luminal type B is characterised by a high level of ki-67 ($\geq 14\%$), frequent p53 mutation rate ($\sim 40\%$) and high expression of proliferation- related genes such as Cyclin B1 (CCNB1), Marker Of Proliferation Ki-67 (MKI67) and myeloblastosis oncogene-like 2 (MYBL2) (Zhang et al, 2014a; Sørli et al, 2001). The 5-year survival rate of luminal type A is

95% and approximately 50% of luminal type B breast cancer (Zhang et al, 2014a; Sørli et al, 2001).

Basal-like subtypes are known to be ER, PR and HER2 negative (triple negative) and likely to be grade 3 tumour (Wu et al, 2012). These types of tumours account for 60% to 90% triple negative cases (Liedtke et al, 2008; Bertucci et al, 2006) and are of particular interest due to their aggressive clinical course and the lack of any form of standard targeted systemic therapy (Badve et al, 2011). The gene expression profiles of these tumours mimic that of the normal breast myoepithelial cells and basal epithelial cells of other parts of the body. Such gene expression profiles include lacking or low expression of ER, PR and HER2, and high expression of basal epithelial cell markers such cytokeratins 5, 6, 14, 17 and EGFR (epidermal growth factor receptor or HER1) (Carey et al, 2006; Wu et al, 2012). These tumours also shows high expression levels of proliferation related genes and are more probable to have low expression levels of BRCA1 and to harbour TP53 mutation (Carey et al, 2006; Wu et al, 2012).

Similar with basal cancers, HER2 over-expression tumours are likely to be of grade 3 tumours (Weigelt et al, 2010; Voduc et al, 2010). HER2 tumours display two patterns of gene expression. The first one is the high expression of HER2 and lack of ER expression (HER+/ER-). The second pattern of HER2 positive tumours is the expression of ER (HER2+/ER +) (Carey et al, 2006). The tyrosine kinase

HER2 receptor is encoded by the HER2 gene, which is a proto-oncogene located on chromosome 17q21 (Yersal and Barutca, 2014). Activated HER receptors undergo dimerization and transphosphorylation on the tyrosine residues of their intracellular domains. The phosphorylated tyrosine residues interact with numerous intracellular signaling molecules, leading to activation of a number signaling pathways that lead to the activation of transcription factors which regulate many

genes involved in cell proliferation, survival, differentiation, angiogenesis, invasion and metastasis (Barnes and Kumar, 2004; Gutierrez and Schiff, 2011). Clinically and biologically, HER2-positive tumours display aggressive behavior, are highly proliferative and more than 40% have TP53 mutations. These tumours account for 15-20% of breast cancer subtypes and are characterised by the high expression levels of genes associated with the HER2 pathway (Barnes and Kumar, 2004; Gutierrez and Schiff, 2011). These tumours show relative resistance to hormonal agents and an increased sensitivity to certain cytotoxic agents such as doxorubicin (Yersal and Barutca, 2014).Doxorubicin sensitivity has been possibly due to amplification of topoisomerase-2 gene which is located near the HER2 locus on chromosome 17 and is the target of this drug (Ross et al, 2003; Gabos et al, 2006). However, the advances in translational science have led to the development of a large spectrum of HER targeted therapies.

Normal breast like tumours are poorly characterised. They account for about 5%-10% of all breast cancers and usually do not respond to neoadjuvant chemotherapy (Yersal and Barutca, 2014).They are characterised by high expression of genes related to adipose tissues presenting an intermediate prognosis between luminal and basal-like tumours (Perou et al.,2000; Smid et al.,2008). These tumours can also be classified as triple negative due to the lack of ER, PR and HER2 expression. However, they are not considered basal type because they lack the expression of cytokeratins 5 and EGFR (HER1) (Yersal and Barutca, 2014).Table 1.1 summaries the characteristics of breast cancer subtypes.

Table 1.1 The molecular taxonomy of breast cancer

Molecular subtype	Tumour characteristics	References
Luminal A	ER positive. Excessive expression of ER related genes GATA binding protein 3(GATA-3), X-box binding protein 1(XBP-1), Forkhead box A1(FOXA1) and Alcohol Dehydrogenase 1B (ADH1B). Low expression of Ki-67 ($\leq 14\%$). P53 mutation rate of 13%. 5-year survival rate is 95% .	Zhang et al, 2014a; Sørlie et al,2001
Luminal B	ER positive. High expression of proliferation- related genes such as CCNB1, MKI67 and (MYBL2). High expression of ki67 ($\geq 14\%$). P53 mutation rate of 40%. 5-year survival rate is 50%.	Zhang et al, 2014a; Sørlie et al,2001
basal-like	ER negative. Represents 10-15% of breast cancers. High expression of genes related to the basal epithelial cells (cytokeratins 5, 6, and 17). Low expression of BRCA1.	Brenton et al, 2005; Zhang et al, 2014a Carey et al,2006 Wu et al,2012
HER2 (ERBB2+)	Represents 10 % of breast cancers Two patterns of expression similar to basal like tumours and to luminal type B breast tumour.	Brenton et al, 2005; Zhang et al, 2014a Carey et al,2006
Normal breast-like cells	Highly expression of genes related to adipose tissues.	Perou et al,2000 Smid et al,2008

Currently, the use of endocrine therapy is considered the best choice for the treatment of breast cancer with ER and PR overexpression. While systematic chemotherapy is considered as the best choice for the treatment of HER2 positive tumours (Viale, 2012 ; Rakha & Green, 2016). For ER positive and HER2 negative breast tumours, the option of using chemotherapy with endocrine therapy is determined according to the tumour size and grade, the rate of proliferation

(determined by the immunohistochemically staining of ki67 antigen), distant metastasis and lymph node involvement (Viale, 2012 ; Rakha and Green, 2016). Gene signatures have been developed as predictors of response to therapy and the protein and non-protein products of the genes that are directly involved in the development of breast cancer are potential targets for the development of novel specific and effective therapeutics.

1.4 Stages of breast cancer

The TNM (Tumour, Node, Metastasis) staging is a scoring system for evaluating the predictive factors of breast cancer management (Benson, 2003; Bagaria et al, 2014; Brierley et al, 2016). This grading system involves the determination of the tumour state according to their size, axillary or local lymph node involvement and whether they are invading to the nearby tissue or distant metastasis (Benson,2003; Bagaria et al,2014; Brierley et al,2016). However, this system is not sufficient to predict a suitable treatment for a certain type of breast cancers, particularly, the triple negative subtype (TNBC). According to the TNM staging system, the stages of breast cancer range from 0 to IV (0 to 4). Table 1.2 summarises the TNM stages depending on the combination of tumour size (T), lymph node status (N) and metastasis (M).

Table 1.2 The TNM stages of breast cancer (Whitman et al, 2006)

Stage	Characteristics
Stage 0 (Tis, N0, M0)	Non-invasive cancer. Localised in the ducts and lobules without spreading to the axillary lymph nodes.
Stage 1A (T1, N0, M0)	Small size of tumour. Invasive but has not spread to the lymph nodes.
Stage 1B (T1, Nmic, M0). Nmic refers to lymph nodes seen by microscope only	Tumour size is larger than 0.2 mm and less than 2mm. Spread to the lymph nodes.
Stage IIA: 1-T0, N1, M0 2-T1, N1, M0 3- T2, N0, M0	1-No evidence of breast tumour but it has spread to the lymph nodes. 2-Tumour size less than 20mm and has spread to the axillary lymph nodes. 3-Tumour size is between 20-50mm and has not spread to the axillary lymph nodes.
Stage IIB: either 1-T2, N1, M0 2- T3, N0, M0	1-Tumour size is between 20-50mm and has spread to 1-3 axillary lymph nodes 2-Tumour size is larger than 50mm without spreading of cancer cells to the lymph nodes.
Stage IIIA (T0, T1, T2, T3, N2, M0)	The tumour presents in any size. Spread to 4-9 axillary lymph nodes but no distant metastasis.
Stage IIIB (T4, N0,N1 or N2, M0)	The tumour spread to the chest wall causing inflammation or ulceration with or without involvement of axillary lymph nodes. No distant metastasis.
Stage IIIC (any T, N3, M0)	The tumour presents in any size. Spread to 10 or more axillary lymph nodes without distant metastasis.
Stage IV (any T, any N, M1)	Tumour presents in any size. Tumour has metastasised to different parts of the body.

1.5 Grades of the breast cancer

The histological grading of breast cancer is responsible for assessing the prognostic factors of the tumours. This grading uses certain criteria that determine the degree of tumour cell differentiation such as cell morphology (glandular or tubular), nuclear pleomorphism and the rate of mitosis by detecting the level of Ki-67, which increases in proliferating cells (Sainsbury et al, 1994; Rakha et al, 2008; Rakha and Green, 2016).

Accordingly, three grades are currently used and these include grade 1, 2 and 3. Grade 1 (low grade or well differentiated) is characterised by the appearance of large number of differentiated cells in well-organized pattern and low rate of mitosis. Grade 2 (intermediate or moderately differentiated) involves the appearance of abnormal cells, which look different from normal cells (variation in size and shape of the cells as well as the nucleus become larger and darker) and have a faster rate of growth (Rakha et al, 2008; Elston et al, 1999). Grade 3 (high grade) is characterised by the presence of poorly differentiated or undifferentiated cells with irregular pattern and high rate of proliferation. Table 1.3 provides summary of the grades of breast cancer and their characteristics.

Table 1.3 The grades of breast cancer (Sainsbury et al, 1994; Rakha et al, 2008; Rakha and Green, 2016)

Grade	Characteristics
Grade 1	Low grade or well differentiated. Large number of differentiated cells in well-organized pattern and low rate of mitosis.
Grade 2	Intermediate or moderately differentiated Cells look different from normal cells and their growth rate is increased.
Grade 3	High grade or poorly differentiated. Cells are poorly differentiated or undifferentiated cells with irregular pattern and characterized by immense rate of proliferation.

1.6 Long non-coding RNA

The Encyclopaedia of DNA Elements (ENCODE) Consortium, an international research consortium aiming to identify the functional elements in the human genome sequence, confirms that 80.4% of human genome displays some functionality in at least one cell type. Their data interpreted main features about the organisation and function of the human genome, including the annotation of coding and noncoding regions and identify the regulatory elements controlling chromatin accessibility, transcription factor binding and DNA methylation (Qu and Fang, 2013). Their results revealed that many of these regulatory elements interact with one another to form a network that affects gene expression (Qu and Fang, 2013).

Results from the ENCODE project included the annotation of 20,687 protein-coding genes, 33,977 noncoding transcripts and 9640 long non-coding RNA (lncRNA) genes (Qu and Fang, 2013).

The term non-coding RNA (ncRNA) is regularly used to describe RNA that does not encode a protein. Although it has been generally assumed that most genetic information that specifies biological form and phenotype is expressed as proteins,

increasing number of evidence suggests that the majority of the genomes of mammals is in fact transcribed into ncRNA, many of which are alternatively spliced, may be processed into smaller products and have a very wide range of biological functions. ncRNA are a class of naturally occurring RNA molecules, transcribed from non-protein coding genes and possess a fundamental role in cell Biology (Mattick and Makunin,2006; Marchese and Huarte, 2014) They are predominantly associated with eukaryotes and reported to encompass many varieties of RNA that have specific but non-coding functions (Mattick and Makunin,2006). These ncRNA are reported to comprise an important role in underpinning the highly controlled, complex pathways involving gene expression and may be significant in disease, particularly cancer (Mattick and Makunin, 2006). Such ncRNA include housekeeping ncRNA (examples include ribosomal RNA, transfer RNA, small nuclear and nucleolar RNAs) and regulatory ncRNA which contribute to the regulation of cellular differentiation and developments by their effect on gene expression at transcriptional and posttranscriptional levels (Dinger et al, 2008; Prasanth and Spector, 2007). Regulatory ncRNAs are generally subdivided according to their length into two classes, either small non-coding RNAs composed of 18-200 nucleotides or long non-coding RNAs (lncRNAs) which are described as transcripts longer than 200 nucleotides (Mattick and Makunin, 2006; Wang and Chang, 2011). Examples of small ncRNAs include microRNAs (miRNAs) and small nucleolar RNAs (snoRNAs). miRNAs are one of the most studied of small ncRNA and act as endogenous post-transcriptional silencing effectors. They serve as guides for either the cleavage or translational inhibition of complementary mRNA target transcripts (Amaral and Mattick, 2008). Their regulatory roles in several critical biological processes such as cellular proliferation, differentiation and developmental timing are well established and several evidence support their

involvement in cancer and many other diseases (Amaral and Mattick, 2008). Small nucleolar RNAs (snoRNAs) act as guide for post-transcriptional modification of ribosomal RNAs (rRNA) and some spliceosome RNAs. These post-transcriptional modifications are very important for the production of efficient and accurate ribosomes, the cell's protein synthesis machinery (Lestrade and Weber, 2006). In addition, the importance of snoRNAs in controlling fate and its role in oncogenesis have been recently highlighted (Reviewed by Williams and Farzaneh, 2012).

LncRNAs, which represent the focus of this thesis, have received attention due to their tissue- and developmental-specific expression patterns and their functional importance in many physiological and pathological processes (Quinn and Chang, 2016). The improvement in RNA sequencing and computational prediction techniques have resulted in the identification of large numbers of these lncRNAs (more than 16.000) and will undoubtedly lead to a further increase in their number (Spector, 2017). Similar to mRNAs, lncRNAs are RNA polymerase II transcripts, processed via capping at the 5' end polyadenylated at the 3' end and spliced. They are predominately located within the nucleus, but they can also be found in the cytoplasm (Dinger et al, 2008). Unlike mRNAs and other small ncRNAs, lncRNAs are poorly conserved between related species and because of this it has been initially suggested that these transcripts are not functional (Wang and Chang, 2011; Ponjavic et al, 2007). However, their strong cell-type specific and temporal expression has confirmed their importance and the fact that they are under transcriptional control rather than "transcriptional noise" (Cabili et al, 2011; Mattick and Makunin, 2006; Ponjavic et al, 2007). It is now well established that several lncRNAs play key roles in the control of multiple biological processes, such as gene expression, epigenetic regulation, chromatin remodeling, organ or tissue development, and innate immune response (Quinn and Chang, 2016).

Accumulating evidence has further supported their roles in many cellular functions relevant to the process of ageing including cellular response to stress, proliferation, differentiation, quiescence, senescence and death (Spector, 2017). .Consequently, mutations and deregulation of these lncRNAs have been associated with the development and progression of many human diseases, including cancer, autoimmune diseases and cardiovascular diseases. Accordingly, lncRNAs provide interesting novel opportunities as potential biomarkers for disease diagnosis, treatment and prognosis, and new therapeutic strategies (Spector, 2017).

1.6.1 Types of long noncoding RNAs

lncRNAs can be divided into three subclasses: natural antisense transcripts (NATs), intronic long non-coding RNAs and long intergenic non-coding RNA (lincRNAs) (Moran et al, 2012).

1.6.1.1 Natural antisense transcripts (NATs)

NATs belong to a large class of lncRNA that have transcripts complementary to other RNAs. NATs are, as their name implies, transcripts coded from the opposite strand of a protein-coding gene in the antisense direction, of which around 40% of coding genes express these lncRNAs (Moran et al, 2012; He et al, 2008). There are two main NAT categories: cis-NATs and trans-NATs. cis-NATs are antisense RNA transcribed from a single locus, due to the existence of a physical overlap of two genes in different strands, usually having specific targets in a one-to-one style. On the other hand, trans-NATs are RNAs transcribed from different loci, displaying imperfect complementarities; therefore, they are able to aim at many sense targets forming complex regulation networks (Lavorgna et al, 2004). Antisense-overlapping

lncRNAs have a tendency to undergo fewer splicing events and the basal expression levels in different tissues and cell lines can be either positively or negatively regulated (Moran et al, 2012; He et al, 2008). These lncRNAs can use transcriptional and post-transcriptional gene regulatory mechanisms to carry out a wide variety of biological roles. They can form duplexes with their corresponding mRNA counterpart to either induce or inhibit their translation (Lavorgna et al, 2004) and they have been implicated in epigenetic silencing of functionally important genes (Morris et al, 2008 ; Morris, 2009). An important example of a functional NAT is ANRIL, an lncRNA involved in cancer progression (Gibb et al, 2011a). ANRIL is a 3.8 kb-long antisense transcript to the INK4 locus that spans an estimated region of 30–40 kb at chromosome 9p21 and its expression correlates with INK4a epigenetic silencing (Gibb et al 2011a; Li and Chen, 2013). The INK4 locus encodes three tumour suppressor genes that are reported to be silenced in prostate cancer. ANRIL is reported to be an initiating factor in cancer formation by causing abnormal silencing of the INK4 (El Messaoudi-Aubert et al, 2010; Pasmant et al, 2007). Studies have shown that ANRIL mediates INK4a transcriptional repression in cis by acting as scaffold molecule and interacting with the Pc/Chromobox 7 (CBX7) protein, a member of the Polycomb Repressive **Complex 1** (PRC1) associated with the remodeling and manipulation of chromatin (Yap et al, 2010).

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1.6.1.2 Intronic lncRNAs

Intronic lncRNAs are transcripts contained within introns of protein coding genes in either the sense or antisense direction and are released during pre-mRNA processing by the action of spliceosomes (Moran et al, 2012). These lncRNAs have the same tissue expressions as their corresponding protein-coding genes and

function either by acting as a regulator of alternate splicing of the protein transcript or by being involved in transcript stabilisation (Moran et al, 2012). Some of these lncRNAs possess a long half-life in the cytoplasm allowing them to contribute to the regulation of translation. (Mattick and Gagen, 2001; Hesselberth, 2013).

For instance, SAF, an lncRNA transcribed from the opposite strand of intron 1 of the human FAS gene, is involved in regulating the expression of Fas alternative splice variants through pre-mRNA processing (Yan et al, 2005). Over-expression of SAF lncRNA in the human T-leukemic cell line Jurkat did modulate the expression of different FAS protein soluble forms, making cells more resistant to FAS-mediated apoptosis (Yan et al, 2005; Louro et al, 2009).

Intronic sense and antisense lncRNAs may regulate the expression of a neighbouring protein-coding gene through a phenomenon termed transcriptional interference, which prevents initiation complex recruitment or transcriptional elongation (Mazo et al, 2007). Evidence has been provided that in human, a partially intronic lncRNA, produced from the genomic locus encoding dihydrofolate reductase (DHFR), directly interacts with the major promoter, decreasing the expression of the protein-coding RNA (Martianov et al, 2007). Another work showed that long spliced intronic antisense transcripts, overlapping the promoter of the progesterone receptor gene (PGR), are necessary for activation of PGR expression (Schwartz et al, 2008).

1.6.1.3 Long intergenic non-coding RNAs (LincRNAs)

LincRNAs, unlike NATs and Intronic lncRNAs, are transcribed in regions outlying protein-coding genes (Moran et al, 2012; Cabili et al, 2011). They were previously considered to be “JUNK DNA” that has no functional importance. However, studies

have shown that these lncRNAs play a myriad of functions that range from epigenetic regulation to post transcriptional genetic modulations. Their roles have been demonstrated in embryonic stem cell pluripotency, cell proliferation and immune surveillance (Amit et al, 2009; Moran et al, 2012). The abnormal expressions some of these lncRNAs in a number of cancer support their role in the formation and progression of different types of cancer ranging from solid tumours to leukaemia (Tsai et al, 2011).

Compared to mRNA expression, lincRNA expression is generally more variable between tissues (Derrien et al, 2012) and many of them have been reported to be preferentially expressed in brain and testis (Ravasi et al, 2006; Cabili et al, 2011). The expression of some of these lincRNAs is reported to be regulated by transcription factors such as p53 and NF κ B (Amit et al, 2009; Moran et al, 2012). For instance, lincRNA-p21 is transcribed from a region ~15 kb upstream of p21 and mediates apoptosis in a p53-dependent manner upon DNA damage response (Huarte et al, 2010).

Another lincRNA, P21 associated ncRNA DNA damage activated (PANDA), is transcribed from the ~5 kb upstream region of p21 in an antisense orientation to p21. Similar to p21, the expression of PANDA is also induced by DNA damage and activated in a p53-dependent manner.(Huarte et al, 2010).

While many of these lincRNAs are found mostly in the cytoplasm, some are almost exclusively found in the nucleus such as Xist, MALAT1, NEAT1, and MIAT (Kino et al, 2010; Hutchinson et al, 2007 ; Sone et al, 2007; Spector, 2017).

1.6.2 Functions of lncRNAs

lncRNAs demonstrate a wide range of functional and structural roles and are involved in many biological processes (Gibb et al, 2011b). They have been implicated in a number of cellular functions. These include acting as regulators of gene expression in both cis and trans directions, guiding chromatin-modifying complexes to specific loci, X-chromosome inactivation, RNA splicing and nuclear organisation (Mercer et al, 2009; Wang and Chang, 2011).

The main ways in which these lncRNAs exert their effects can be described by four mechanistic models, including acting as signals, decoys, guides or scaffolds (Wang and Chang, 2011; Deniz and Erman, 2017).

1.6.2.1 lncRNA as signals

lncRNAs can act as a signal in regulation of gene expression since they are characterised by cell-type specific expression that occurs in a specific time and place in addition to their ability to respond to different external stimuli (Figure 1.1) (Wang and Chang, 2011).

A number of lncRNAs was found to be activated in response to specific stimuli and subsequently activated or down regulated the corresponding subsets of genes (Wang and Chang, 2011; Moran et al, 2012). These signal lncRNAs appear to have critical roles in epigenetic regulation, whereby transcriptional silencing of groups of genes on a particular chromosome is mediated by interactions with the chromatin or by the recruitment of chromatin-modifying proteins (Wang and Chang, 2011; Moran et al, 2012). Some lncRNAs can bind the transcription factors themselves, in order to regulate gene expression, resulting in the inability of the factor to bind its target sequence (Wang and Chang, 2011; Moran et al, 2012).

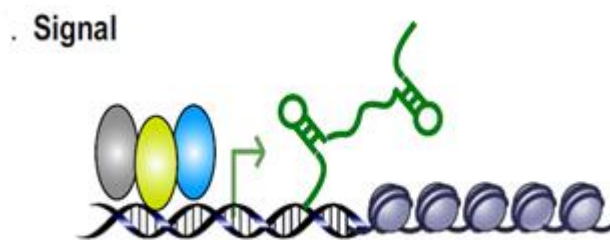


Figure 1.1 lncRNAs exert their effects by diverse mechanisms. In this case, the lncRNA acts as a single to enhance gene expression, as well as recruiting transcription factors thereby regulating gene expression in a spatio-temporal manner. Adapted from Chang and Wang (2011)

Signal lncRNA expression cannot only act as markers of transcriptional elements and their abundance, but also as a reflection of spatio-temporal gene regulation (Wang and Chang, 2011; Moran et al, 2012). The best example is Xist, which is an lncRNA about 17kb in length, involved in the inactivation of female X chromosome. Initially, during female cell differentiation, a small region within Xist known as Rep A (Repeat A) binds to one of the X chromosomes in the X inactivation center (Xi) in association with Polycomb Repressive Complex 2 (PRC2). This will lead to further repression in the Xist gene on the intended X chromosome, causing a whole silence of the chromosome in cis (Brosnan and Vionnet, 2009). Meanwhile Tsix, which is an antisense lncRNA, plays an important role in protecting the other X chromosome

from silencing by the repressive effect of Rep A through preventing their binding to the Xi (Moran et al, 2012).

1.6.2.2 LncRNAs as decoys

Different non-coding RNAs were found to interact and some lncRNAs demonstrated binding capabilities to miRNAs, consequently preventing further binding of the miRNAs to their target mRNAs (Wang and Chang, 2011; Moran et al, 2012). It was found that lncRNAs can also act as decoys to remove transcription factors from gene promoters so as to prevent binding and transcription (Figure 1.2) (Wang and Chang, 2011). In this case, the function of the lncRNAs is probably as a negative regulator of a specific effector to control transcription repression (Wang and Chang, 2011). These lncRNAs act as molecular decoys in order to regulate gene transcription, post-transcriptionally, thus protein synthesis and possibly in a tissue-specific capacity (Wang and Chang, 2011; Moran et al, 2012).

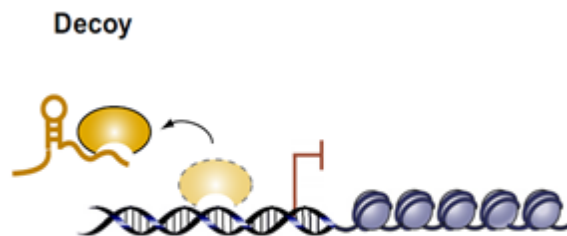


Figure 1.2 LncRNAs exert their effects by diverse mechanisms. These lncRNA can bind to transcription factors and other proteins preventing them from binding their target DNA sequence acting as molecular decoys. Adapted from Chang and Wang (2011)

Growth Arrest 5 (GAS5) is a lncRNA reported to act like a “riborepressor” of the glucocorticoid receptor (GR). It binds to the DNA-binding domain of the GR by acting

as a decoy glucocorticoid response element (GRE), thus competing with DNA GREs for binding to the GR and modulating the transcriptional activity of the GR (Kino et al, 2010). P21 associated ncRNA DNA damage activated (PANDA) is another lncRNA that act as a decoy and prevents the p53-mediated apoptosis because of DNA damage (Hung et al, 2011). PANDA inhibits the expression of apoptotic genes by sequestering the transcription factor NF- κ B of its promoters (Hung et al, 2011). Studies have shown that inhibiting the interaction between PANDA and NF- κ B interaction leads to an increased transcription activity of NF- κ B and restored apoptosis

1.6.2.3 LncRNAs as guides

Epigenetic factors can determine cellular identity by modulating specific gene expression in both cis and trans, using enzymes to modify the chromatin, either activating or repressing the gene (Figure 1.3) (Moran et al, 2012). LncRNAs potentially have a role in guiding these epigenetic factors, for example, directing chromatin-remodelling complexes to their specific loci where they exert their effect (Moran et al, 2012). The process by which this occurs has yet to be elucidated, however, one suggested mechanism is that some lncRNAs may bind the chromatin first and mediate binding of chromatin-modifying complexes by acting as a docking station (Chu et al, 2011). The other one is proposed by the interaction of DNA-binding proteins to lncRNA, which in turn guiding them to their target sites in chromatin (Kanhare and Jenner, 2012). Guiding lncRNAs have been found to be involved in important biological processes including, X inactivation and roles in the establishment of chromatin states (Wang and Chang, 2011).

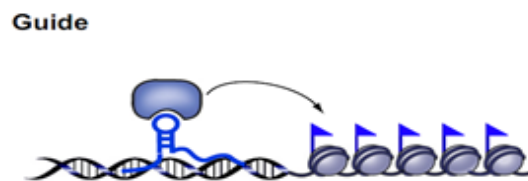


Figure 1.3 LncRNAs exert their effects by diverse mechanisms. These lncRNA can act as guides and are able to recruit chromatin-modifying complexes to specific genes, and thus contribute to tissue-specific gene expression. Adapted from Chang and Wang (2011)

Both AIR (Antisense to insulin-like growth factor type 2 receptor (Igf2r)) and HOTAIR (HOX Antisense Intergenic RNA) are examples of lncRNAs that act as guides for the regulation of gene expression. Air is a 108 kb, polyadenylated, non-coding RNA that transcribed from an antisense promoter located in intron 2 of the Igf2r (insulin-like growth factor type 2 receptor) in the mouse chromosome 17 (Hung and Chang, 2010 ; Bonasio et al, 2010). The Igf2r gene cluster contains three imprinted genes: Igf2r, Slc22a2, and Slc22a3. Unlike Igf2r, Slc22a2, and Slc22a3 maternal transcription, the AIR ncRNA is only expressed from the paternal allele (Nagano et al, 2008). Expression of the AIR results in a "cloud" nuclear pattern over the imprinted DNA locus during embryonic development of the placenta and the adult heart (Hung and Chang, 2010 ; Bonasio et al, 2010). HOTAIR is located at the boundary of two chromatin domains in the HOXC locus. HOTAIR is transcribed antisense to the HOXC genes. HOTAIR distally regulates the chromosomal domain in trans on HOXD locus (Rinn et al, 2007; Biswas and Desai, 2017). Another study revealed that the 5' domain of HOTAIR physically interacts with PRC2 methylase and increases its activity, which facilitates histone H3 lysine-27 trimethylation on the HOXD locus and results in silencing of the HOXD gene (Rinn et al, 2007).

1.6.2.4 LncRNAs as scaffolds

LncRNAs can interact with chromatin-modifying complexes, transcription factors and splicing factors to form ribonucleoprotein complexes, acting to some extent as a scaffold. Such ribonucleoprotein complexes may act on chromatin to affect histone modifications or may play a structural role and stabilise nuclear structures or signaling complexes (Figure 1.4) (Wang and Chang, 2011; Moran et al, 2012).

The function of scaffold lncRNAs is essential to provide an infrastructure, allowing proteins and various transcriptional factors to assemble around in order to carry out their effect (Khalil et al, 2009; Wang and Chang, 2011). This class of lncRNAs must presumably possess different binding domains to allow the degree of coordination to occur, and in the binding of the constituents bring these effector molecules together to result in their interaction (Wang and Chang, 2011). In many cases, the disruption of these scaffold lncRNAs leads to detrimental biological effects inferring these lncRNAs are required for co-localisation and for the precise dynamics of interactions to occur (Khalil et al, 2009; Taft et al, 2010).

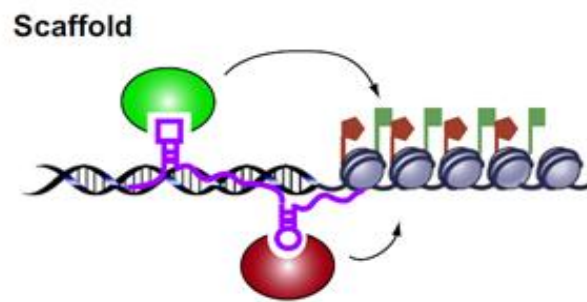


Figure 1.4 lncRNAs exert their effects by diverse mechanisms. These lncRNA can act as molecular scaffolds for protein complexes and act in part as a stabiliser between proteins that may lack protein interaction domains. Adapted from Chang and Wang (2011)

An example of lncRNA that acts as a scaffold is ANRIL, an antisense non-coding RNA transcribed from INK4 locus, which encodes three tumour suppressors (p15INK4b, ARF and p16INK4a). Studies have revealed that ANRIL is able to bind to chromobox 7 (CBX7) within the PRC1 complexes, leading to the repression of the expression of the genes located on the INK4 (Kotake et al, 2011; Yap et al, 2010).

LncRNAs that act as scaffolds and bring together different proteins or bridging protein complexes includes Nuclear Enriched Abundant Transcript (NEAT1) and Metastasis-Associated in Lung Adenocarcinoma Transcript (MALAT1) (Guttman and Rinn, 2012). Both NEAT1 and MALAT1 bind multiple proteins localising to the paraspeckles and nuclear speckles, respectively (Clemson et al, 2009; Sunwoo et al, 2009; Murthy and Rangarajan, 2010; Spector, 2017).

1.7 The hallmarks of cancer

Cancer is defined as the uncontrollable division and proliferation of abnormal cells forming malignant growths, which are caused by mutations involving DNA (Hudson, 2011). These cancerous cells proliferate rapidly, resist apoptosis and can metastasize to various parts of the body, making cancer a challenging disease to treat (Hudson, 2011). Although there are many different types of cancer, all cancer cells share six biological capabilities that are acquired during the multistep development of a tumour; these shared characteristics known as the six hallmarks of cancer, which aid carcinogenesis (Hanahan and Weinberg, 2000).

The most fundamental trait of cancer cells is their ability to proliferate without a controlled signalling input. This is possible by several mechanisms, including the increase in growth factor production such as vascular endothelial growth factor (VEGF) and transforming growth factor (TGF) (Hanahan and Weinberg, 2000). Cancer cells can also increase the number of receptors on their cell surface and structurally alter existing ones in order to enable cancer cell signalling, which causes other cells to proliferate out of control (Hanahan and Weinberg, 2000). The second important hallmark of cancer cells is the ability to evade growth suppression. In normal cells, proliferation is a highly controlled process, in which different signals

are involved in specific phases of the cell cycle (Hanahan and Weinberg, 2000). In particular, the G1 phase of the cell cycle is a vital point in which anti-growth signals have the ability to block proliferation and prevent further growth. However, most cancer cells can evade these signals in order to continue proliferating (Hanahan and Weinberg, 2000). The two most common tumour suppressors down regulated in cancer include the retinoblastoma protein (Rb) and p53. The loss of these tumour suppressor genes aids cancer cells in their third distinctive trait of replicative immortality (Hanahan and Weinberg, 2000). Normal cells possess an intrinsic mechanism, which blocks cell division to a certain limit. However, cancer cells have the ability to over express telomerase, which allows the cells to continue to proliferate (Hanahan and Weinberg, 2000). In addition to their ability to proliferate out of control, cancer cells also have the ability to resist apoptosis by down regulating signalling pathways, through the over expression of anti-apoptotic (E.g. Bcl -2) and the silencing of pro-apoptotic proteins (E.g. Bax/ Bak). The extrinsic pathway in particular is widely implicated in tumour formation and is triggered from within the cell because of DNA damage (Hanahan and Weinberg, 2000).

A critical trait of cancer cells required for the progression of cancer is the ability to activate metastasis and tissue invasion (Hanahan and Weinberg, 2000). This is a key component in the spreading of cancer from the primary site to distant organs. The process involves changes to the ways in which cells attach to other cells and to the extracellular matrix (Hanahan and Weinberg, 2000). There are several steps involved, including local tissue invasion, intravasation, transition through the blood and lymphatic tissue and finally colonisation in foreign tissues. Finally, in order to maintain tumour growth and metastasis, cancer cells have the ability to induce angiogenesis, the formation of new blood vessels (Hanahan and Weinberg, 2000). Tumour angiogenesis is a multi-step process, which involves signalling from several

pro-angiogenic growth factors such as VEGF. Angiogenesis enable the tumour to grow and expand through the delivery of oxygen and nutrients to the cells and it is suggested that metastases can migrate through the new tumour vessels into the circulation allowing aberrant cells to colonize other tissues (Hanahan and Weinberg, 2000)

1.8 LncRNAs and cancer

There is a number of evidence that implicates lncRNAs in a wide range of cancers, as many have been found to be dysregulated in these cells (Hudson, 2011; Moran et al, 2012). A substantial amount of the evidence acquired so far suggests that lncRNAs play important roles in each of the hallmarks of cancer and therefore contribute to the carcinogenesis process, invasion and metastasis. Some lncRNAs are classified as oncogenic transcripts because they regulate cellular pathways that lead to oncogenesis and metastasis. Identified oncogenic or pro-oncogenic lncRNAs include prostate cancer antigen 3 (PCA3), prostate cancer gene expression marker 1 (PCGEM1), and prostate cancer associated ncRNA transcript 1 (PCAT1) which are highly expressed in prostate cancer, posing as attractive biomarkers Reviewed by (Layfield et al, 2015). PCGEM1 is also overexpressed in breast cancer (Ginger et al, 2006). KRASP, HULC, HOTAIR, MALAT1/NEAT1, p15AS, ANRIL, H19, SRA1, p21NAT, and RICTOR have also been described as oncogenic lncRNAs. On the other hand, others lncRNAs are found to function as tumour suppressors, including MEG3, GAS5, LincRNA-p21, PTENP1, TERRA, CCND1/Cyclin D1, and TUG1. Some of these lncRNAs may have oncogenic and/or tumour suppressive effects depending on the cellular context. For example, XIST

transcript is upregulated in some male cancers, but down-regulated in female cancers (Weakley et al, 2011).

Accumulating evidence suggests that some lncRNAs have critical roles in carcinogenesis by regulating tumour cell proliferation. A particular example of these lncRNAs is the steroid receptor RNA activator (SRA) (Lanz et al, 1999). SRA expression is reported in normal and malignant human mammary tissues. However, elevated levels of SRA are found in breast tumours and the increased SRA levels might contribute to the altered ER/PR action that occurs during breast tumorigenesis (Leygue et al, 1999). The lncRNA, PCAT-1 (prostate cancer associated transcript 1) was identified to be upregulated in a subset of metastatic and high-grade localized prostate cancers (Fu et al, 2006). Overexpression of PCAT-1 causes an increase in LNCaP prostate cancer cell proliferation, whereas its siRNA mediated knockdown caused a 50 % reduction in cell proliferation rate and resulted in the upregulation of genes associated with mitosis and cell cycle (Prensner et al, 2011). Another example of lncRNA that alter cell proliferation is small nuclear RNA7SK also known RN7SK (Yang et al, 2001; Chiappetta et al, 1996). RNA7SK regulates the transcription elongation by binding to the positive transcription elongation factor b (P-TEFb) and inhibiting its positive effects on RNA polymerase II transcription elongation (Nguyen et al, 2001; Yang et al, 2001). RNA7SK also interacts with the transcription factor and chromatin regulator HMGA1 (high mobility group AT-hook 1) which regulates the expression of growth related genes. RNA7SK compete with HMGA1 binding to DNA and therefore inhibiting its function (Chiappetta et al, 1996; Chiappetta et al, 2001). A number of studies have implicated lncRNAs in the inhibition of tumour suppressor genes and therefore allowing the cell to acquire the ability to evade growth suppressors. Five ncRNA fragments have been shown to interact with the tumour suppressor PSF (Li et al, 2009). PSF protein is involved in

repressing the transcription of proto-oncogenes by binding to their regulatory regions. Li et al. (2009) identified these lncRNAs in a screen aimed at identifying RNA that interact with PSF. Their results showed that these lncRNAs promote the release of PSF from the human proto-oncogene GAGE6 regulatory region resulting in an activation of GAGE6 expression (Li et al, 2009). Increased expression of these RNA fragments in human melanoma cell line promoted their tumorigenic phenotype, confirming their role in tumorigenesis and the importance of lncRNA-protein interaction (Li et al, 2009). ANRIL, an antisense non-coding RNA in the INK4 locus, is another lncRNA reported to inhibit the activity of tumour suppressor genes (Kotake et al, 2011). ANRIL functions by interacting with SUZ12 (suppressor of zeste 12 homolog), one of the subunits of the Polycomb repression complex 2 (PRC2) promoting the recruitment of the PRC2 complex to the well-known tumour suppressor gene p15 (INK4B) suppressing its expression (Kotake et al, 2011). Silencing of ANRIL expression in WI38 normal embryonic lung cells leads to an increase in the expression of p15 (INK4B) and inhibition of cell proliferation (Kotake et al, 2011). ANRIL also interacts with a subunit of Polycomb repressive complex 1 (PRC1), CBX7 (chromobox resulting in the recruitment of PRC1 to the p16 (INK4A)/p14 (the tumour suppressor ARF) locus and subsequent silencing of this gene locus by H3K27-trimethylation (Yap et al, 2010). Both, CBX7 and ANRIL are overexpressed in human prostate cancer, highlighting the importance of such interaction for tumour development (Yap et al, 2010).

In addition to lncRNAs that act as oncogenes, others act as tumour suppressors. These include GAS5 (Growth Arrest-Specific 5) and lincRNA-p21. GAS5 was originally isolated due to its increased levels in growth-arrested mouse NIH3T3 fibroblasts (Schneider et al, 1988). Further studies supported these findings by showing that in human leukemic cells, the level of GAS5 expression increased in

density-induced cell cycle arrest and greatly reduced in actively growing cells (Coccia et al, 1992). GAS5 has been implicated in many cancers and its expression has been shown to be altered in many cancers including prostate and breast cancers (Pickard and Williams, 2014). GAS5 has been showed to act as a “riborepressor”: Its exon 12- encoded sequence contains hairpin structure that contains two glucocorticoid response elements (GRE) -like sequences, termed GRE-1 and GRE-2, which are complementary to each other (Raho et al, 2000; Muller et al, 1998). These sequences interact with the DNA binding domain of the glucocorticoid receptors, thus competing with the GRE in the genome for binding to these receptors. This leads to the suppression of several responsive genes including cellular inhibitor of apoptosis 2 (cIAP2) and consequently sensitizes cells to apoptosis (Raho et al 2000). Another example of a lncRNA that acts as tumour suppressor is lincRNA-p21 which was identified in the attempt to identify novel ncRNA involved in the regulation of TP53 function (Huarte et al, 2010). LincRNA-p21 is a p53 target gene with its gene located directly next to the p21 (Cdkn1a) gene on mouse chromosome 17. Its expression has been shown to be activated upon DNA damage in different mouse tumour models (Huarte et al, 2010). LincRNA-p21 associates with the RNA binding protein hnRNP K and mediates its binding to its target gene. hnRNP K is a transcriptional repressor. Binding of lincRNA-p21 to hnRNP K leads to gene silencing and the induction of apoptosis (Huarte et al, 2010). LincRNA-p21 appears to be conserved and is induced in human fibroblasts after DNA damage induction. Further studies have shown that lincRNA-p21 is down-regulated in human prostate cancer, and low levels of lincRNA-p21 correlated with high disease stage and prediction of poor survival (Wang et al, 2017b). Low expression level of lincRNA-p21 was found to correlate with low expression of p53-associated genes (Wang et al, 2017b). In vivo studies showed that overexpression

of lincRNA-p21 inhibited prostate cancer cell proliferation and long-term survival partly by regulating p53 downstream gene expression and by promoting apoptosis (Wang et al, 2017b).

The above examples clearly support a role for lncRNAs in two traits of cancer: sustaining proliferative signalling and evading growth suppressors. LncRNAs also play a role in enabling replicative immortality, the third hallmark of cancer, which is also related to cell proliferation. This trait is due to the fact that tumour cells avoid the shortening of telomeres and cell senescence by expressing the specialised enzyme telomerase which is able to add telomeric repeat to the end of the chromosome (Shay and Wright, 2000). The lncRNA TERRA (telomeric repeat-containing RNA) transcripts are derived from several subtelomeric loci. TERRA localises to telomeres and is involved in telomeric heterochromatin Formation (Deng et al 2009). TERRA is believed to act as a negative regulator of telomerase (Redon et al 2010). TERRA binds to its interacting protein partner hnRNP A1 and together with POT1 (protection of telomeres 1), they act to displace RPA (replication protein A) from telomeric ssDNA after DNA replication to promote telomere capping and preserve genomic integrity (Flynn et al, 2011). TERRA suppresses the activity of the telomerase, therefore low expression levels of TERRA transcription is necessary for the telomerase function and telomere lengthening. Accordingly, low expression level of TERRA is reported in telomerase-positive cancer cells (Ng et al, 2009). In fact, TERRA is downregulated in many cancers, providing a possible link to the longevity of cancer cells by telomerase-mediated lengthening of telomeres (Ng et al, 2009).

Resisting cell death is another hallmark of cancer. A number of lncRNAs have been identified has been showed to play an important role in the control of cell death decisions. PCGEM1 (Prostate-specific transcript 1) is a prostate tissue-specific and

prostate cancer-associated lncRNA involved in inhibiting apoptosis (Petrovics et al, 2004). Overexpression of PCGEM1 in LNCaP cells lead to delayed induction of P53 and p21(Waf1/Cip1) and subsequent inhibition of apoptosis (Petrovics et al, 2004). The PCGEM1-associated anti-apoptotic responses was reported to be androgen-dependent, as androgen-independent variants of LNCaP cells did not exhibit this effects (Petrovics et al, 2004). SPRY4-IT1 is another lncRNAs involved in the control of cell death. SPRY4-IT1 is derived from an intron of the SPRY4 (Sprouty RTK Signalling Antagonist 4) gene. SPRY4-IT1 is predominantly localized in the cytoplasm of melanoma cells, and its knockdown results in defects in cell growth, differentiation and higher rates of apoptosis in melanoma cell lines (Khaitan et al, 2011).

lncRNAs have been implicated in the fifth hallmark of cancer, which allows the tumour cells to invade and form distant metastases. The lncRNA MALAT1 (Metastasis-Associated Lung Adenocarcinoma Transcript 1, MALAT1) has been shown to be involved in metastasis and therefore it is considered as a prognostic marker for metastasis and patient survival in non-small cell lung cancer (NSCLC) (Ji et al, 2003). MALAT1 is extremely abundant in many human cell types and is highly conserved across several species highlighting its functional importance (Gutschner et al, 2011). MALAT1 is retained in the nucleus and specifically localises to nuclear speckles which play a role in pre-mRNA processing (Hutchinson et al, 2007). Studies have shown that MALAT1 regulates alternative splicing of pre-mRNAs by modulating the levels of active serine/arginine splicing factors (Tripathi et al, 2010). High expression level of MALAT1 is associated with metastasis in NSCLC patients. It is also up-regulated in several cancers including lung cancer, uterine endometrial stromal sarcoma, cervical cancer and hepatocellular carcinoma (HCC) (Ji et al, 2003). Studies have shown that MALAT1 promotes cell motility through

transcriptional or post-transcriptional regulation of metastasis-related genes. Additionally, MALAT1 has also been shown to support proliferation and invasion of cervical cancer cells and its knockdown in CaSki cells led to an upregulation of caspase-8 and -3 and Bax and the downregulation of Bcl-2 and Bcl-xL (Guo et al, 2010). HOTAIR (HOX Antisense Intergenic RNA) is another lncRNA involved in cancer metastasis. HOTAIR is a 2.2 kb lncRNA transcribed in antisense direction from the HOXC gene cluster (Rinn et al, 2007). It plays an important role in epigenetic regulation of gene expression by interacting and recruiting the PRC2 to the HOXD locus which leads to transcriptional silencing across (Martianov et al, 2007). HOTAIR also interacts with another histone modification complex, the LSD1/CoREST/ REST complex, which coordinates targeting of PRC2 and LSD1 to chromatin for coupled histone H3K27 methylation and K4 demethylation (Tsai et al, 2010). HOTAIR expression is deregulated in different types of cancer (Yang et al, 2011b). In human breast cancer, HOTAIR is over-expressed and its expression positively correlates with metastasis and poor outcome. HOTAIR depletion inhibits invasiveness in epithelial cancer cells and its overexpression alters H3K27 methylation via PRC2 and therefore alters target gene expression leading to increased cancer invasiveness and metastasis (Gupta et al, 2010).

HOTAIR levels are increased compared with non-cancerous and its high expression is considered as an independent prognostic marker for HCC recurrence and shorter survival (Yang et al, 2011b). In addition, HOTAIR suppression in liver cancer cells sensitizes cancer cells to tumour necrosis factor α induced apoptosis and to the chemotherapeutic drugs cisplatin and doxorubicin (Yang et al, 2011b). Another hallmark of cancer is acquiring the ability to induce angiogenesis. The lncRNA α HIF is a natural antisense transcript (NAT) complementary to the 3' untranslated region of the hypoxia inducible factor α (HIF1 α), negatively regulates the expression of

HIF1 α , a critical regulator of angiogenesis (Rossignol et al, 2002). Overexpression of α HIF triggers HIF1 α mRNA decay (Uchida et al, 2004). α HIF transcripts are detected in several human cancers and it is considered a marker for poor prognosis in breast cancer (Uchida et al, 2004). Another NAT associated with angiogenesis is termed sONE or eNOS antisense (NOS3AS) which regulates the expression of nitric-oxide synthase (eNOS) in a post-transcriptional manner under normoxia and hypoxic conditions (Fish et al, 2007).

Over all, the presented evidence strongly supports the functional importance of long ncRNAs and provides mechanistic understandings how lncRNAs can contribute to the hallmark capacities of cancer cells.

1.9 Long non-coding RNAs in breast cancer

Several lncRNAs have been reported to play an important role in various mechanisms that contribute to the development of breast cancer, including tumorigenesis, proliferation, apoptosis, invasion, angiogenesis and drug resistance. Among these lncRNAs, seven have been investigated intensively. These include HOTAIR, MALAT1, H19, BCAR4, SRA, XIST, GAS5 (Wang et al, 2017a).

Increased expression levels of HOTAIR have been reported in primary breast cancer (Gupta et al, 2010). Further studies have shown that HOTAIR promotes breast cancer metastasis and can be used as metastatic biomarker (Chisholm et al, 2012; Sørensen et al, 2013). According, to its radio-genomic feature, HOTAIR overexpression leads to elevation the ERF (enhancing rim fraction) score, a quantitative dynamic contrast material-enhanced (DCE) breast magnetic resonance (MR) imaging biomarker, and hence increase the possibility of detection early metastasis in breast cancer (Wang et al., 2016a).

As discussed above, HOTAIR acts as scaffold and binds to different functional complexes leading to the modification of specific histone proteins and epigenetically controlling gene expression. The 5' end of HOTAIR binds to PRC2 facilitating H3K27 methylation and the silencing of targeted genes (Sørensen et al, 2013). On the other hand, the binding of 3' end of HOTAIR to the LSD1/ CoREST/REST complex leading to the demethylation of H3K4 and the activation of genes expression (Tsai et al, 2010). Many genes affected by the changes in HOTAIR expression are involved in different cell signalling pathways (Gupta et al, 2010). One of the HOTAIR targets genes is miR-568, which control the expression of nuclear factor of activated T cells 5 (NAFT5) (Li et al, 2014b). Epigenetically silenced miR-568 leads to the increased expression of NAFT5, which activates the expression of several metastatic-related genes, such as S100A4 and VEGF-C (vascular endothelial growth factor C) and promotes the epithelial-mesenchymal transition (EMT) and angiogenesis of breast epithelial cells (Li et al, 2014b).

In addition to its function in the regulation of alternative splicing of pre-mRNA, MALAT1 was reported to form a repressive complex with the RNA-binding protein HuR, which is involved in the regulation of CD133 expression. CD133 is a cancer cell stem marker CD133 that promotes the EMT-program in various cancers, including breast cancer (Latorre et al, 2016). Accordingly, HuR silencing MCF-7 breast cancer cells resulted in an increase in N-cadherin (CDH2) and CD133 expression with a migratory and mesenchymal-like phenotype (Latorre et al, 2016). MALAT1 also interacts with Polycomb 2 protein leading to the activation of the transcription of growth related genes (Yang et al, 2011a). Both full length MALAT1 and an alternatively spliced variant of MALAT1 were found to be highly expressed in ER positive cell lines (Latorre et al, 2016; Ellis et al, 2012).

Breast cancer anti-estrogen resistance 4 (BCAR4) is a lncRNA associated with tamoxifen resistance (Meijer et al, 2006). Over-expression of BCAR4 causes tamoxifen resistance in tamoxifen-sensitive breast cancer cells, anchorage-independent cell growth and an increase in the phosphorylation of ERBB2 (HER2), indicating the involvement of ERBB2 signalling pathway in BCAR4 mediated effects (Godinho et al, 2011; Godinho et al, 2010). BCAR4 is reported to increase cell migration by regulating the transcription of glioma-associated oncogene homolog 2 (GLI2)-dependent target gene (Godinho et al, 2010). The C-C chemokine ligand 21 (CCL21) and its receptor, chemokine receptor 7 (CCL21/CCR7) promotes growth and metastasis of many tumour types including breast cancer (Tutunea-Fatan et al, 2015). The important role of BCAR4 in CCL21-induced hypo-phosphorylation of RNA Pol II Ser5 was further demonstrated in mouse models where locked nuclear acid (LNA) was used to target BCAR4 resulted in the hyper-phosphorylation of RNA polymerase II (Pol II) Ser5 and suppression of breast cancer metastasis in mouse models (Xing, et al.,2014).

H19 is an imprinting lncRNA involved in breast cancer. In humans, this lncRNA is transcribed from the maternal allele located on chromosome 11p15.5 (Pachnis et al, 1984; Zemel et al, 1992). Accumulating evidence demonstrates an oncogenic role for H19 in breast cancer. Over-expression of H19 MDA-MB-231 cells promotes anchorage independent growth (Lottin et al, 2002). In addition, injection of H19-transfected cells into nude mice leads to an increase in tumour progression (Matouk et al, 2007). Studies have also demonstrated increased levels of H19 expression in either invasive breast cancer or ductal carcinoma in situ (DCIS) compared with normal adjacent breast tissues (Zhang et al, 2015). A number of evidence supports a role for H19 in multiple stages of tumour progression including proliferation and metastasis (Raveh et al, 2015). Over-expression of H19 which mediated by the

binding of the transcription factor E2F1 to its promoter, leads to accelerated G1-S transition and cell cycle progression (Berteaux et al, 2005). H19 stimulates tumour cell proliferation by down-regulating tumour suppressors such as p57kip2 or up-regulation of oncogenes such as cyclin E2, facilitating the transcription of angiogenic genes or inhibits apoptotic-related genes (Raveh et al, 2015). Additionally, H19 was found to be highly expressed (ten-fold higher) in ER-positive breast cancer compared to ER-negative tumour tissues (Sun et al, 2015). Inhibition of ER in luminal progenitors lead to a decrease in H19 expression and smaller colony formation, highlighting the presence of ER-H19 axis in ER positive breast cancer cells involved in promoting cell survival (Basak et al, 2015).

The lncRNA SRA modulates the function of ER and PR steroid receptors, is also involved in breast cancer (Klinge et al, 2004). SRA mediates the transactivation of the steroid receptors by binding to their N-terminal AF1 (activation function 1 domain) and forming a co-active complex with SRC-1 (steroid receptor co-activator 1) (Lanz et al, 1999). Genes affected by SRA includes these involved in cell proliferation and apoptosis. In addition, SRA is a part of nuclear receptor-mediated transcription and miRNA processing (Redfern et al, 2013). It also interacts with the RNA helicase P68 and participates in CTCF (CCCTC-binding factor) - mediated chromosome organization (Yao et al, 2010). SRA expression level in breast cancer is much higher than its level in normal breast tissues (Lanz et al, 1999). Several SNPs (single nucleotide polymorphisms; rs10463297, rs801460) in SRA genes have been identified to be associated with an increase susceptibility to breast cancer (Yan et al, 2016). SRA transcript is subject to differential splicing in addition to the lncRNA product, some of the splice variants encode a conserved protein, the SRA protein (SRAP). SRAP is highly expressed in primary breast tumours (Emberley et al, 2003). Studies have shown that SRAP is expressed in breast tumour and that its

levels could be used as a predictive marker in younger patients with ER-positive/node-negative breast cancer (Yan et al, 2009). Microarray studies have shown that SRAP expression is increased in both ER positive and ER negative breast tumour. However, the results showed that the high level of SRAP expression was associated with poor prognosis in ER positive breast cancer patients (Yan et al, 2009).

While the lncRNAs discussed above play an oncogenic role in breast cancer, GAS5 is one of the lncRNAs that act as tumour suppressor. GAS5 encodes different splice variants of non-coding transcripts as well as small nucleolar RNAs (snoRNAs), microRNAs, and PIWI-interacting RNAs (Xu et al, 2016). GAS5 accumulates in growth-arrested cells due to interaction with the mechanistic Target of Rapamycin (mTOR) pathway and through nonsense-mediated decay (Lv et al, 2016). In active growing cells, GAS5 mRNA is degraded through the nonsense-mediated RNA decay (NMD) pathway (Yamashita et al, 2009). The involvement of GAS5 in human cancers was first studied in breast cancer, when it was found to be downregulated in breast cancer tissues (Mourtada-Maarabouni et al, 2009). Further studies reported GAS5 is found to be downregulated in various cancers and its low expression levels were often found to be predictive of poor prognosis in cancer patients (Hayes and Lewis-Wambi, 2015). Moreover, GAS5 silencing was found to promote breast cancer cell proliferation and its overexpression promoted apoptosis and inhibited cell growth in different types of cell including breast cancer cells (Mourtada-Maarabouni et al, 2008; 2009).

The involvement of XIST in breast cancer is now well documented. XIST plays an essential role in X-chromosome silencing in female cells. XIST is expressed in all female somatic cells. However, the loss of X inactivation and expression of XIST transcript have been noticed in breast and ovarian cancers (Pageau et al, 2007).

XIST expression was found to be significantly reduced in breast cancer tissues compared with normal breast tissues (Huang et al 2016). XIST interacts with SHARP/SPEN and SMRT co-repressor and acts as decoy preventing histone deacetylase 3 (HDAC3) from binding to the promoter of PH domain and leucine-rich repeat phosphatase 1 (PHLPP1), so that PHLPP1 is transcribed. PHLPP1 is a phosphatase, which dephosphorylates AKT leading to the inhibition of its activity and decreased cell growth and viability. Proteins such as BRCA1 (breast cancer 1) and the stem cell pluripotency transcription factors NANOG and Oct4 (octamer-binding transcription factor 4) are involved in the regulation XIST expression (Galupa and Heard, 2015). In addition to the seven lncRNAs discussed above, recent studies have identified novel lncRNAs associated with breast cancer. These include LINP1, LINK-A (long intergenic non-coding RNA for kinase activation) and NKILA (NF-Kappa B interacting lncRNA). LINP1 which is overexpressed in human TNBC (triple negative breast cancer), is involved in the regulation of the non-homologous end joining pathway (NHEJ) in TNBC (Zhang et al, 2016b). LINP1 acts as a scaffold and linking Ku80/70 and DNA-PKcs to broken ends and thereby enhancing the repair of DNA double-strand breaks. LINK-A plays an important role in the glycolysis reprogramming of TNBC (Lin et al, 2016). LINK-A interacts with breast tumour kinase (BRK) and leucine-rich repeat kinase 2 (LRRK2), promoting their phosphorylation and activation of HIF1 α (Hypoxia-inducible factor 1-alpha). Active HIF1 α promotes transcriptional programs resulting in the activation of glycolysis reprogramming in TNBC (Lin et al, 2016). NKILA is up regulated by NF- κ B, which is critical link between inflammation and cancer. NKILA binds to NF- κ B /I κ B and prevent NF- κ B activation. NKILA is essential to prevent over-activation of NF- κ B pathway in inflammation-stimulated breast epithelial cells. Low NKILA

expression is associated with breast cancer metastasis and poor patient prognosis (Liu et al, 2016).

In conclusion, an increasing number of evidence supports a crucial role for lncRNAs in the development and progression of breast cancer and highlights the potential of these lncRNAs as therapeutic targets in addition to diagnostic and prognostic (Cerk et al, 2016; Liu et al, 2016). lncRNAs have been shown to be involved in affecting epigenetic regulation, chromatin remodelling, gene expression and signalling pathways that demonstrated to affect various processes in breast cancer events. These include cell proliferation, cell survival, metastasis, angiogenesis and response to therapy. Therefore, characterization of lncRNAs modes of action will allow their future use for therapeutic purposes and as potential biomarkers for diagnostic and prognostic purposes.

The above information has outlined some examples of lncRNAs involved in breast cancer. The focus of this thesis is to investigate the roles of the two nuclear non-coding RNAs, Nuclear Enriched Abundant Transcript 1 (NEAT1) and Myocardial Infarction Associated Transcript (MIAT) in breast cancer. Therefore, the biology of these two lncRNAs will be outlined below.

1.10 NEAT1 (Nuclear Enriched Autosomal Transcript 1)

Nuclear Enriched Abundant Transcript 1 (NEAT 1), also known as Virus Inducible Noncoding RNA (VINC) or MEN ϵ/β RNA (Multiple Endocrine Neoplasia ϵ/β), is a long noncoding RNA encoded on chromosome 11q13.1 by the multiple endocrine neoplasia locus. Its chromosomal location is separated from its genomic neighbor MALAT1 by just 55 kb (Figure 1.5) (Hutchinson et al, 2007). MALAT1 also called NEAT2 (Nuclear enriched Abundant Transcript 2) which was one of the first

lncRNAs that was demonstrated to be associated with a disease, namely non-small cell lung cancer (NSCLC) (Hutchinson et al, 2007). MALAT1 was subsequently identified to play a pivotal role in cell proliferation, migration, and invasion by regulating processes such as alternative splicing, nuclear organization, epigenetic control of gene expression (Hutchinson et al, 2007; Tripathi et al, 2010). NEAT1 shows no homology with NEAT2 but both lncRNAs have been shown to be highly conserved within the mammalian lineage, which suggests that they have significant function (Hutchinson et al, 2007). In addition to another lncRNA GOMAFU/MIAT (Myocardial Infarction Associated Transcript), both NEAT1 and NEAT2 are examples of long noncoding RNAs that accumulate abundantly within the nucleus as RNA components of specific nuclear bodies (Clemson et al, 2009).

NEAT1 is a stable intergenic lncRNA molecule, transcribed by RNA polymerase II into an unspliced structure containing a polyadenylated tail motif at the 3' end of the sequence. It is specifically found within the paraspeckles nuclear compartments, where it localises (Clark and Mattick, 2011; Clemson et al, 2009). There are thought to be at least two isoforms of NEAT1; a small, widely expressed 3.7kb isoform (Hutchinson et al, 2007) NEAT1_1 and a much larger, 23kb isoform termed NEAT1_2 (Clark and Mattick, 2011) which is expressed at slightly lower levels. Interestingly, the NEAT1_2 isoform contains a conserved tRNA-like structure, which can be cleaved by RNaseP to generate long and short noncoding RNA (ncRNA) (Clark and Mattick, 2011; Cornelis et al, 2016). The expression levels of both transcripts are up regulated upon differentiation of human embryonic stem cells (Chen and Carmichael, 2009), muscle differentiation (Sunwoo et al, 2009) and in vitro neuronal differentiation (Mercer et al, 2010). The function of NEAT1 is unique for a lncRNA molecule and therefore is thought to have an important role in the biology of the cell. It is essential in the formation of paraspeckles, the distinct nuclear

structures that localise within the sub- compartments of the nucleus (Clemson, et al, 2009; Cornelis et al, 2016).

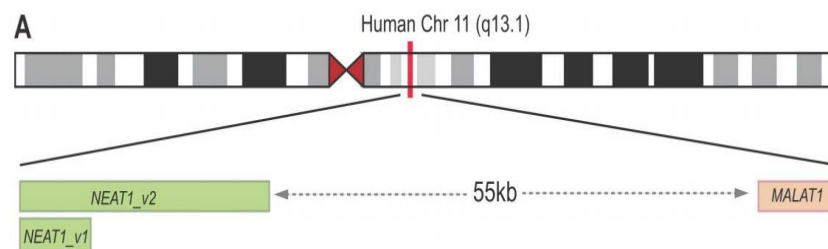


Figure 1.5 diagram showing the location of the NEAT1 gene on chromosome 11q13.1, about 55kb nearby to MALAT1 locus. NEAT1 transcribed into NEAT1_1 (short isoform) and NEAT1_2 (long isoform). Adapted from Bond and Fox, 2009.

1.10.1 NEAT1 and nuclear paraspeckles

Nuclear paraspeckles are mammalian specific ribonucleoprotein nuclear bodies named due to their speckled appearance and they are approximately 0.5-1 Microns in size and their number between 5-20 foci per nucleus (Fox and Lamond, 2010; Clark and Mattick, 2011). They are located in the interchromatin nucleoplasmic space within the cell, near to but distinct from splicing factor-enriched nuclear speckles (Bond and Fox, 2009). Figure 1.6 shows fluorescent images of the stained paraspeckles within the nuclei and their relation to other nuclear speckles found in the adjacent area.

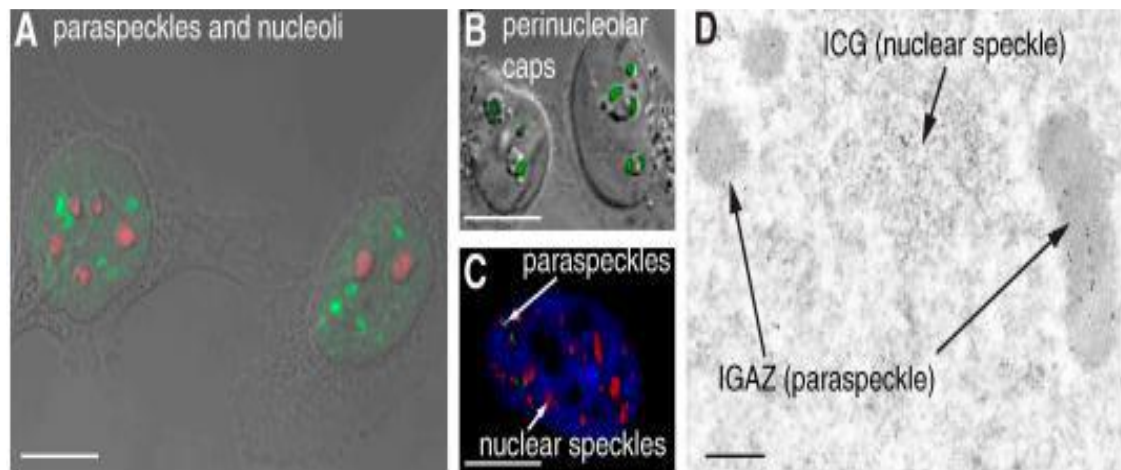


Figure 1.6 the fluorescence graphs of paraspeckles. (A) the combined interference contrast and fluorescence micrograph of HeLa cells stained with anti-PSPC1(anti-paraspeckles protein 1) reveals the paraspeckles (green) as nucleoplasmic domain, which distinct from nucleoli (stained with B23 antibody; red). (B) HeLa cells showing reorganization of the DBHS protein PSPC1 (green) to perinucleolar caps after treatment with actinomycin D to inhibit RNA Pol II transcription. (C) HeLa cell stained with anti-PSPC1 (green), anti-SC35 (red), and DAPI (blue) to show the relationship between paraspeckles abutting nuclear speckles in the interchromatin space. (D) TEM image of a HeLa cell section immune gold labelled with anti-PSPC1. The labelled IGAZs are usually found in close proximity to the interchromatin granules (ICGs; nuclear speckles). This image is adapted from the Bond and Fox , 2009.

The relationship between NEAT1 and paraspeckles can be seen by their correlation in expression levels, an increase in the level of NEAT1 expression results in an increased number of paraspeckle bodies within the cell (Clemson et al, 2009; Cornelis et al. 2016). NEAT1 is essential for the formation of paraspeckles since NEAT1 knockout mouse were found to be devoid of paraspeckles and showed a loss in female fertility. Knockout females were not able to form the corpus luteum in a stable proportion resulting in infertility, as well as a lactation defect (Nakagawa, *et al*, 2014; Standaert et al, 2014). The suppression of NEAT1 using small interference RNAs (siRNA) eliminates the presence of paraspeckles but does not affect the expression of other nuclear structures such as Cajal bodies or nuclear speckles (Clemson et al, 2009). Interestingly, a recent study has dissected the role of the two

NEAT1 isoforms by using CRISPR-Cas9 genome editing to create total NEAT1 knockout cell line, cell line with expression of short isoform NEAT1_1, and cell line that express twofold more of NEAT1_2 long isoform (Li et al, 2017b). This study has provided evidence that the long NEAT1 isoform, NEAT1_2 is the major component of the paraspeckles while NEAT1_1 is not and co-localises in numerous non-paraspeckles foci named "micro speckles," suggesting that it may carry paraspeckles-independent functions (Li et al, 2017b).

1.10.2 Function of paraspeckles

Paraspeckles are stress-induced nuclear bodies. Their function is to retain RNA molecules edited through processes such as Adenosine-Inosine editing (Clark and Mattick, 2011; Clemson, et al, 2009; Cornelis et al, 2016). They also function to sequester the transcription/splicing factor SFPQ, thereby depleting SFPQ from promoters and affecting expression of critical immune genes (Hirose et al, 2014; Imamura et al, 2014). It is therefore suggested that paraspeckles act as a sequestration 'sponge' for proteins (Mang et al, 2017). Such sequestration mechanism is similar to other nuclear structures such as nucleoli that retain certain cell cycle regulators and nuclear stress bodies that trap specific splicing factors (Visintin and Amon, 2000; Biamonti and Vourc'h, 2010). Paraspeckles proteins are common in both human and mice cells, and contain the structures required for cellular functions including the splicing of pre-mRNA, nuclear retention of RNA and the regulation of transcription (Clark and Mattick, 2011; Clemson, et al, 2009). The involvement of paraspeckles in mRNA regulation has been shown to be due to the protein p54 forming complexes, which causes adenosine-inosine edited RNA molecules to be retained within the nucleus, therefore storing molecules, such as

splicing factors (Hutchinson et al, 2007). A molecule found in paraspeckles, which suggests its involvement in pre-mRNA splicing, is CFIm68, a pre-mRNA 3' end-processing factor, which would facilitate the release of any transcripts retained by p54 through the cleavage of these molecules (Clemson et al, 2009).

1.10.3 Paraspeckles proteins

The paraspeckles contain more than 40 RNA binding proteins assembled on the scaffolding RNA NEAT1 (Naganuma et al, 2012). Almost all these proteins have a role in transcriptional and post-transcriptional gene regulation and have DNA or RNA binding domains (Naganuma et al, 2012). Some of these proteins are members of the multifunctional *Drosophila Melanogaster* behaviour/human splicing proteins (DBHS family) (Bond and Fox, 2009). These proteins contain RNA recognition motifs (RRM) and RNA binding domains required for their localisation to the paraspeckles and are usually used as markers for the paraspeckles (Clemson et al, 2009; Clark and Mattick, 2011).

They are reported to form heterodimers and extended long oligomers, which are essential for the formation of the paraspeckles (Clemson et al, 2009; Clark and Mattick, 2011). siRNA mediated knockdown of two of the DBHS proteins, NONO (Non-POU domain-containing octamer-binding) and SPFQ (Splicing factor proline and glutamine rich), resulted in the loss of paraspeckles in HeLa cells, providing evidence that NONO and SPQF are essential for the formation and stability of paraspeckles (Sasaki et al, 2009; Naganuma et al, 2012). In addition, of NONO and SPQF, five other proteins were identified to be essential for the formation and the integrity of the paraspeckles. These include RBM14 (RNA binding motif protein 14), HNRNPK (Heterogeneous nuclear ribonucleoprotein K), DAZAP1 (DAZ associated

protein 1), FUS (Fused in sarcoma) and HNRNPH3 (Heterogeneous nuclear ribonucleoprotein H3) (Naganuma et al, 2012)

1.10.4 Formation of paraspeckles

The formation of paraspeckles may occur initially during the transcription of NEAT1 by RNA polymerase II, once at least two foci of the RNA are present, before dispersing into distinct locations within the nucleus as the NEAT1 transcripts are released (Clemson et al, 2009). This diffusion of the structures occurs in response to the movement of NEAT1 foci away from chromosome 11 and its site of transcription, into the nucleus (Hutchinson et al, 2007). As the cells progress through the cell cycle, the levels and location of both the NEAT1 RNA foci and the paraspeckles change (Clemson et al, 2009). The foci are the large localised structures of the RNA, which are widely distributed throughout the cell (Hutchinson, et al, 2007). During the interphase stage of the cycle, NEAT1 RNA and the paraspeckles appear in a bipolar formation. In the early G1 stage of the cycle, there are only low numbers of NEAT1 RNA foci, which are located close to the transcription sites. The levels of the paraspeckle proteins such PSP1 (PSPC1, paraspeckle protein component 1), NONO and SPQF within the paraspeckle begin to rise as the cell cycle progresses, allowing the structures to be identified. This correlates with the simultaneous increase in NEAT1 RNA foci due to the transcription of gene that immediately results in visible NEAT1 foci (Clemson et al, 2009). The correlation between the two structures as they progress through the cell cycle indicated how closely related their functions are. This can be further demonstrated through the disassembly of the paraspeckle bodies due to the

transcriptional arrest of NEAT1, and their inability to reassemble unless NEAT1 RNA is present (Clark and Mattick, 2011).

1.10.5 NEAT1 function

In addition to its role in the formation and maintenance of paraspeckles, NEAT1 plays an important role in regulating different cellular functions (Lo et al, 2016a). It has been reported to play a critical role in mouse mammary gland development (Lo et al, 2016a). Recent studies implicated NEAT1 in adipogenesis (Gernapudi et al, 2016). The study identified a microRNA-140 (miR-140)/NEAT1 non-coding RNA signalling networks involved in adipogenesis which could be a potential target in the prevention or treatment of obesity. Adipocyte-derived stem cells isolated from miR-140 knockout mice showed a strong reduction in their adipogenic capabilities, which was associated with a decrease in the expression level of NEAT1. Transfecting NEAT1 into the cells restored the adipogenesis process and differentiation (Gernapudi et al, 2016).

NEAT1 has been reported to be significantly up regulated in Huntington's disease (Johnson, 2012). NEAT1 have been associated with drug addiction. For instance, NEAT1 up-regulation was detected in the nucleus accumbens of heroin abusers (Johnson, 2012). NEAT1 was also identified as one of the RNA that binds to TDP-43, a predominantly nuclear RNA-binding protein that forms inclusion bodies in frontotemporal lobar degeneration and amyotrophic lateral sclerosis (Tollervey et al, 2011). The significance of these results is unclear as the NEAT1 knockout mice are viable, despite the absence of paraspeckles (Nakagawa et al, 2011). Further studies in model organisms will be required to determine the significance of NEAT1 in the neurodegenerative process.

During the course of this study, a number of evidence has emerged implicating NEAT1 in oncogenesis. A number of studies have identified NEAT1 function as an oncogene in multiple types of cancer, including breast cancer. NEAT1 overexpression has been reported in different types of solid tumours like malignancies of digestive system, hepatocellular carcinoma, lung cancer and oesophageal squamous cell cancer (Yang et al,2017; Xiong et al ,2017; Guo et al, 2015; sun et al, 2016; Chen et al, 2015). Its mode of action is not very clear, but it has been reported that in non-small lung cancer (NSCLC) the contribution of NEAT1 in progression of this cancer via its role as a competing endogenous RNA (ceRNA) (Sun et al, 2016). NEAT1 overexpression caused a repression of has-miR-377-3p and prevented its effect on the transcription factor E2F3 resulting an increase in the expression levels of E2F3 which plays a crucial role in enhancing the progression of NSCLC (Sun et al, 2016). In gastric cancer, NEAT1 was found to be over-expressed in gastric cancer tissues and cell lines, and its expression positively correlated with clinical stage, histological type, lymph node metastasis, and distant metastasis (Song et al, 2017). Cox regression analyses also showed that NEAT1 overexpression was a poor independent prognostic factor for gastric cancer patients (Song et al, 2017). In vitro studies showed that NEAT silencing significantly suppressed the gastric cancer cell migration and invasion and reduced epithelial-mesenchymal transition (EMT)-associated proteins expression (Song et al, 2017). NEAT1 involvement in breast cancer has also been reported during the course of the current study. NEAT1 is reported to be a direct transcriptional target of hypoxia-inducible factor 2 (HIF-2) in a number of breast cancer cell lines and in solid tumours and its levels were shown to dramatically increased in hypoxia (Choudhry et al, 2015). Induction of NEAT1 in hypoxia was associated with an increase in cellular proliferation, improved clonogenic survival and reduced apoptosis, all of which are

hallmarks of increased tumorigenesis (Choudhry et al, 2015). NEAT1 was found to be highly expressed in breast cancer tissues and its high expression was closely related to the tumour size and lymph node metastasis (Choudhry et al, 2015; Zhang et al, 2017b). In vitro studies have also shown that NEAT1 silencing caused a decrease in cell proliferation and metastasis in breast cancer cells and decreased the protein expression levels of EMT-associated proteins (Zhang et al, 2017b). More interestingly, recent genomic studies have shown that NEAT1 promoter carry recurrent mutations in breast cancer. Such mutations affect the protein binding to the NEAT1 promoter leading to the alteration of expression levels (Rheinbay et al, 2017).

While high expression levels of NEAT1 was reported in different solid tumours and was associated with poor prognosis, other studies have shown that NEAT1 is downregulated in oesophageal cancers, retinal cancers and acute promyelocytic leukaemia where it promotes leucocyte differentiation (Gibb et al, 2011b; Johnson, 2012; Gao et al,2016). NEAT1 expression levels were found to be considerably downregulated in leukaemia patient samples compared with those from healthy donors (Gao et al, 2016). NEAT1 low expression levels was also reported in leukemic cell lines such as K562, THP-1, HL-60 and Jurkat, which goes along with the expression observed in leukemic patients (Gao et al, 2016). In addition, NEAT1 overexpression into K562 and THP-1 leukemic cells lead to the inhibition of ATP-binding cassette G2, which is involved in promoting the mechanism of multidrug resistance to the chemotherapy, and alleviated the multidrug resistance induced by cytotoxic agents (Gao et al, 2016). In addition to the evidence discussed above, the importance of NEAT1 in the regulation of cell death and survival is also highlighted by its identification as a potential gene that control cell survival using an unbiased functional screen to identify genes regulating apoptosis (Williams et al, 2006). Such

screen has identified several genes, each of which opened up a venue for apoptosis research. One of the genes identified using this approach was the lncRNA GAS5 that plays very important role in the control of cell death and survival (Mourtada-Maarabouni et al, 2009; Mourtada-Maarabouni et al, 2010). Based on the accumulating evidence of the significant role of NEAT1, It is therefore important to study its function in the control of programmed cell death and survival and its involvement in cancer.

1.11 MIAT (Myocardial Infarction Associated Transcript)

MIAT is one of the noncoding RNAs associated with nuclear structures namely nuclear bodies (Ishizuka et al, 2014). MIAT was previously known as RNCR2 (retinal non-coding RNA2) and GOMAFU. Increasing number of evidence confirms the role of MIAT lncRNA in a number of cellular processes, like the formation of nuclear bodies (Ishizuka et al, 2014) and neurogenic commitment (Aprea et al, 2013). In addition MIAT lncRNA is involved in a number of diseases and cellular processes, including myocardial infarction (Ishii et al, 2006; Liao et al, 2016), diabetic retinopathy (Vausort et al, 2014), microvascular dysfunction (Yan et al, 2015) and paranoid schizophrenia (Rao et al, 2015). MIAT was originally identified in the neurons of the mouse retina and was later found to be highly expressed in the nervous system throughout development and its expression was reported to continue into adulthood (Sone et al, 2007). It was later found to be conserved among higher vertebrates, including human and chicken, in terms of both its nuclear localisation and expression pattern in the nervous system (Sone et al, 2007; Tsuiji et al, 2011). MIAT gene is located on chromosome 22q12.1 and its transcript has the characteristics of mRNA, which include 5' capping, polyadenylation at the 3'

ends and splicing. However, unlike protein coding mRNA, MIAT transcript escapes nuclear transport and accumulates within the nucleus, where it forms a unique nuclear structure (Sone et al, 2007; Sattari et al, 2016; Xuefeng et al, 2017).

1.11.1 Role of MIAT in cardiovascular, microvascular and related diseases

The association of MIAT with cardiovascular diseases was first highlighted by identifying a single nucleotide polymorphism in the human homologue associated with an increased risk of myocardial infarction. Hence, the gene has been named myocardial infarction associated transcript instead of GOMAFU (Ohnishi et al, 2000). Further clinical trials studies comparing 414 myocardial infarction (MI) patients with 86 healthy volunteers have shown that patients with ST segment elevation myocardial infarction (STEMI) had lower expression levels of MIAT compared to those with non- ST segment elevation myocardial infarction (NSTEMI). Among all the cardiovascular risk factors, expression of MIAT was positively associated only with smoking (Vausort et al, 2014). Xuefeng et al. (2017) showed that MIAT acts as a pro-fibrotic factor in MI. The studies demonstrated that MIAT expression level in cardiac muscles increased myocardial attack. MIAT acts as a sponge for miR-24. An increase in MIAT leads to the decrease in miR-24 and the increase in the expression levels of miR-24 target genes including the fibrosis-related regulators, Furin (furin, paired basic amino acid cleaving enzyme) and TGF- β 1 (transforming growth factor beta 1), leading to increase in cardiac fibrosis and hence insufficiency in cardiac function (Xuefeng et al, 2017). Accordingly, MIAT knockout caused an up-regulation in miR-24, which prevented fibrosis and enhanced cardiac function (Xuefeng et al, 2017). Therefore, normalisation of MIAT

expression post-myocardial infarction can be considered as a therapeutic target that could lead to the decrease in fibrosis and the improvement in cardiac function (Xuefeng et al, 2016; Liao et al, 2016).

MIAT was also reported to play an important role in the regulation of mammalian retinal cell differentiation (Rapicavoli et al, 2010). A number of evidence has implicated MIAT lncRNA in the development of microvascular dysfunction. Studies have shown that MIAT expression level is increased in the retina of diabetic rats and humans and in vitro experiments demonstrated that its expression was induced by high glucose (Yan et al, 2015). Decreasing the expression level of MIAT in diabetic rats led to the improvement in the visual functions, the reduction in the pro-inflammatory proteins related to diabetes mellitus and the decrease in endothelial inflammatory responses, suggesting that inhibition of MIAT expression might improve retinal vessel impairment (Yan et al, 2015). MIAT was shown to function in retinal endothelial cell as a competing endogenous RNA (ceRNA) which acts as a sponge for miR-150-5p leading to an increase in the expression of vascular endothelial growth factor (VEGF), a miR-150-5p target gene and the maintenance of retinal and corneal vascularization (Jiang et al, 2016b; Yan et al, 2015). Recent studies have shown that MIAT is associated with the development of age-related cataract as it was found to be specifically up-regulated both in the plasma fraction of whole blood and aqueous humour of cataract patients (Shen et al, 2016). The studies showed that MIAT knockdown in human lens epithelial cells suppressed tumour necrosis factor- α expression, stimulated of an atypical growth of these cells and increased the rate of migration, suggesting a potential role of MIAT in Posterior capsule opacification (PCO)-related pathological process. This result elucidates the role of MIAT in the pathological process of posterior capsule opacification (PCO), a post-operative complication of cataract (Shen et al, 2016).

1.11.2 Role of MIAT in neuron development and mental disorders

In addition to its expression in different types of neurons and through the neurogenesis process, MIAT has been reported to be involved in neuronal development and its abnormal expression might result in particular nervous dysfunction (Sone et al, 2007; Ishizuka et al, 2014). Microarray analysis of transcripts associated with neural stem cell proliferation and differentiation revealed that MIAT

is expressed during neurogenesis an oligodendrocyte lineage specification (Mercer et al, 2010). Further studies have showed that MIAT controls the differentiation of neural progenitors, the survival of new neurons and the splicing of Wnt7b, a protein involved in different steps of neurogenesis, indicating the importance of MIAT in neuronal commitment and survival (Aprea et al, 2013).

Studies performed by Barry et al. (2014) to investigate the role of MIAT in SZ pathogenesis involved stimulation of the neuronal depolarization using mouse primary cortical neurons and determining the changes in gene expression associated with such stimulation at different time points. Their results showed that MIAT was one of the most strongly downregulated non-coding transcripts at different time points. Down-regulation of MIAT expression in human-induced pluripotent stem cells derived neurons was shown to result in alternative splicing patterns that resemble those observed in SZ for the two SZ-associated genes disrupted-in-schizophrenia 1 (DISC1) and Receptor tyrosine-protein kinase (ERBB4) (Barry et al,2014). MIAT was shown to be important in anxiety, a common symptom in SZ. Spadaro et al. (2015) have investigated the relationship between MIAT (Gomafu) expression and anxiety-like behavior in mice. The studies reported that MIAT was significantly downregulated in fear conditioned group of mice and siRNA mediated

silencing of MIAT in the pre-limbic region of the prefrontal cortex enhanced fear response during behavioral training (Spadaro et al, 2015). However, the fear enhancement caused by down-regulation of MIAT disappeared after 24h, indicating that MIAT has no effect on long-term memory (Spadaro et al, 2015). The study showed that MIAT down-regulation leads to its dissociation from BMI1, a key member of the Polycomb repressive complex 1, and relieves its repressive control over the schizophrenia-related gene crystallin beta 1 (Crybb1) promoter, resulting in an increase in Crybb1 gene expression. Collectively, these results highlight the importance of MIAT in neuronal function and strongly suggest that their dysregulation in MIAT expression and function may contribute to the development of neurological and neuropsychiatric disorders.

1.11.3 Role of MIAT and cancer

Evidence implicating MIAT in cancer is now emerging. MIAT is selectively upregulated in neuroendocrine prostate cancer, which is the most lethal prostatic neoplasm (Crea et al, 2016). MIAT has been shown to interact with Polycomb genes enhancing histone modification and playing a role in cancer cells' plasticity, thereby promoting the emergence of metastatic, drug-resistant neoplasms (Crea et al, 2016). Data mining aimed to perform lncRNA profiling on a cohort of 213 glioblastoma patients from The Cancer Genome Atlas as well as independent data sets from the Gene Expression Omnibus (GEO), identified six-lncRNA signature that were significantly associated with the overall patient survival (Zhang et al, 2013). These lncRNAs include ART1, MGC21881, GAS5, PAR5, and MIAT (Zhang et al, 2013). MIAT expression was reported to be significantly down-regulated in glioma brains compared to normal and its expression of MIAT was found to be associated

with long survival (Zhang et al, 2013). While MIAT expression in glioblastoma is associated with long survival and appears to act as a tumour suppressor, recent studies have reported MIAT over-expression in an aggressive form of chronic lymphocytic leukaemia (CLL) (Sattari et al, 2016). The studies showed upregulation of MIAT in lymphoid but not in myeloid cell lineage with mature B cell phenotype. MIAT was found to be significantly up regulated in primary leukemic cells from patients with aggressive form of CLL carrying either 17p-deletion, 11q-deletion, or Trisomy 12. Furthermore, upregulated MIAT levels were associated with rapid death cases (Sattari et al, 2016). The mouse homologue of MIAT has been shown to bind to Oct4 gene (octamer-binding transcription factor 4), a marker of stemness, leading to an increase in Oct4 expression (Mohamed et al, 2010; Zeindene et al, 2014). Oct4 also binds to MIAT and positively regulates its transcription in mouse embryonic stem cells, and thus, they constitute a regulatory feedback loop (Mohamed et al, 2010). The relationship between MIAT and Oct4 was investigated in malignant B cells. The results showed that siRNA mediated down-regulation of MIAT in malignant B cell lines was associated with a concomitant downregulation of Oct4 (Sattari et al, 2016). These results indicated that as in mouse embryonic stem cells, MIAT in malignant B cells positively regulates Oct4 transcription and Oct4 regulated MIAT expression, and thus both molecules make up a positive feedback loop and are essential for cell survival (Sattari et al, 2016). These studies highlighted the anti-apoptotic role of MIAT in malignant mature B cells and its potential as a biomarker for aggressiveness of chronic lymphocytic leukemia

1.12 Aims and objectives

Currently, the study of LncRNAs is becoming one of the most popular fields in the biological and medical sciences. LncRNAs have emerged as important regulatory molecules in developmental, tumour suppressor and oncogenic pathways and other diseases. Some of these lncRNAs can regulate cell survival and cell death, suggesting a key role for these molecular regulators. Others have become diagnostic markers and potential therapeutic targets. Understanding of the mechanism(s) by which lncRNAs regulate cell death survival will lead to new markers of cancer diagnosis and identification of novel therapeutic targets. The aims of this study are to investigate the role of two nuclear lncRNAs, NEAT1 and MIAT/GOMAFU in the regulation of cell death and survival of breast cancer cells. The present study specifically investigates the functional activity of NEAT1 and MIAT in breast cancer cells and aims to address the gap in our understanding of the role and mechanisms of action for these two lncRNAs.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1. Cell culture, transfection and functional analysis

The human breast cancer cell lines MCF7 and MDA-MB-231 were purchased from ATCC-LGC Promochem (Teddington, UK). RPMI-1640 growth medium containing [phenol Red as pH indicator (# R0883), L-glutamine (# G7513), sodium pyruvate (# S8636), HEPES Buffer (# H0887), gentamicin (# G1272)], Trypsin/EDTA (# 59418C) and Phosphate-buffered saline (PBS) (# 101584397) were from Sigma-Aldrich Company Ltd. (Gillingham, UK). Foetal bovine serum (FBS) (# FB-1001S) was from Biosera (East Sussex, UK). Some NEAT1 siRNAs (Nominated according to their targeting site) were obtained from Qiagen (Crawley, UK), NEAT1a (ID: 2163122, # S105189751), NEAT1c (ID: 2163121, # S103682126). Ambion Select NEAT1 siRNAs were obtained from Life Technologies Ltd (Paisley, UK). NEAT1, 1 (ID: s238175, # 4399665) and NEAT1, 2 (ID: s238174, # 4399666). MIAT2 siRNA (ID: 2164377, #SI04314919), MIAT3 siRNA (ID: 2163124, # SI00582799) were purchased from Qiagen (Crawley, UK). All siRNAs were HPLC purified, annealed and ready to use. Silencer®siRNA labelling kit-Cy3 (#1632) and Negative Control siRNA (# AM4635) were purchased from Life Technologies. The Negative Control siRNA has no significant sequences similar to mouse, rat, or human gene sequences. Custom antisense oligonucleotides were ordered from GE Healthcare Dharmacon Inc., Little Chalfont, UK. The single stranded RNA/DNA oligonucleotides were converted to the 2' hydroxyl form and desalted.

The following kits were obtained from Merck Millipore: Muse cell count and viability kit (# MCH100102), Muse annexin V and dead cell kit (# MCH100105) and Muse cell cycle kit (# MCH100106). The MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay) was from Promega (Southampton, UK). HiPerFect

transfection reagent (# 301704) was from Qiagen and the Nucleofector solution (Mirus Ingenio®kit, # MIR 50115) was from Mirus Bio LLC, Madison, WI, USA. The chemotherapeutic drugs (Docetaxel, 5-Fluorouracil, Nutlin-3a and Mitoxantrone) were purchased from Sigma-Aldrich Company Ltd (Gillingham, UK).

2.1.2 Materials for RNA isolation and gene expression analysis

TRIzol (#15596018), TaqMan assays, Random primer (# N48190-011) and Ribonuclease inhibitor were from Life Technologies Ltd. SensiFast Probe Hi-ROX kit was from Bioline (London, UK), RQ1 RNase-free DNase was from Promega (Southampton, UK). Omniscript Reverse Transcription (RT) Kit, (# 205111) was from Qiagen. Ribonuclease inhibitor (Invitrogen, # 10777-018). RQ1 DNase 10x Reaction Buffer (# M198A), RQ1 RNase-free DNase (# M 610A) and RQ1 DNase stop reaction (# M199A) were purchased from Promega (Southampton, UK).

Isopropanol and Ethanol were purchased from Fisher Scientific UK Ltd (Loughborough, UK). Chloroform was from BDH laboratory supply, England.

RT² Profiler PCR Array (96-Well Format) for Human Cell Cycle (# 330231 PAHS-020A) and Human Breast Cancer (# 330231 PAHS-131ZA), RT2 First Strand Kit (# 330401) and RT² SYBR® Green qPCR Mastermix (# 330520) were purchased from QIAGEN. TissueScan™ Cancer and Normal Tissue cDNA Arrays (# BCRT102) were purchased from OriGene (Rockville, USA).

2.2 Methods

2.2.1 Cell culture

Two types of breast cancer cell lines MCF7 and MDA-MB- 231 were used in this study. Both cell lines were derived from plural effusion of patients with breast cancer. The first cancer cell line MCF7 was established in 1973 in the Michigan cancer foundation and is characterized by low level expression of the proliferation marker Ki67 and high hormonal sensitivity via expression of oestrogen receptor (Soule, et al, 1973). In contrast, the triple negative MDA-MB- 231 cell line lacks the expression of oestrogen, progesterone and HER2 receptors and shows high expression level of Ki67 (Cailleau et al, 1974; Holiday and Speirs, 2011).

Cells were routinely cultured in complete RPMI-1640 growth medium supplemented with 10% heat inactivated foetal calf serum, 2mM L-glutamine and 200 µg/ml gentamycin. Cells were incubated at 37° C in a 5% CO₂ humidified incubator. After a confluence of 80% ,cell detachment were proceeded by rinsing the flask with 3 ml of Phosphate buffered saline (PBS) followed by adding 2 ml of 0.25% Trypsin /EDTA minutes before returning the flask to the incubator for 3- 5 minutes to facilitate detachment of the cells. An equal volume of medium was added to the flask to inactivate the trypsin and the content of the flask was transferred to a 15 ml centrifuge tube and centrifuged at 1500 rpm for 5 minutes in order to get a pellet. The supernatant was discarded and the cell pellet was re-suspended in fresh RPMI medium and seeded into new flasks.

2.2.2 Freezing and thawing of MCF7 and MDA-MB-231 cell lines

MCF7 and MDA-MB-231 cells were stored for long period at -140°C in liquid nitrogen. Cells were re suspended in 1ml of cryoprotectant medium (40% FBS, 50%

complete RPMI growth medium and 10% dimethyl sulphoxide (DMSO). before transferring them to a cryo-tube. They were immediately stored in an isopropanol chamber at -80 before being transferred to liquid nitrogen for long-term storage. For the recovery of the cells, the cryopreserved cells were thawed at 37°C and re-suspended in 10 ml of RPMI complete medium. Cells were centrifuged at 1500 rpm for 5 min and re-suspended with appropriate volume of RPMI complete growth medium and maintained in a 5% CO₂ humidified incubator at 37°C. Cell lines were replaced with fresh stocks from liquid nitrogen after a maximum culture period of 2 months.

2.2.3 siRNA labelling

To determine siRNA transfection efficiency, siRNA duplexes were labelled with Cy3 using the Cy3 Silencer™ siRNA labelling kit, following the manufacturer's instructions. . The Cy3-labelling siRNA reaction was prepared in a sterile, nuclease-free tube and with a limited exposure to light for the entire procedure. The reaction consisted of 18.3µl of nuclease free water, 5 µl of 10x labelling buffer, 19.2 µl siRNA (20 µM) and 7.5 µl of Cy3 labelling reagent (100 µM) which was added last. After mixing by vortexing, the labelling reaction was incubated at 37°C for 1hr. Labelled siRNAs were stored in freezer at -20°C.

2.2.4 Optimisation of Transfection by HiPerFect transfection reagent

To obtain the highest transfection efficiency with minimal effects on cell viability, optimisation trials for HiPerFect transfection reagent were carried out for every cell

line using Cy3 labelled scrambled siRNA, according to the supplier protocol. Breast cancer cells were plated at a concentration of 1.5×10^5 cells/well in a 6-well plate with added 2.5 ml growth medium and incubated at 37°C for 30 minutes. siRNA complex was prepared in 0.5 ml Eppendorf tube by mixing different volumes of HiPerFect reagent with 6 μ l of 7.68 μ M Cy3 labelled siRNA diluted in 94 μ l Opti-MEM I. Opti-MEM I is a reduced serum media which is ideal for cationic lipid transfection. It is buffered with HEPES and sodium bicarbonate; and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factors. Each complex was added drop-wise to the cells in a 12 well plate before being incubated at 37°C in a 5% CO₂. The efficiency of transfection was determined after 72 hr using fluorescence microscopy. 1ml cells transfected with Cy3 labelled negative siRNA were centrifuged at 1500 rpm for 6 minutes. Supernatant was discarded and the pellet washed with 1ml of PBS. Cells were centrifuged as above, and re-suspended in 20 μ l of RPMI medium. The whole sample was placed on a slide and covered with a coverslip and viewed under the microscope. Transfection efficiency was calculated as percentage of transfected cells from all cells by counting transfected cells holding a red fluorescent signal as a result of the successful transfection with Cy3 labelled siRNA, using Nikon Eclipse E400 Binocular Fluorescence Microscope with FITC filter (Figure 2.1). Viability was determined using vital dye staining, as described in section 2.2.7.1. The results showed that HiPerFect did not affect cell viability. High transfection efficiency was obtained using 4.5 μ L of transfection reagent, (83.5% and 90% in MCF7 and MDA-MB-231) respectively (Table 2.1).

Table 2.1 Optimisation of transfection by HiPerFect transfection reagent. Different volumes of HiPerFect transfection reagent was used with Cy3 labelled siRNA. Transfection complexes were added into MCF7 and MDA-MB-231 cells. The efficiency of transfection and cell viability were determined 72h post transfection.

Cell line	Volume of the transfection reagent	Transfection efficiency 72 hours post-transfection	Cell viability
MCF7	1.5 µL	73%	89%
	3 µL	67%	90%
	4.5 µL	83%	90%
MDA-MB-231	1.5 µL	64.3%	84%
	3 µL	72%	79%
	4.5 µL	90%	87%

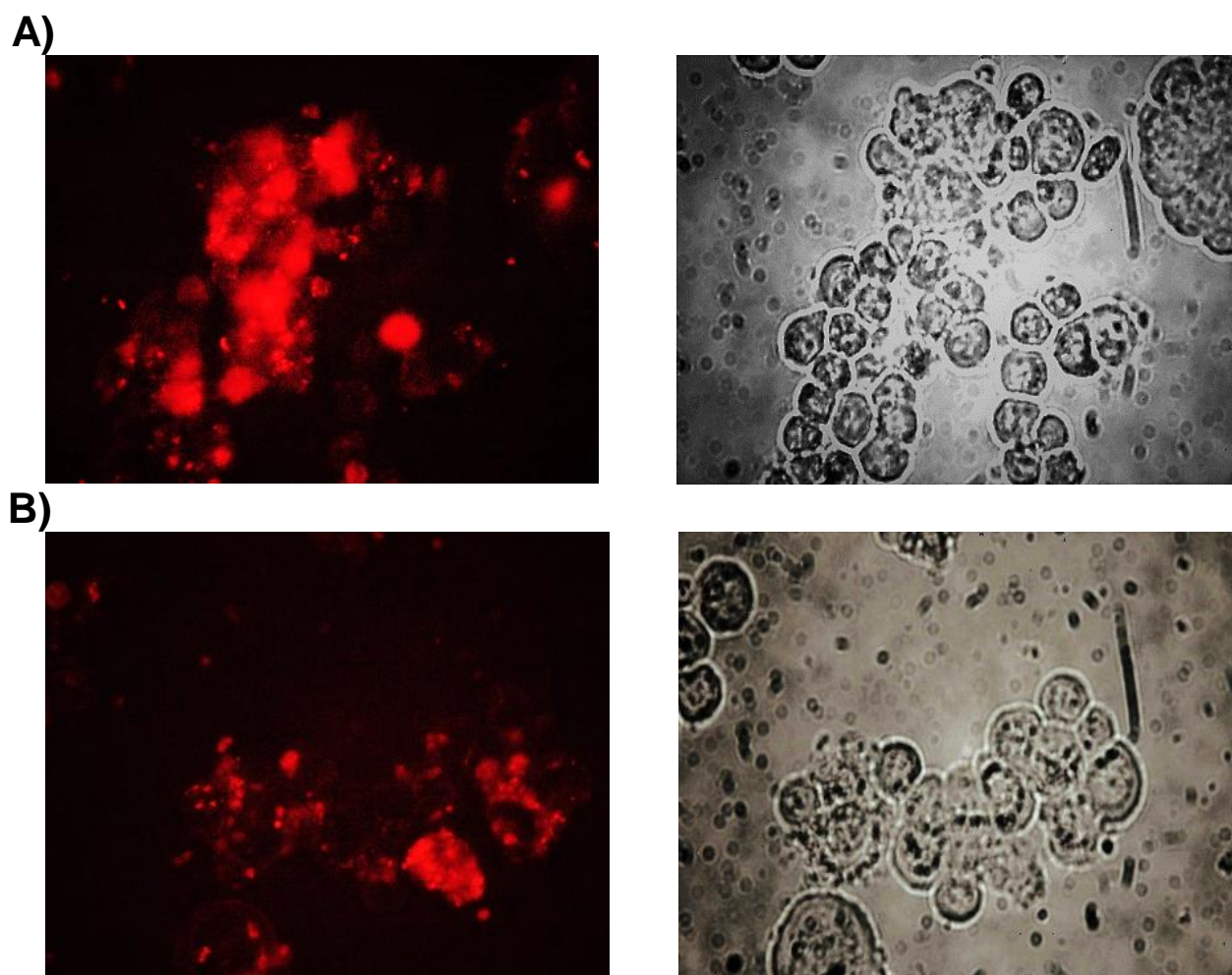


Figure 2.1 A representative image of transfection efficiency in breast cancer cells using HiPerFect transfection reagent. (A) The transfection efficiency of MCF7 cells and (B) MDA-MB-231 cells, 72 hours post-transfection. The transfection was carried out using 4.5 μ l of HiPerFect transfecting reagent and the images were taken using fluorescent microscope.

2.2.5 RNA interference by small interference RNA (siRNA)

Cells were transfected with gene specific Ambion Select or Qiagen siRNAs using HiPerFect transfection reagent or Nucleofection. Controls received negative control siRNA. Details on the gene specific siRNAs and their target location are presented in Table 2.2 and Table 2.3

Transfection using HiPerFect transfection reagent was carried out according to standard protocol (Qiagen, 2010). Breast cancer cells were seeded in 6 well plate (1.5×10^5 cells/well) and incubated at 37°C for 30 minutes. siRNA and HiPerFect complex was prepared in 0.5 ml Eppendorf tube by mixing 4.8 µl of 20 µM of either (-)siRNA or gene specific siRNA and 95.2 µl Opti-MEM I. The mixture was vortexed before adding 4.5 µl of HiPerFect transfection reagent and mixed gently by pipetting. The mixture was incubated at room temperature for 10 minutes before being added in a dropwise manner whilst swirling the plate. Cells were incubated at 37°C for 72 h in 5% CO₂. Efficiency of transfection and the levels of gene expression were determined 72h post transfection by fluorescence microscopy and qRT-PCR, respectively. Harvested cells were re-plated 24h for functional analysis and assessment of cell survival.

Nucleofection was also used for the transfection of siRNAs. MCF7 and MDA-MB-231 were nucleofected with gene specific siRNA or negative control siRNA using Ingenio electroporation solution. The cells were sub-cultured one day before Nucleofection. On the day of the nucleofection, 2×10^6 cells were re-suspended in 100 µl of Ingenio electroporation solution and 5 µl of siRNA (3.3 µM). Cell suspension was transferred into nucleofector certified cuvettes and was nucleofected using Amaxa™ Nucleofector™ II, P-020 and X-013 for MCF7 (ATCC) high efficiency and MDA-MB-231 (ATCC) respectively. Cells were recovered and

plated in 5 ml RPMI medium in 6-well plates. The plate was incubated in a humidified incubator at 37°C and 5% CO₂ for 48h. 48h after transfection the transfected cells were trypsinised counted and re-plated for further 24 and 48 hours before assessment of the cell survival.

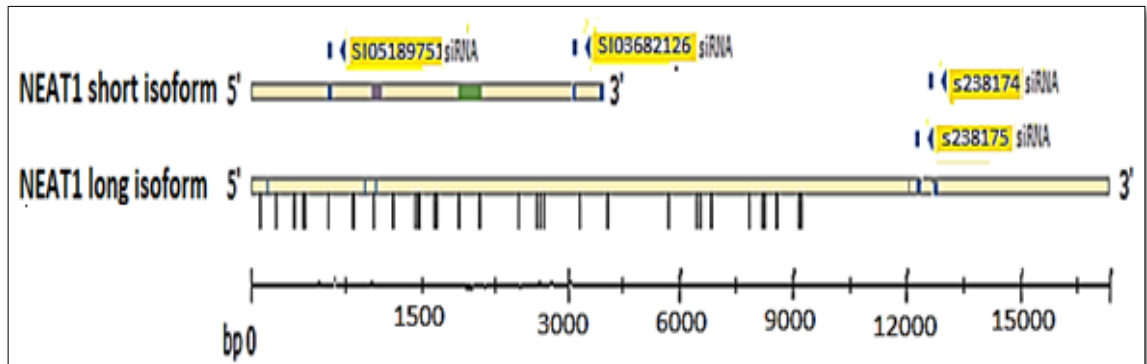
Table 2.2 Details of NEAT1siRNAs and Antisense Oligonucleotides (ASOs) and their target sequences.

	Name	Sequence ID	Cat. No.	Targeting site	Target sequences (5'--3')
NEAT1 siRNAs	NEAT1a	NR_00280 2	SI0518975 1	445-465	5'- GCCGGGAGGGCTA ATCTTCAA-3'
	NEAT1c	NR_00280 2	SI0368212 6	3310-3330	5'- CTGCGTCTATTGAA TTGGTAA-3'
	NEAT1, 1	gb/GQ859 162.1	S238175	12013- 12033	5'- TGCTGCCAAAATAG AATAA-3'
	NEAT1, 2	gb/GQ859 162.1	S238174	12084- 12104	5'- GACTGTAATTGGTA CAGTA -3'
NEAT1 (ASOs)	NEAT1, 1	NR_13101 2.1	GEHCU- 000379	1934-1953	5'- GCAGATTACTAGGA GAAGGG-3'
	NEAT1, 2	NR_13101 2.1	GEHCU- 000380	3163-3182	5'- AATGAGCCAAGACT AGAGGG- 3'

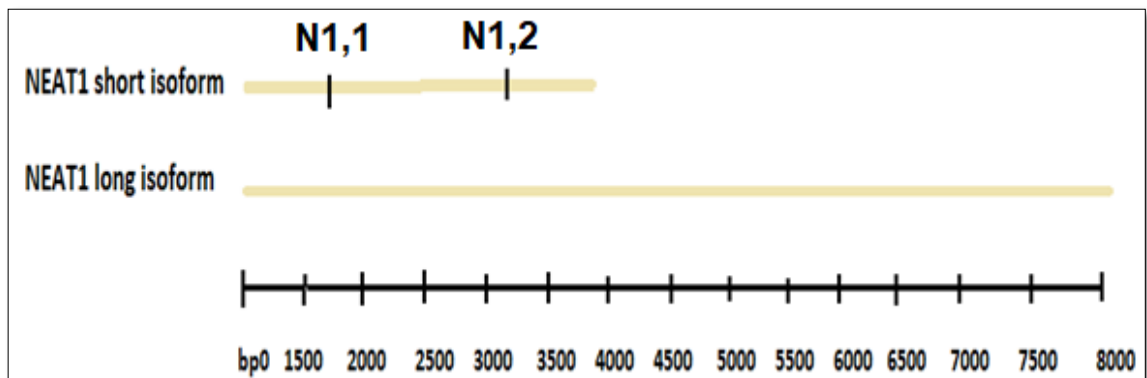
Table 2.3 MIAT siRNAs and their target sequences

siRNAs	Sequence ID	Cat. No.	Targeting site	Target sequences (5'-3')
MIAT2	NR_003491	SI04314919	6488-6508	5'- GCGGGUCUUUCCUAC GCUATT-3'
MIAT3	NR_003491	SI204072831	9735-9755	5'- GGAGUCUACUGAACAU CAATT-3'

A) NEAT1 siRNAs



B) NEAT1 Antisense Oligonucleotide



D) MIAT 2 and MIAT 3 siRNAs

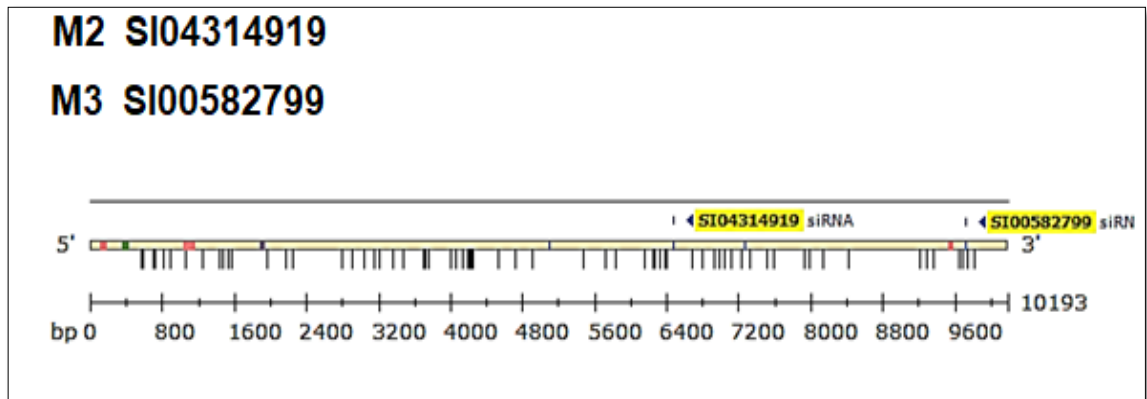


Figure 2.2 Schematic diagram representing the targeting sites of small interference RNAs (siRNAs) and antisense oligonucleotide (ASO). (A) Represents the targeting sites of NEAT1 siRNAs, in which the targeting sites of N1a (SI05189571) and N1c (SI03682126) are (445-465) and (3310-3330) respectively. While, the targeting sites for N1, 1(s238175) and N1, 2 (s238174) are (12013-12033) and (12084-12104) respectively (Qiagen). (B) Represents the targeting sites of NEAT1 antisense Oligonucleotides and in both NEAT1 short and long isoforms, in which the targeting site for N1, 1 and N1, 2 are (1934-1953) and (3163-3182) respectively (NCBI). (C) Shows the targeting sites of MIAT siRNAs, where the target sites of M2 (SI04314919) and M3 (SI00582799) are (6488-6508) and (9735-9755) respectively (Qiagen).

2.2.6 Nucleofection of antisense oligonucleotides

The antisense oligonucleotides (ASOs) consisted of DNA and RNA phosphorothioate-modified oligonucleotides containing selective phosphorothioate backbone modifications (as indicated by * and 2' O-methyl RNA bases (indicated by 'm' as presented in Table 2.2). Cells at density of (2×10^6) in 0.1 ml Ingenio electroporation solution were nucleofected with 5 pmol oligonucleotide using programmes P-020 and X-013 for MCF7 and MDA-MB-231 respectively. Then, the cells were re-plated in 3 ml RPMI medium in 6-well plates. The plate was incubated in a humidified incubator at 37°C and 5% CO₂ for 48h.

2.2.7 Determination of apoptosis and cell survival

2.2.7.1 Vital dye exclusion assay

Cell viability was determined by vital dye staining using trypan blue and a haemocytometer. The intact membranes of the live cells exclude the vital dye and appear bright under light microscope. Whereas, dead cells absorb trypan blue dye through their damaged membrane and selectively show to have blue colour. 20 µl of the cell suspension was mixed with an equal volume of 0.4% trypan blue stain. Cell suspension was loaded in the edge of the chamber between the coverslip and the groove in the chamber and allowed to be drawn into the chamber by capillary action. Viable and non-viable cells were counted under the light microscope and cell number per ml was calculated using the following equation:

“Average Number of Cells in four large squares X dilution factor X 10^4 ”

2.2.7.2 Cell viability using flow cytometry

Total and viable cell counts were determined by flow cytometry using the Muse® Count and Viability Assay kit and the Muse system, a mini flow cytometer. The Muse Count and Viability reagent contains two DNA binding dyes, which allow the differential staining of viable and non-viable cells based on their membrane integrity and permeability to these dyes. One of the DNA-binding dyes in the reagent specifically stains the nucleus of dead and dying cells, the cells that have lost their membrane integrity. This parameter is used to discriminate between the live cells (that do not take up the dye) from the stained non-viable or dying cells and is displayed on the Muse system as “VIABILITY”. The other dye is membrane-permeant and stains the DNA in all viable nucleated cells with a nucleus (Muse™ user’s guide, # MCH100102,). This parameter is used to discriminate cells with a nucleus from debris and non-nucleated cells. The Muse™ System counts the stained nucleated events and uses the cellular size properties to distinguish cellular debris from cells to determine a precise total cell count. Using these data, the Muse™ Count and Viability Software Module automatically performs calculations and displays data in two dot plots (Figure 2.3).

Determination of total and viable cell number of transfected cells using the Count and Viability Assay was carried out following the manufacturer’s instructions. Trypsinised cells were diluted 20 fold with the reagent (for example, 20 µl of cell suspension into 380 µl of Muse Count and Viability Reagent). Cells were allowed to stain for a minimum of 5 minutes at room temperature and were counted using the Muse cell analyser.

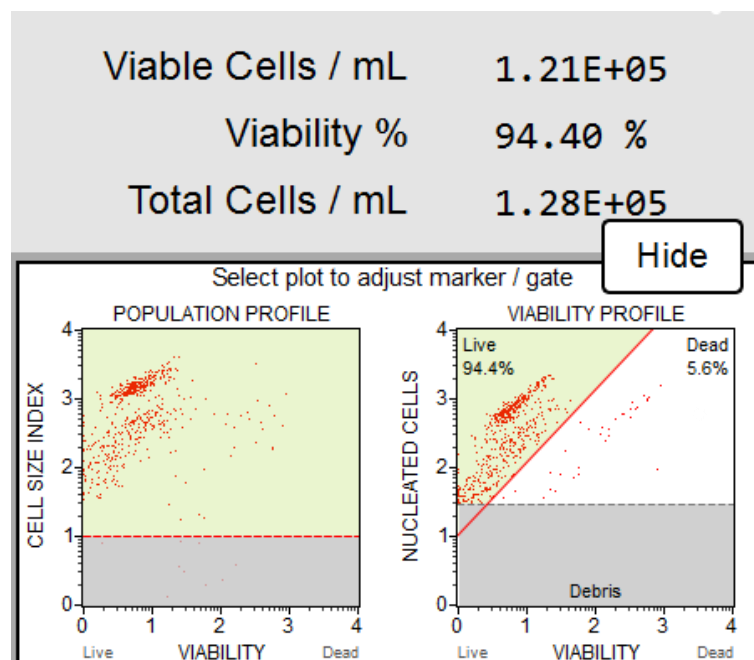


Figure 2.3 The output of Muse displaying the results of the number of viable cells and viability. These results are obtained after completion of acquisition using the Muse™ Cell Count and Viability Software, which automatically performs calculations and displays data in two dot plots.

2.2.7.3 45 MTS cell viability assay

Cell viability was also determined using The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS assay). The CellTiter 96® AQueous One Solution Reagent contains tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine ethosulfate; PES). The enhanced chemical stability of PES allows it to be combined with MTS to form a stable solution. The MTS tetrazolium compound is bio-reduced by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells, resulting in the development of coloured soluble formazan product in tissue culture medium (Cell Proliferation Assay, Promega).

MTS assay was performed by directly adding 20 µl of the MTS solution to the cells cultured in 96 well plates. Cells were incubated for 1-3 h at 37°C in 5% CO₂ before recording the absorbance at 490nm with the multi plate reader TECAN (Infinite).

2.2.7.4 Determination of apoptosis using flow cytometry

Apoptosis level was measured by flow cytometry using Apoptosis was the Muse cell analyser system and the Muse® Annexin V and Dead Cell Assay kit. The characteristics physiological changes that occur because of the initiation of apoptosis include the externalization of phosphatidylserine (PS) to the cell surface, cleavage and degradation of specific cellular proteins, condensation of nuclear chromatin, and the loss of membrane, which occurs in the late stage (Kerr et al, 1972). PS is a membrane component that normally localised in the internal side of the cell membrane. PS translocate to the outer side of the membrane in the early events of the apoptosis process (Kerr et al, 1972). Annexin V is a calcium-

dependent phospholipid-binding protein with a high affinity for PS, and can readily bind to externalised PS and can therefore label early apoptotic cells (Kerr et al, 1972).

The Muse™ Annexin V and Dead Cell assay utilizes Annexin V to detect externalised PS on apoptotic cells. The assay also contains a fluorescent dead cell marker, 7-aminoactinomycin D (7-AAD) which is used as an indicator of cell membrane structural integrity. 7-AAD is excluded from live, healthy cells, as well as early apoptotic cells. Using this assay allows the distinction between four populations of cells (Muse™ user's guide, # MCH100105). As present in Table 2.4 and Figure 2.4

Table 2.4 The distinction of cell population after using Muse™ Annexin V and Dead Cell assay. Annexin V was used to detect externalised PS on apoptotic cells and 7-aminoactinomycin D (7-AAD), a fluorescent dead cell marker, which was an indicator of cell membrane integrity.

Types of cell population	Annexin V	Dead cell maker (7-AAD)
non-apoptotic cells	-ve	-ve
early apoptotic cells	+ve	-ve
late stage apoptotic and dead cells	+ve	+ve
nuclear debris	-ve	+ve

The level of apoptosis was measured using the Muse™ Annexin V and Dead Cell assay according to the manufacturer's instructions. 100 µl of Trypsinised cells were mixed with 100 µl of Muse® Annexin V and Dead Cell reagent. The mixture was incubated for 20 minutes at room temperature in the dark before data acquisition using the Muse cell analyser system.

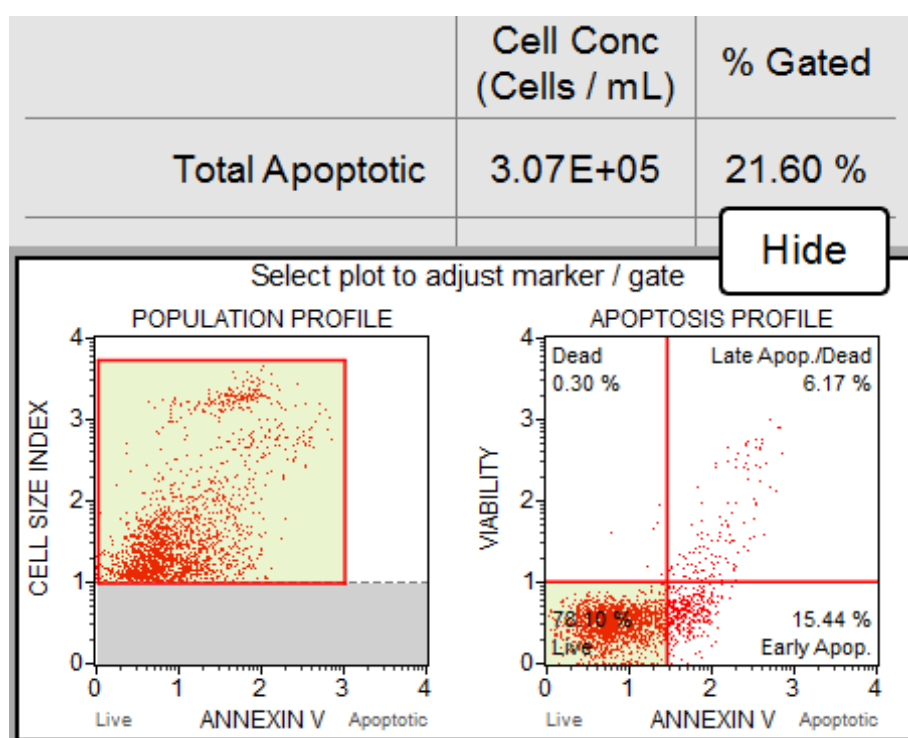


Figure 2.4 A display of the ratio of apoptotic and dead cells after completion of data acquisition by Muse. The Muse™ Annexin V & dead cell Software automatically performs calculations and displays data in two dot plots

2.2.8 Cell cycle analysis

Cell cycle analysis was carried by flow cytometry using the Muse™ Cell Cycle Assay and the Muse cell analyser. The assay reagent consists of a mixture of the nuclear DNA intercalating stain propidium iodide (PI) and RNase A. PI allows the discrimination between the cells at different stages of the cell cycle, based on their differential DNA content. The use of RNase in the reagent increases the specificity of DNA staining (Muse™ user's guide, # MCH100106). Resting cells in G0/G1 phase contain two copies of each chromosome. As cells enter the cell cycle, they start the synthesis of other copies of their chromosomal DNA (S phase). The increase in the DNA content will result in the increase in fluorescence intensity from PI. Fluorescence intensity increases until all chromosomal DNA has doubled in G2/M phase. At this stage, cells in the G2/M cells fluorescence with twice the intensity of the G0/G1 population. The G2/M cells eventually divide into two daughter cells (Muse™ user's guide, # MCH100106). The Muse Cell Cycle Software Module performs calculations automatically and display the data in two plots. The DNA content index and cell size index is displayed as dot plot. DNA content index histogram is also displayed with markers available to analyse the cell populations in each phase of the cycle Figure 2.5.

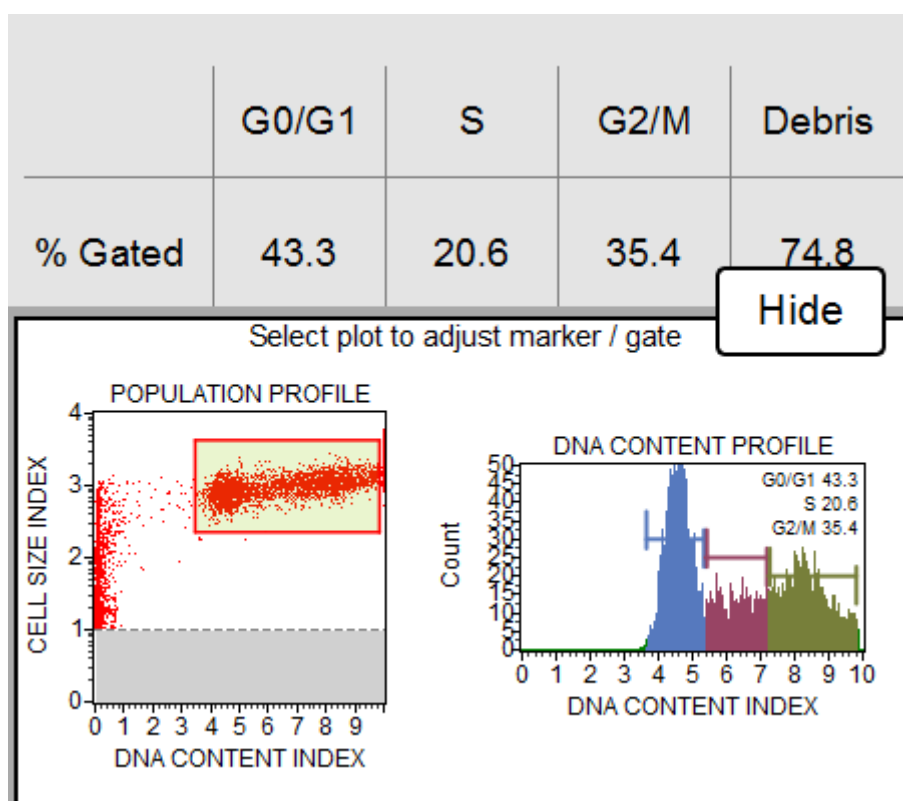


Figure 2.5 A display of the percentage of cells in different phase of cell cycle using the **Muse**. After completion of data acquisition, the Muse™ cell cycle Software automatically performs calculations and displays data in histogram and dot plots.

The transfected cells were harvested 48 hours post-transfection and re-plated in fresh medium 2×10^5 cells/well in a 6-well plate. Following incubation for 24 hours, 1 million cells were washed in PBS before re-suspending the pellet in 200 μ l PBS. Cells were then fixed in 1 ml ice cold (70% ethanol / 30% PBS) while vortexing at medium speed. Cells were stored at -20°C for at least 3 hours prior the cell cycle analysis. On the day of the analysis, cells were centrifuged for 5 minutes at 1500 rpm. The supernatant was discarded and the cell pellet was re-suspended in 200 μ l of Muse Cell Cycle Reagent.

Cells were incubated for 30 minutes in the dark and data acquisition was carried out using the Millipore Muse cell analyser.

2.2.9 Anchorage- dependent clonogenic assay

The effect of gene silencing on the long-term survival was determined by assessing the ability of the cells to form colonies. Anchorage- dependent Clonogenic assay was carried out 48 hours post-transfection. Cells were seeded at a density of 1×10^3 cells/well in triplicate in a 6-well plate in 2 ml RPMI medium containing 10% conditioned growth medium. Plates were incubated at 37°C in 5% CO₂ for 2-3 weeks. Number of the colonies was determined after staining with crystal violet stain (0.5% (w/v) in methanol) for 10 minutes.

2.2.10 Cell migration

The migration ability of MCF7 and MDA-MB-231 cells was assessed using the in vitro scratch assay. The technique is based on creating a "scratch" in the cells grown in monolayer. Using light microscope, images are captured at the beginning and at regular intervals during cell migration to close the scratch. Captured images are compared and used to quantify the migration rate of the cells (Liang et al, 2007). Cells transfected with gene specific siRNAs were plated in 6-well plate at a density of 2×10^5 cells/ml in RPMI 1640 growth medium. Cells were incubated in 5% CO₂ at 37°C until they reached 80%-90% confluence. A scratch line was made using 10 µl pipette tip and the plates were rinsed with RPMI 1640 growth medium to remove detached cells, followed by the addition of 3 ml medium. The gap generated by the scratching was measured at zero time before incubating the cells in 5% CO₂ at 37°C. Measurement of the distance between the two edges of the wound was measured at 18 hours interval using the light Microscope. The percentage of the wound closure was calculated using the following formula:

$$\frac{(\text{Pre-migration})_{\text{area}} - (\text{Migration})_{\text{area}}}{(\text{Pre-migration})_{\text{area}}} \times 100$$

2.2.11 Induction of cell death and cell survival assays

After transfection, cells were trypsinised before being irradiated with Ultraviolet-C (UV-C). 0.8×10^5 cells were exposed to the UV-C light, using a UVG-54 hand-held lamp (UVP, Cambridge, UK). The used dose was 40 J/m^2 for MCF7 and MDA-MB 231 cells. Cells were then collected washed in RPMI 1640 growth medium and re-suspended with appropriate volume of growth medium, and re-plated in 6-well plate's cell survival assay at 24 and 48 hours.

For drug treatments, the post transfect cells were cultured for minimum 20 h in 96 well plates at 0.8×10^5 cell/ml, before addition of 5-fluorouracil (5-FU; $175 \mu\text{M}$), docetaxel ($10 \mu\text{M}$), Nutlin-3 ($5 \mu\text{M}$), mitoxantrone ($50 \mu\text{M}$) or vehicle (0.25 % dimethyl sulphoxide). Cells were cultured for 48 h post-treatment and cell viability was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS assay) (Section 2.2.7.3). However, the growth inhibitory effect of chemotherapeutic drugs in MCF7 and MDA-MB 231 was determined at 24h and 48h of drug treatment and calculated according to the equation.

$$\% \text{ of cytotoxicity} = 100 - [\text{OD}_{490} \text{ of treated sample} / \text{OD}_{490} \text{ of untreated sample (vehicle)}] \times 10$$

2.2.12 RNA isolation

Total RNA was isolated from the parental cells and the transfected cells in order to determine gene expression; using TRIzol. TRIzol is a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously solubilizes DNA and RNA and denatures proteins. Addition of chloroform after solubilisation allows phase separation of the DNA, RNA and proteins. Proteins are extracted to the organic phase, DNA resolves at the interface, whereas the RNA remains in the aqueous phase. After transferring the aqueous phase, RNA can be recovered by precipitation with isopropyl alcohol (Chomczynski and Sacchi, 1987). Cells were lysed by adding 1 ml of TRIzol and the cell lysate was pipetted several times to ensure sufficient cell disruption. 200 µl of chloroform was added 5 minutes post incubation at room temperature. The samples were shaken vigorously for 15 seconds before being incubated for 5 minutes at room temperature followed by centrifugation at 13,000 RPM for 15 minutes at 4°C. This step allows the separation of a clear upper aqueous layer which contains the RNA, an interphase and a red lower organic layer which contains the DNA and proteins. The upper aqueous layer containing the RNA was collected in another tube to be precipitated by adding 0.5 ml cold isopropyl alcohol. After incubation for 10 minutes at room temperature, the samples were centrifuged at 13000 rpm for 15 minutes and at 4°C. The supernatant was discarded and the formed pellet was washed once by adding 1 ml of 75% ethanol, vortexing and centrifuging at 8000 rpm for 5 minutes at 4°C. The resultant pellet was air-dried for 10 minutes, before being re-suspended in RNAs free water, pure deionized water that prepared via certain processes without adding any chemical additives (Qiagen, 2008).

The integrity of the isolated RNA was assessed by gel electrophoresis and by determining the ratio of absorbance at 260 nm and 280 nm using NanoDrop 1000 spectrophotometer (Thermo, scientific)

Furthermore, purification of cytoplasmic RNA from animal cells using the RNeasy Mini Kit (Qiagen). According to the manufacturer's instructions, the purification steps started by lysis of cultured cells at density of 1×10^6 with 175 μ l of precooled (4°C) buffer RLN (a non-ionic detergent that lysis the cell membrane without affecting the nucleus) and prepared from (50mM TrisCl, pH 8, 140mM NaCl, 1.5mM MgCl_2 , 0.5%(v/v) Nonidet P-40 (1.06 g/ml). After 5 min of incubation in ice the homogenised cells were centrifuged at 4°C for 2min to precipitate the nucleus, then transfer the supernatant to another centrifuge tube and adding 350 μ l of buffer RLT (a guanidine-thiocyanate containing lysis buffer) with 430 μ l of ethanol. After that, 700 μ l of the sample were transferred into the RNeasy mini spin column placed in 2ml collection tube and centrifuged. This was followed by the addition of , 700 μ l of buffer RW1 and centrifugation to wash the spin column membrane. Frequent washing followed this step using 500 μ l of buffer RPE, a washing buffer, and the final step was adding 30-50 μ l of RNase free water to the spin column membrane and centrifuge for 1min to elute the RNA. The obtained RNA was kept at -80°C .

2.2.12.1 Gel electrophoresis.

The integrity of RNA was determined by gel electrophoresis which allows the examination of the 28S and 18S ribosomal RNA bands. The upper ribosomal band (28S in eukaryotic cells and 23S in bacterial cells) should be about twice the intensity of the lower band (18S in eukaryotic cells). 1% standard agarose gel was prepared by using 0.5 g of agarose and 50 ml of TAE buffer (Tris base, acetic acid and EDTA

with the final composition of 40 mM Tris acetate; 1 mM EDTA; pH 8.2 - 8.4). The mixture was then microwaved for 2 min on high power. After cooling, the gel was poured into a casting tray after adding 4µl of Ethidium bromide and left to set. Samples of RNAs were prepared by adding 2µl of loading buffer to 1µg of RNA (Final volume 7 µl). The gel was run on 98 Volt for 35 minutes before being visualised with UV light box and photographed (Figure 2.6).



Figure 2.6 A representative gel Electrophoresis of RNA samples. RNA extracted from MCF7 and MDA-MB231 transfected cells. The figure shows a 2:1 intensity ratio between 28S(5kb) and 18S (2kb) rRNA in the hyperladder,a molecular weight marker composed of RNA fragments of known length , which is considered a benchmark for intact RNA

2.2.13 Real time RT-PCR (RT-qPCR)

2.2.13.1 Reverse transcription

Reverse transcription (RT) was carried using the Omniscript Reverse Transcription kit from Qiagen. 2 µg of total cellular RNA was reverse transcribed using Omni script RT kit (Qiagen. # 205111), Random primer (Invitrogen # N 48190-011) and Ribonuclease inhibitor (Invitrogen. #10777-018), following the manufacturers' instructions. RNA samples were first treated at 37°C for 30 minutes with 3 µl of RNase-free DNase (Promega) to remove any residual DNA in the samples.

Following the incubation, 1 µl of RQ1 DNase stop solution was added to the samples and the samples were further incubated for 10 minutes at 65°C. then, the reverse transcriptase mixture was added to the samples as illustrated in table 2.5 and further incubate at 37 °C for 1h. After that, the cDNA samples were kept in -20 °C.

Table 2.5 The reverse transcriptase mixture

Component	Volume in µl / reaction
10X buffer RT	2 µl
dNTP Mix(5mM each dNTP)	2 µl
Random primer 10 µM	2 µl
RNase inhibitor (10 units/ µl)	1 µl
Omniscript Reverse Transcriptase	1 µl
RNase free water	variable
Total reaction volume	20 µl

2.2.13.2 Real time PCR

The real time PCR (qPCR) was carried out using SensiFast Probe Hi-ROX kit and gene specific TaqMan Gene Expression Assays. Table 2.7 contains information on the different assays used .The real time PCR (qPCR) was carried out using SensiFast Probe Hi-ROX kit, gene specific TaqMan Gene Expression Assays and the ABI Prism 7000 sequence detection system (Warrington, UK). Table 2.7 contains information on the different assays used . Each PCR reactions contained 1 µl (10 ng) of cDNA, 10 µl Sensifast, 1 µl TaqMan Gene Expression Assays and 8 µl nuclease free water in a final volume of 20 µl. A standard curve was included with each run to allow relative quantitation. A standard curve was made by preparing serial dilution of 0.1–30 ng cDNA (prepared from cDNA from different types of breast

cancer cells) (Table 2.6). Nuclease-free water containing 100 ng/μl yeast tRNA was used as a diluent; it acts as a carrier molecule that adheres to nucleic acid binding sites in the micro centrifuge tube and prevents binding of the nucleic acid of interest. Input amounts of samples were calculated from their respective threshold cycle (CT) values, using the standard curves generated with each assay. Data were expressed relative to 18S rRNA.

Table 2.6 Serial dilution of standards that prepared from cDNA from different types of breast cancer cells

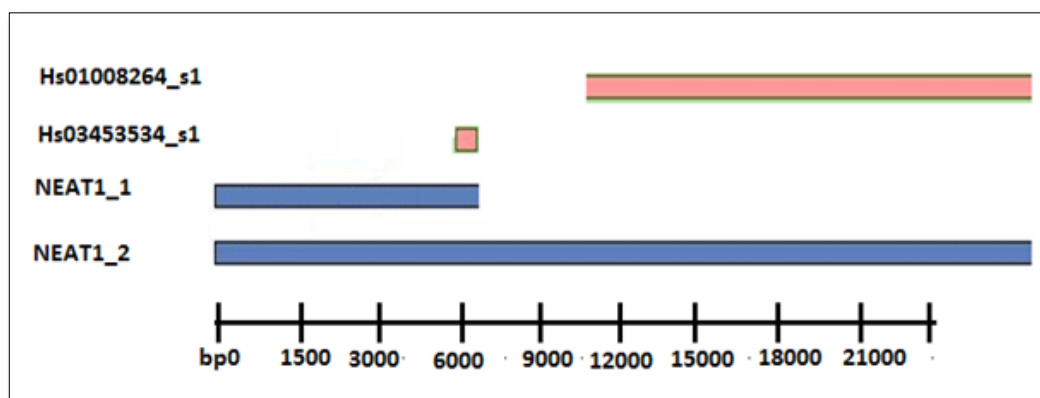
Standard	Conc.ng/ μl	μl Standard	μl dilution
S1	30	50	116.7
S2	10	60	120
S3	3	50	116.7
S4	1	60	120
S5	0.3	50	116.7
S6	0.1	60	120

Table 2.7 The details of TaqMan gene expression assay specific for human NEAT1 (short and long isoforms), MIAT and MALAT1 expression.

Probe	RefSeq	ID	Exon Boundary	Assay location
NEAT1 Short isoform	NR_028272.1	Hs03453534_s1	1-1	3310
NEAT1 long isoform	NR_131012.1	Hs01008264_s1	1-1	7996
MIAT	NR_003491.3	Hs00402814_m1	5	1864
MALAT1	NR_002819.3	Hs00273907_s1	1-1	4952

RT-qPCR was also performed on TissueScan™ Cancer and Normal Tissue cDNA Arrays using Breast Cancer cDNA Array II. The array is ready-to-use panel of cDNA samples derived from patients with breast cancer of different stages, grades and hormone receptors expression together with cDNA samples derived from healthy individuals. The panel contains 48 samples and includes 5-normal and 43 breast cancer samples. The samples were collected from females of 31-84 year of age with breast cancer of grade 3-9 according to Nottingham grading system. The tumour ranges from carcinoma in situ to metastatic ductal or lobular adenocarcinoma of the breast. The panel contains samples of different stages; 11-stage I, 8-IIA, 6-IIB, 8-IIIA, 2-IIIB, 4-IIIC, 4-IV. The panel contains samples show different hormone receptor expression; some are oestrogen positive, progesterone positive, oestrogen/progesterone positive, HER2 positive, triple positive or triple negative. For each assay, a standard curve of threshold cycle (CT) value versus log input standard cDNA was constructed by linear regression, and the equation of the line was used to calculate input amounts of samples from their respective CT values. Data were expressed relative to 18S

A) NEAT1 probes



B) MIAT

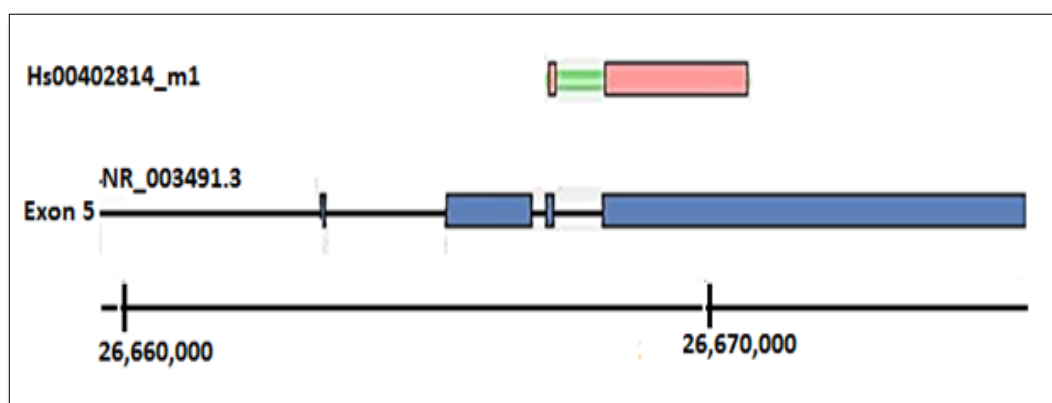


Figure 2.7 schematic diagrams representing the location of Taqman gene expression assay. (A) Represents the location of NEAT1 probes that cause amplification of short isoform (Hs03453534_s1) and long isoform (Hs01008264_s1) respectively. (B) Represents the location of MIAT Taqman probe (Hs00402814_m1), which results in the amplification of exon 5 (Thermofisher scientific for gene expression assay).

2.2.14 RT2 profiler PCR array

The effect of NEAT1 expression on the other genes was determined by using the RT2 Profiler PCR Array (96-Well Format) kit for Human Cell Cycle (# 330231 PAHS-020A), which define the expression of 84 genes responsible for positive and negative cell cycle regulation (cell cycle – Qiagen). While, Human Breast Cancer array (# 330231 PAHS-131ZA) responsible for profile the expression of 84 genes that involved in dysregulation of signal transduction and other biological processes

of breast cancer (Breast Cancer – Qiagen). Following the manufacturers' instructions, 25 µl of the PCR component mixture was added to each well, containing a dried assay, of the RT2 profiler plate. tightly covered with optical thin wall 8-Cap strips, to be run in PCR cycling program (ABI Prism 7000 sequence detection system) as in Table 2.7.

Table 2.8 The cycling program for RT2 Profiler PCR Array

Cycles	Duration	Temperature
1	10min	95 °C
40	15 s	95 °C
	1 min	60 °C

2.3 Statistical analysis

Data are presented as mean \pm slandered error of the mean (S.E.), where (n) represent the number of experiments. Statistical analysis was determined by Student's t-test and two-way ANOVA with Bonferroni's multiple comparison test (MCT) using the Graph Pad 7 software. The results of Real-Time PCR were analysed by using the standard curve method. A p-value of <0.05 was considered to be statistically significant. Web-based PCR Array Data analysis software at www.SABioscience.com was used to analyse RT2 profiler assay results. The results of RNA sequencing were analysed by using the IPathway Guide for Next-gen pathway analysis at www.advaitabio.com.

Chapter 3

Expression analysis of NEAT1 and MIAT in breast cancer

3.1 Introduction

Despite its heterogeneous characteristics, breast cancer is one of the few cancer types in which molecular classification has successfully been utilised for the use of personalised therapies, leading to significant improvements in disease-specific survival (West et al, 2001). Gene expression profiling has allowed the classification of breast tumours into the well-known major subtypes that exhibit different response to treatment, risk of disease progression, and preferential organ sites of metastasis. While the ER and PR positive luminal types respond well to hormonal intervention and HER2+ tumours can be effectively controlled with a diverse array of anti-HER2 therapies, the molecular-based signature for the basal-like TNBC tumours is still lacking and only 20% of these tumours respond well to standard chemotherapy. Determining the specific gene expression signature of different breast cancer types, including TNBC, is very important because it will allow the identification of specific diagnostic and prognostic biomarkers that will provide a more precise classification of the disease (West et al.,2001). Indeed, many multi-gene prognostic signatures from microarray gene expression analysis of either mRNA or miRNA levels were recently shown to predict prognosis and metastatic risk with greater accuracy than the traditional prognostic criteria (Bao and Davidson, 2008; Perou et al, 2000). In addition to their diverse functional roles, accumulating evidence confirms that the expression of many lncRNAs is dysregulated in multiple human cancers (Fu et al,2016; Qian et al, 2016; Arun et al, 2016). The expression of some of these lncRNAs is also associated with cancer metastasis and prognosis (Arun et al, 2016; Fu et al,2016). These include HOTAIR, which is upregulated in primary breast tumours and its overexpression is associated with enhanced breast cancer metastasis (Zhang, et al,2014). MALAT expression levels have been found to be

elevated in many solid tumours, such as lung (Schmidt et al, 2011), liver (Lai et al, 2012), and prostate cancers (Ren et al, 2013). Several lncRNA signatures have also been developed as novel predictors of survival in glioblastoma multiform (Zhang et al, 2013), breast cancer (Meng et al, 2014) and colorectal cancer (Hu et al, 2014). The aims of this chapter are to investigate if NEAT1 and MIAT expression levels are altered in breast cancer. The expression levels of the two lncRNAs were therefore evaluated in samples from different stages of breast cancer.

3.2 Methods

The expression of NEAT1, MIAT and MALAT1 were determined in the commercially available TissueScan qPCR Breast Cancer Disease Panels II (BCRT102), as described in Section 2.2.13.2. The panel was purchased from OriGene Technologies. Breast cancer complementary DNA (cDNA) array included 48 samples covering cDNA from 5-normal, 11-Stage I, 14-II, 14-III and 4-IV samples, whose clinical and pathological features are freely available at the following address:

<http://www.origene.com/qPCR/Tissue-qPCR-Arrays.aspx>.

Real-time PCR was conducted using SensiFast Probe Hi-ROX kit and TaqMan gene expression assays. Assay codes for Hs99999901_m1 for 18S, Hs03453534_s1 for NEAT1 short isoform (NEAT1_1), Hs01008264_s1 for the long isoform of NEAT1 (NEAT1_2), Hs00273907_s1 for MALAT1 and Hs00402814_m1 for MIAT) were employed as recommended by the manufacturers and were run on an ABI Prism Sequence Detection System model 7000. Assays usually contained 10 ng sample cDNA in a final volume of 25 µl. A standard curve, comprising 0.3–60 ng cDNA (prepared from MCF7, MDA-MB 231, T47D, MDA-MB 361 and Hs5T cells) was included with each run to allow relative quantitation. For each assay, a standard

curve of threshold cycle (CT) value versus log input standard cDNA was constructed by linear regression, and the equation of the line was used to calculate input amounts of samples from their respective CT values. Data were expressed relative to 18S rRNA. The expression levels of NEAT1, MALAT1 and MIAT were determined in both tumour and normal tissue samples and then differentiated according to their clinical stages and molecular classification of breast tumour. Data were analysed by one-way analysis of variance with Bonferroni's multiple comparison test for post hoc analysis of selected groups, depending on the number of groups to be compared. Homogeneity of variance was checked by Bartlett's test. Correlation between expression levels of the three long non-coding RNAs was analysed using Pearson's linear correlation. Two-sided P-values were calculated, and a probability level of less than 0.05 was chosen for statistical significance.

3.3 Results

3.3.1 Expression of NEAT1 transcripts in breast cancer

To analyse the expression of NEAT1 transcripts in breast cancer, NEAT1 short and long isoform transcript levels in samples from the Breast Cancer Disease Panels II (BCRT102) from OriGene, were assayed by real-time PCR. The assay used Taqman gene expression assays targeted against the two different isoforms of NEAT1, and 18S as endogenous control gene. The use of 18S was verified in preliminary experiments, which showed that the mean of its expression levels was found to be similar in tumour and normal samples (respective mean \pm S.E. values were 1.42 ± 0.36 and 1.33 ± 0.29). Levels of the short isoform of NEAT1 (NEAT1_1) in tumour samples, relative to 18S, were significantly increased compared to the normal breast samples. The results revealed up to 4 fold increase in the expression

levels of NEAT1_1 in tumour samples (Figure 3.1A). Interestingly, the degree of increase in the levels of NEAT1_1 varied according to the clinical stages of breast cancer (Figure 3.1B). While there was a 7 fold increase in the transcript levels in samples from patients with stage III and stage IV disease compared to control samples, only a 3 fold increase was seen in the samples from patients with stage I and stage II disease (Figure 3.1B). Stratification of patients into groups according to the molecular subtypes confirmed statistically significantly higher levels of NEAT1_1 in the breast cancer samples positive for both ER and PR and negative for HER2 when compared to normal breast tissue (Figure 3.1C). There was no statistical difference between NEAT1_1 expression levels in triple positive samples and samples from normal tissues. Whereas, the level of NEAT1_1 was significant lower in TNBC samples (Figure 3.1C).

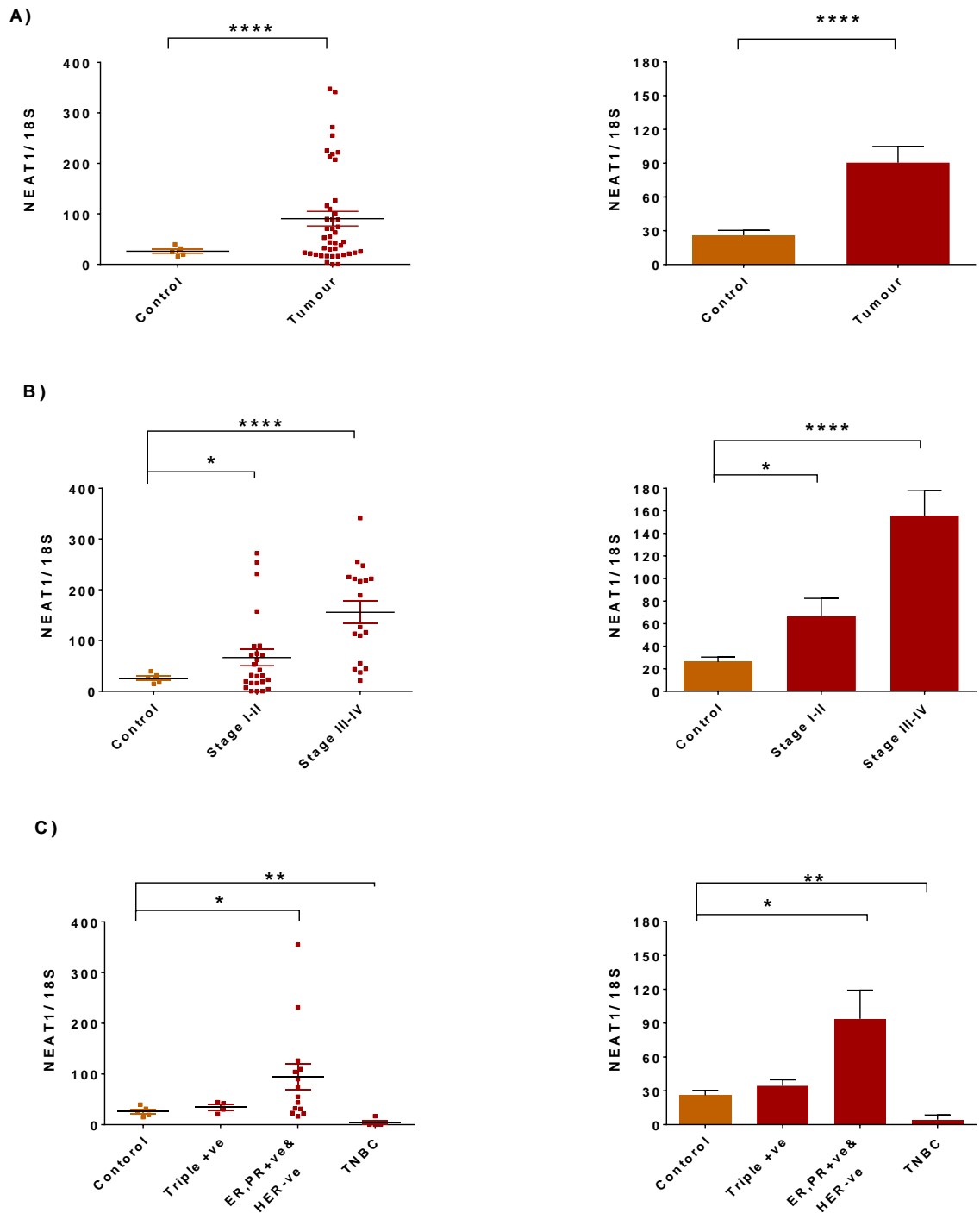
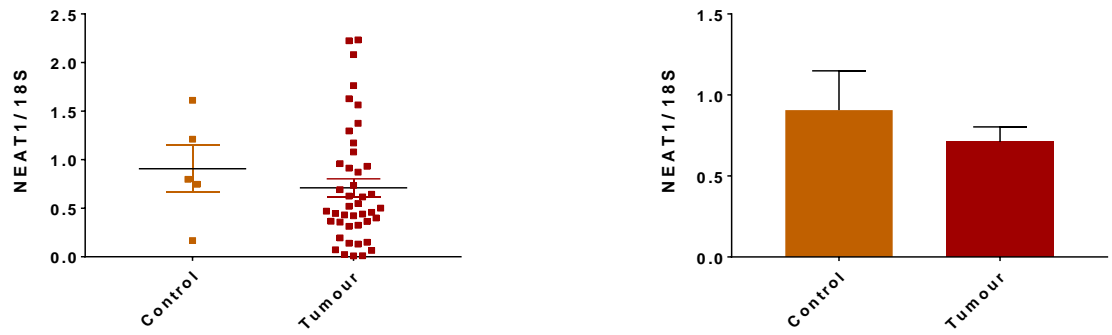


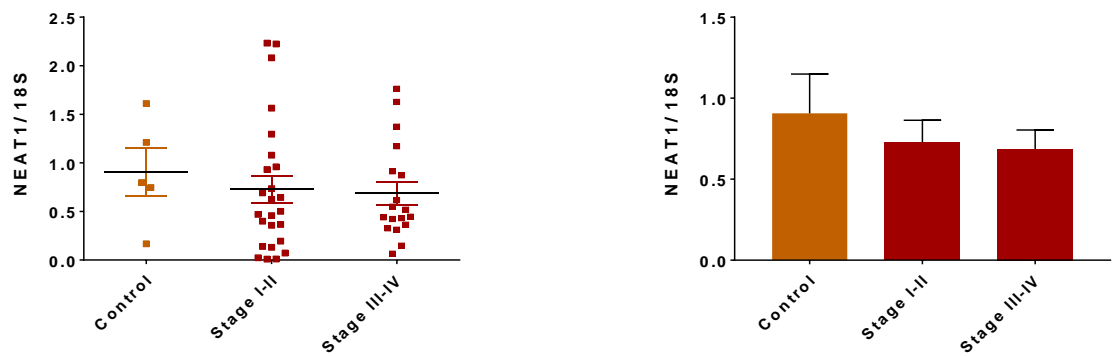
Figure 3.1 The expression levels of NEAT1 short isoform (NEAT1_1) in normal and breast cancer samples. Expression levels of NEAT1_1 were normalised to the levels of 18S. A) In the complete data set (n=48), NEAT1_1 levels are increased (*P< 0.0001) in tumour (n=43) relative to normal tissue samples (n=5). B) A subset of the data showing increased transcript levels of NEAT1_1 which is significant in patients with stages I-II disease (*P<0.05; n=25) and highly significant in stage III-IV disease (**P<0.001; n=18). C) Relative NEAT1_1 expression levels following stratification of samples into groups according to molecular subtypes. Results showed a significant (*P< 0.05) up-regulation of NEAT1_1 in ER, PR +ve & HER-ve samples and highly significant downregulation (**P< 0.01) in TNBC. (One way Anova test with Bonferroni's multiple comparisons)

The expression levels of the long isoform of NEAT1 (NEAT1_2) were also determined in the samples from the Breast Cancer Disease Panels II (BCRT102). The results revealed a lower level of NEAT1_2 in breast cancer samples when compared to samples from normal breast tissues but the statistical analyses were not significant (Figure 3.2 A). Further analysis showed that the expression level of NEAT1_2 is lower in all the stages of the disease compared to the control; however, the decrease in the expression levels was not statistically significant (Figure 3.2B). Analysis of the levels of NEAT1_2 in the different molecular subtypes of breast cancer showed a decrease in the levels of NEAT1_2 in the different subtypes in comparison to the normal tissue samples; however, such decrease was statistically significant only in TNBC samples (Figure 3.2C).

A)



B)



C)

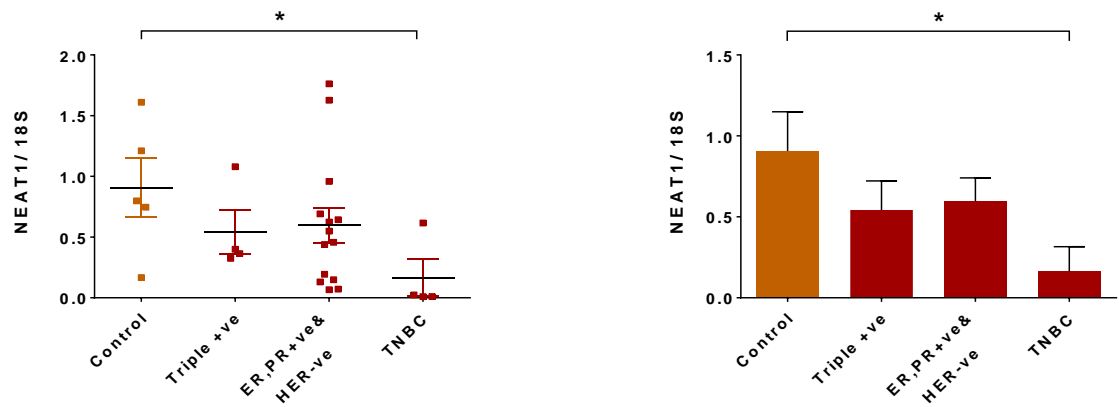


Figure 3.2 The expression levels of NEAT1_2 in breast cancer. (A) The results of the full data set (n=48) showed an insignificant down regulation of NEAT1_2 in breast tumour (n=43) samples compared to control (n=5). (B) A slight decrease in the expression levels in all the stages of breast cancer, such decrease was not statistically significant. (C) The expression levels of the long isoform showed reduced expression in all molecular subtypes of breast cancer, however such down-regulation was statistically significant only in TNBC (*P< 0.05) . (One way Anova test with Bonferroni's multiple comparisons)

3.3.2 Expression of MIAT lncRNA in breast cancer

To examine whether the mRNA expression levels of MIAT exhibit changes in breast cancer, the Breast Cancer Disease Panels II was used to determine MIAT levels in breast cancer and normal tissues. Real time PCR was performed using TaqMan gene expression assay targeted at MIAT gene and 18S, which was used as endogenous control gene. The results revealed that the overall mean of MIAT expression levels was slightly lower in breast tumour compared with the mean of MIAT expression levels in normal breast tissues; however, the results were not statistically significant (Figure 3.3A). Figure 3A shows that some breast tumours samples express high levels of MIAT compared to the control. Further analysis of the data revealed that MIAT expression levels some breast samples in stage II and II expressed higher levels of MIAT. However there was no statistical significant between the expression of MIAT in control samples and samples from stage II and III. MIAT expression levels were slightly lower in the advanced stages of the disease, stage III and stage IV (Figure 3.3B). Examination of MIAT expression in the different molecular subtypes of the disease showed that compared to the normal breast samples, MIAT expression is not changed in the triple positive (ER, PR and HER2 positive) samples, significantly reduced in ER, PR +ve, HER –ve breast cancer subtypes and significantly increased (2 fold increase) in TNBC (Figure 3.3C)

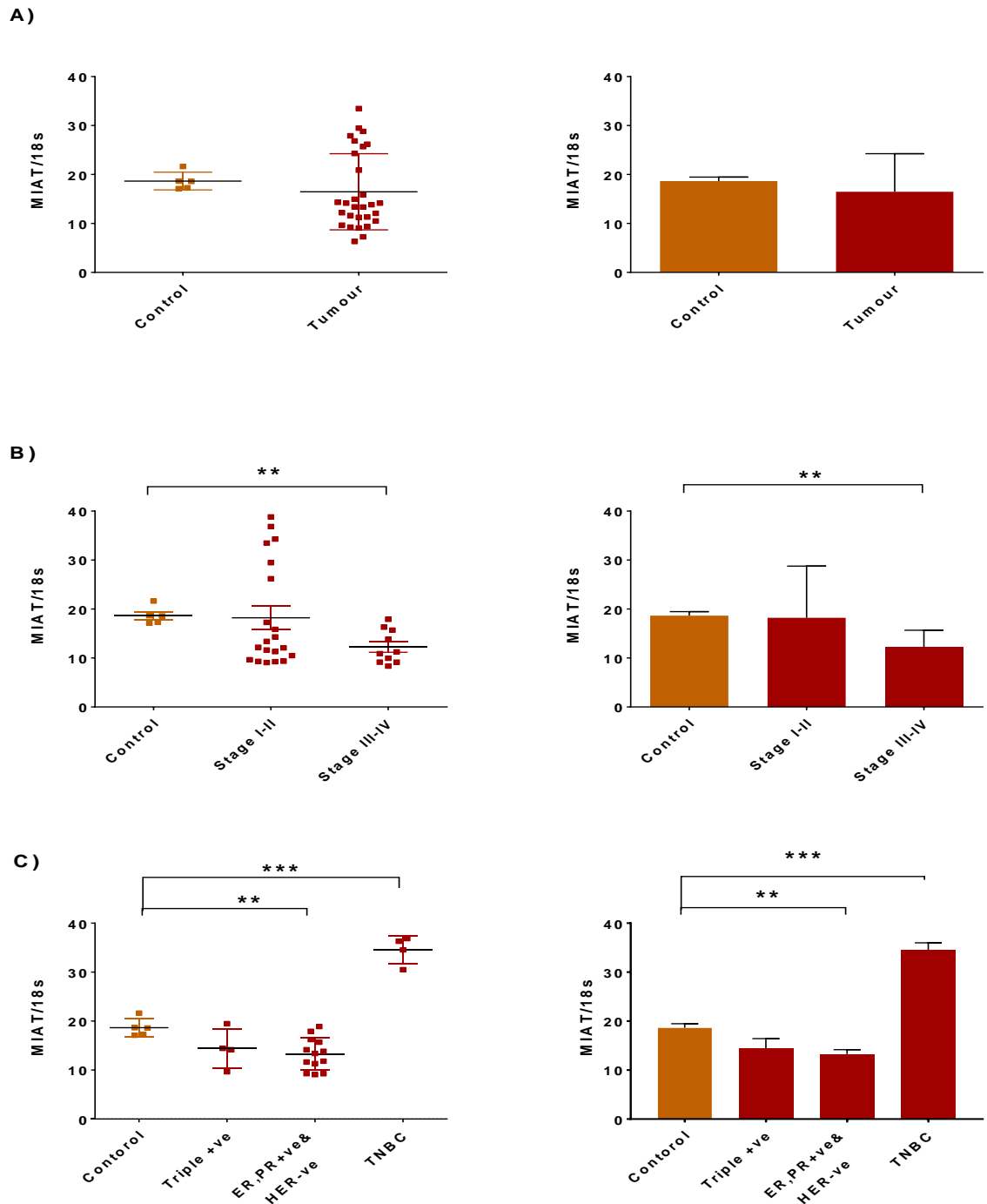


Figure 3.3 MIAT expression levels in breast cancer. The level of MIAT lncRNA was determined in 43 tumour samples and compared to 5 normal breast tissue samples. A) MIAT expression showed no significant change in the full data sets of breast tumour compared to normal. B) Analysis of the data revealed that there is a highly significant (** $P<0.01$) downregulation of MIAT lncRNA in stage III-IV disease. C) Stratification of MIAT expression levels according to the molecular subtypes showed the variation in MIAT expression according to the molecular breast cancer types. A highly significant (*** $P<0.001$) overexpression of MIAT was found in TNBC, and a significant (** $P<0.01$) downregulation in ER, PR +ve & HER-ve breast cancer. (One way Anova test with Bonferroni's multiple comparisons)

3.3.3 Correlation analysis of NEAT1, MALAT and MIAT expression levels

Neat1 gene is located 55 kb upstream from the lncRNA MALAT1/NEAT2. The two genes share common regulatory DNA elements and their expression is reported to be co-regulated in certain tissues such as the intestine and colon (Nakagawa et al, 2012). It was therefore important to determine if there is a relationship between the two lncRNAs in breast cancer. In order to determine if there is a correlation between the expression of NEAT1 and MALAT1, the expression levels of MALAT1 in breast cancer samples and normal tissues samples in the OriGene breast cancer array (BCRT102) was determined using real time PCR and 18S as endogenous control. As shown in Figure 3.4A, MALAT1 expression levels were more than two fold higher in breast cancer samples compared to control (**P< 0.01). This up-regulation is significant (*P< 0.05) in stage I-II and highly significant (**P< 0.01) in stage III-IV, as illustrated in Figure 3.4B. The results also showed that similar to NEAT1, the expression levels of MALAT1 were significantly up-regulated in ER, PR +ve, HER – ve breast tumour subtype samples and significantly down-regulated in TNBC samples (Figure 3.4C).

To explore the correlation between each of the NEAT1 isoforms and MALAT1, a Pearson's linear correlation test was performed. The results revealed there was positive correlation (*P< 0.05) between NEAT1 _1 and MALAT1 expression as shown in (Figure 3.5A), suggesting that MALAT1 expression might be positively regulated by the short isoform of NEAT1 or both lncRNAs might be involved in one pathway. The correlation analysis between the long isoform NEAT1_2 and MALAT1 expression showed positive correlation but this correlation was statistically not significant (Figure 3.5B).

Analysis of the NEAT1_1 and MIAT expression levels showed positive significant (* $P < 0.05$) correlation (Figure 3.6A). Similarly, there was no significant correlation between MIAT and NEAT1_2 expression, as shown in (Figure 3.6B).

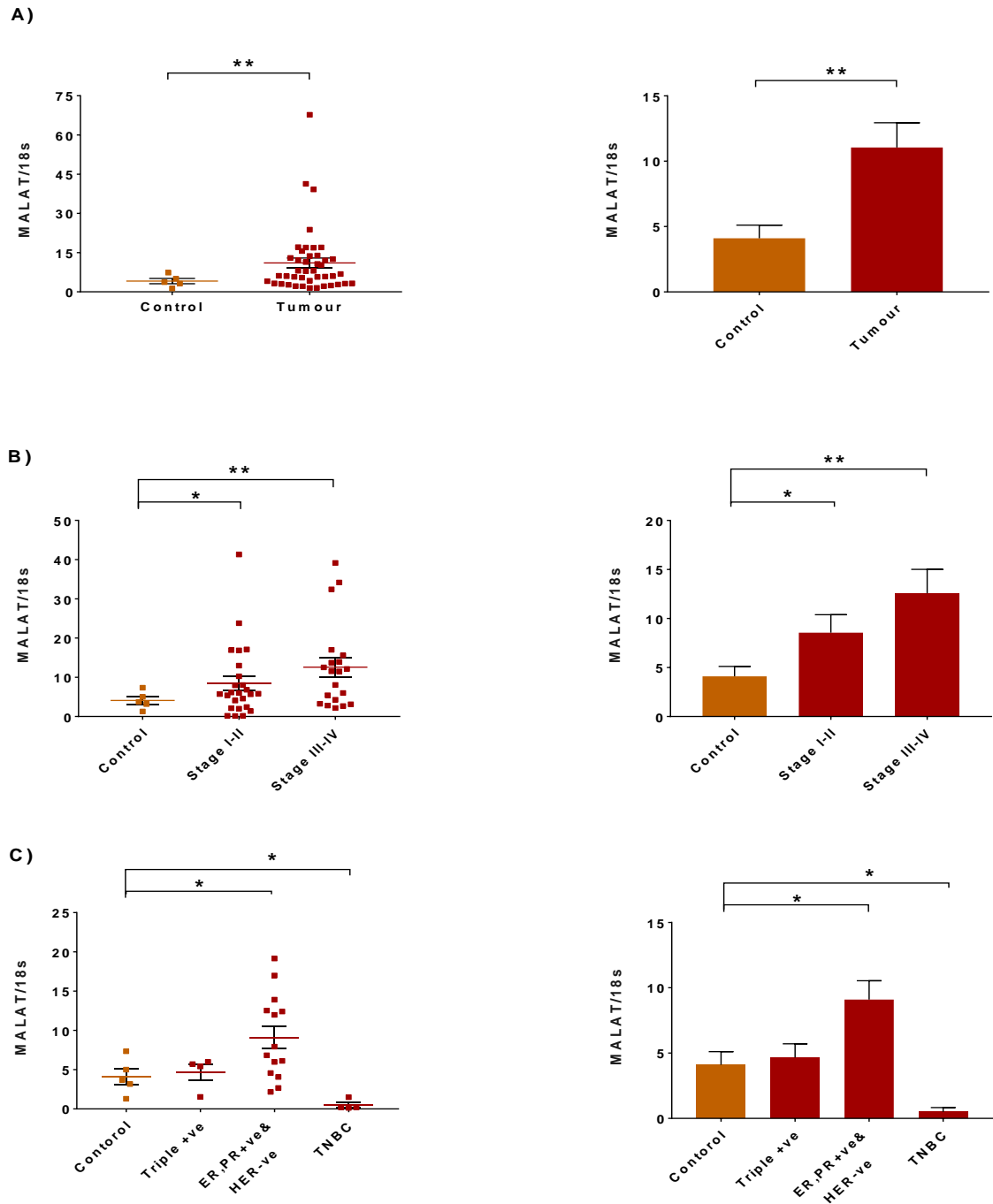


Figure 3.4 The expression levels of MALAT1 lncRNA in breast cancer. A) The results revealed significant increase in the expression levels in breast tumour samples (n=43) compared to the control (normal breast tissue n=5) (**P<0.01). B) Represents the expression stratified according to breast cancer stages, which showed a significant (*P<0.05; n=25) and highly significant (**P<0.01; n=18) increase in MALAT1 expression levels in stage I-II and stage III-IV, respectively. C) Further analysis of the results according to the molecular subtypes of the cancer revealed a significant (*P<0.05) increase in MALAT1 expression ER, PR +ve & HER-ve and a significant down-regulation in TNBC (*P<0.05). (One-way Anova test with Bonferroni's multiple comparisons)

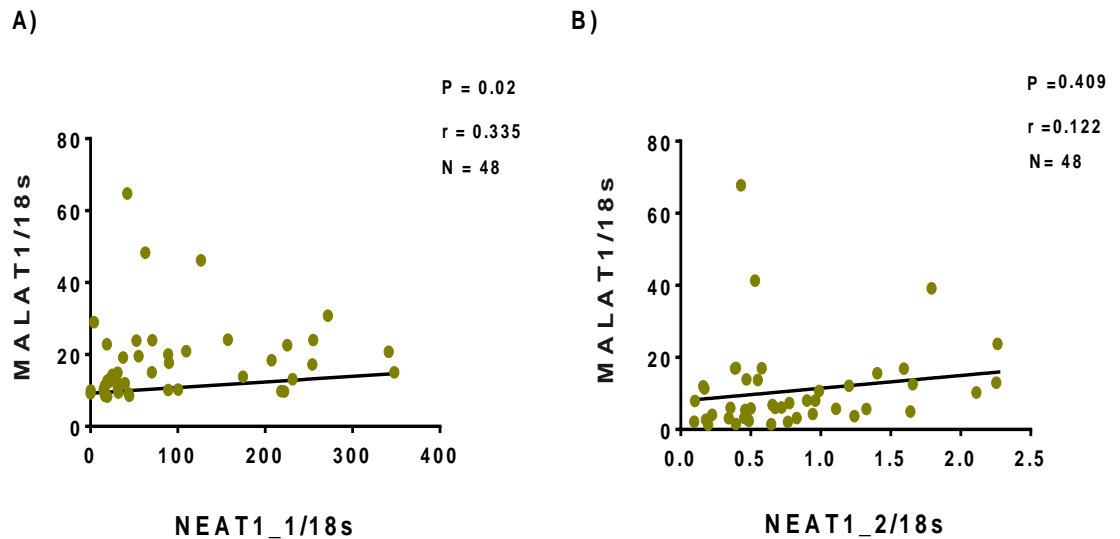


Figure 3.5 Correlation analysis of NEAT1 isoforms and MALAT1 lncRNA expression in breast cancer samples. The analysis of correlation coefficient was done by Pearson's linear test. A) The results show a significant positive correlation ($*P < 0.05$) in the expression levels of NEAT1 short isoform and MALAT1. B) Analysis of the correlation coefficient of NEAT1_2 isoform and MALAT1 showed a statistically non-significant correlation.

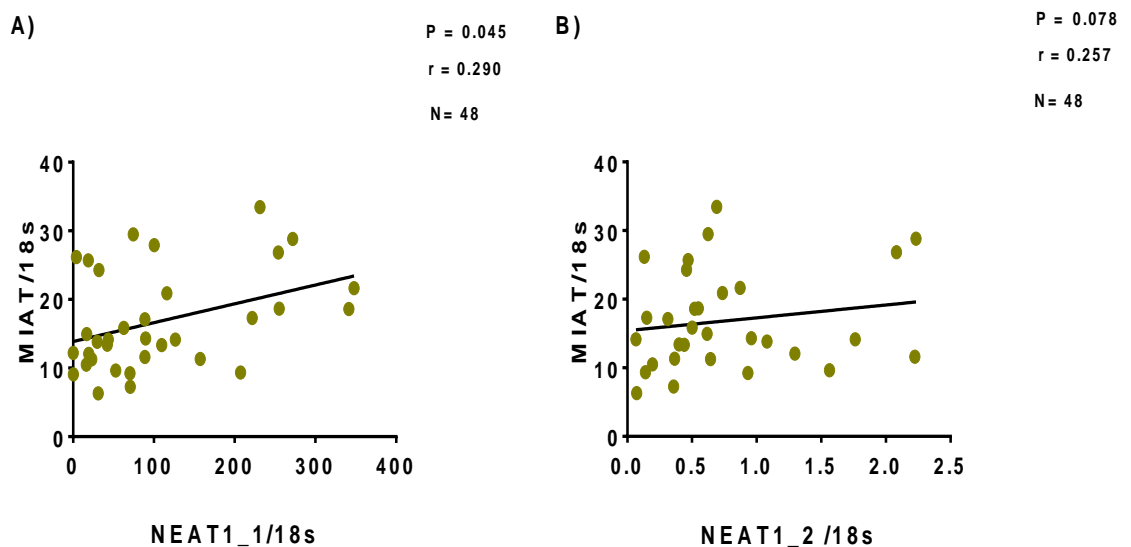


Figure 3.6 Correlation analysis of NEAT1 isoforms and MIAT lncRNA expression in breast cancer samples. A) The results show a significant positive correlation ($*P < 0.05$) in the expression levels of NEAT1 short isoform and MIAT. B) No significant correlation was found in the expression of NEAT1 long isoform and MIAT in breast cancer samples

3.4 Discussion

Comprehensive analysis of lncRNAs gene expression in multiple human tissues showed that their expression is tissue-specific and lower than the protein coding genes (Derrien et al, 2012). Expression of lncRNAs has been reported to be deregulated in many cancers (Malih et al, 2016). Such expression in a tissue and disease specific manner makes them ideal candidates to be used as diagnostic and prognostic biomarkers for different cancers including breast cancer, and could contribute to the improvement in the management of the disease (Fu et al, 2016; Malih et al, 2016). So far, HOTAIR, H19 and KCNQ1OT1 lncRNAs were considered as the important biomarkers in breast cancer diagnosis, in which HOTAIR and H19 show a high expression rate in invasive carcinoma (IC) rather than ductal carcinoma in situ (DCIS), while HOTAIR and KCNQ1OT1 lncRNAs show high expression in tumour cells (Zhang, et al.,2015). Wu et al. (2016) reported a number of tissue specific lncRNAs that are associated with breast cancer development. The expression of these lncRNAs correlated with a set of mRNAs. This co-expression of lncRNAs-mRNAs complexes contributes to the control gene expression that might be a factor in the imitation of breast cancer. One of these lncRNAs is AC145110.1, which is encoded on chromosome 8p12. AC145110.1 and was found to be co-expressed with 127 mRNAs, and such expression differs in tumour and normal breast tissue (Wu et al, 2016). Accordingly, the present study revealed a specific expression pattern of the two nuclear lncRNAs, NEAT1 and MIAT in breast cancer. This study revealed that MIAT and NEAT1 are differentially expressed in different stages of the disease and different molecular subtypes. The results revealed distinct NEAT1 signatures in breast cancer. NEAT1 expression levels showed a moderate increase in stage I and II disease and a significant up-regulation in the advanced

stages, stage III-IV disease. This suggests that overexpression of NEAT1 is important at the earliest stages of breast oncogenesis and may play a crucial role in the transition from pre-invasive to invasive growth. The present work also demonstrates for the first time that the two NEAT1 isoforms display a difference in their expression in breast cancer and confirms that the two isoforms might exert different functions. NEAT1_1, the short isoform, was found to be up regulated in breast cancer and the increase in the expression was strongly associated with the advanced stages of the disease (stage III-IV) and with ER, PR +ve and HER (-) subtype of the disease. NEAT1_1 was found to be significantly down regulated in TNBC. However, the long isoform NEAT1_2 showed no changes in breast cancer apart from significant down-regulation in TNBC, similar to the NEAT1_1. Results presented in this chapter also confirm a role for MIAT in breast cancer. MIAT was found to be slightly down regulated in the advanced stages of the disease (stage III-IV) and an increase in its expression was associated with TNBC. However, it is worth noting that the number of TNBC samples is small and therefore more studies required to confirm these observations.

Many studies have shown that NEAT1 is extensively expressed in human tissues, particularly after being exposed to a stress factor like proteasome degradation and viral infection (Barry et al, 2017). In addition, over-expression of NEAT1 in different tumours and particularly breast cancer was confirmed in different studies (Qian et al.,2016; Choudhry et al.,2015). However, in this study, a variation in expression of NEAT1 isoforms in breast cancer was identified. A significant overexpression of NEAT1 _1 in tumour samples was observed, while NEAT1 _2 expression levels did not show major changes. These findings highlight the fact that the two isoforms might be involved in different cellular function. Indeed, Li et al, (2017b) have successfully dissected the function of the two isoforms using CRISPR-Cas9 genome

editing approach. The study confirmed that only the long isoform is the essential component of the paraspeckles, whereas the short isoform was found to co-localise in non-paraspeckle structures called micro speckles and may carry paraspeckle-independent functions (Li et al, 2017b). Variations in NEAT1 isoforms expression have also been reported in different cell types in various tissues (Nakagawa et al, 2011). NEAT1 _1 is widely expressed in different cell types, whereas strong expression of NEAT1 _2 was found to be associated with prominent paraspeckle formation and restricted to a sub-population of cells in certain tissues, particularly in the digestive tissues such as the stomach and colon, where natural cell loss occurs (Nakagawa et al, 2011). Chai et al. (2016) have provided more evidence to support a differential expression pattern for the two isoforms. The study showed the interaction between HuR, an RNA binding protein, and the miR-124-3p is responsible for regulation of NEAT1 _1 stability and therefore its expression level. A dysregulation of HUR- miR-124-3p axis leads to an increase of NEAT1_1 expression in ovarian cancer (chai et al, 2016). An increase in HuR expression and activity promote ovarian cancer cells growth and invasion by enhancing the over expression of NEAT1 _1, whilst an increase in miR-124-3p expression leads to the decrease in NEAT1 _1 expression levels and inhibition of ovarian cancer cell growth (chai et al, 2016). Furthermore, Wu et al. (2015) have also confirmed the overexpression of NEAT1 _1 in advanced stages of colorectal cancer and in metastatic tissues. The over-expression of NEAT1_1 was associated with poor prognosis of the disease, whereas NEAT1_2 expression level was not affected (Wu et al.,2015).

Stratification of NEAT1_1 expression levels according to the molecular subtypes of breast cancer confirmed a predominant increase of NEAT1_1 ER, PR +ve and HER –ve subtype and a significant decrease in TNBC subtypes. The increase of

NEAT1_1 in ER, PR +ve and HER –ve subtype might be explained by the fact that ER is a transcriptional regulator of NEAT1 (Chakravarty et al, 2014). ER α is reported to regulate the transcription of different lncRNAs involved in prostate cancer including NEAT1 (Chakravarty et al, 2014; Romano et al, 2010). ER α has also been shown to regulate NEAT1 expression in prostate cancer which was the most significantly overexpressed lncRNA in prostate cancer and its expression was associated with prostate cancer progression (Chakravarty et al, 2014; Romano et al, 2010; Lin et al, 2004). Chakravarty et al. (2014) have shown that knockout of NEAT1 compromised the expression of ER α target genes, suggesting that NEAT1 is not only a downstream target but also a mediator of ER α signalling in prostate cancer cells. It is therefore possible that the interaction of NEAT1 and ER observed in prostate cancer is also present in breast cancer. Accordingly, the decrease of NEAT1_1 expression levels in TNBC might be due to the fact that these tumours are ER negative and therefore NEAT1 levels are decreased. However, further studies are required to confirm these findings because of the small sample size.

MALAT1 was first identified as a prognosis marker in early-stage metastasizing lung cancer (Wu et al, 2016). In addition, MALAT1 suppresses expression of anti-metastasis genes such as MIA2 (melanoma inhibitory activity 2) and ROBO1 (roundabout 1), while induces pro-metastasis genes including LPHN2 (latrophilin 2) and ABCA1 (ATP-binding cassette, sub-family A, member 1) to accelerate metastasis (Gutschner et al, 2013). MALAT is over-expressed in different solid tumours including lung, breast, colon, hepatocarcinoma, pancreatic and prostate cancer (Tripathi et al., 2013; Meseure et al, 2016; Ma et al, 2015). Both NEAT1 and MALAT1 genes are located on chromosome 11q. Neat1 gene is located 55 kb upstream from the MALAT1. The expression of both genes is demonstrated to be co-regulated in certain tissues such as the intestine and colon and certain cancers

such prostate cancer (Nakagawa et al, 2012). The present study confirmed that there is a correlation of the NEAT1_1 and MALAT1 expression in breast cancer. Similar to NEAT1_1, MALAT was found to be up regulated in ER, PR +ve and HER –ve subtype and its levels decreased in TNBC subtype. MALAT1 cellular effects on the alternative splicing of pre-mRNA lead to an aberrant expression of genes that are responsible for cell cycle regulation, thereby enhancing the proliferation of tumour cells (Tripathi et al, 2013). MALAT1 is an oestrogen dependent transcript and its expression was regulated by oestrogen receptors, mainly ER α and ER β in breast and prostate cancer respectively (Aiello et al, 2016). Therefore, such interaction might explain the over-expression of MALAT1 lncRNA in ER, PR +ve and HER -ve samples and its decrease in TNBC. Nonetheless, the co-expression patterns in NEAT1_1 and MALAT gene expression suggest a coordinated dysregulation of these nuclear lncRNA loci in cancer.

Analysis of MIAT expression in the breast cancer samples suggested that MIAT might be down-regulated in stages III-IV of the disease and its levels showed a significant increase in TNBC samples. Further experiments are required to confirm the up-regulation in TNBC because of the small sample size. However, the results are in agreement with other studies. Previous work has already implicated MIAT in cancer. Crea et al. (2016) reported a significant relationship between MIAT lncRNA expression and neuroendocrine prostate cancer (NEPC), an androgen receptor (AR)-negative metastatic neoplasm. Therefore, this finding might explain the reason of MIAT upregulation in TNBC, which is characterized by negative endocrine receptors (estrogen and progesterone, and HER2), and by being highly metastatic and resistant to most of the chemotherapeutic treatments and presented with poor prognosis (Zhang et al, 2012). The other possible cause of MIAT overexpression could be related to the over expression of tumour necrosis factor (TNF α) in triple

negative breast cells, a pro inflammatory cytokine that promotes tumour growth (Pileczki et al.,2012). Jin et al (2017) revealed a positive correlation between MIAT and TNF α expression in osteogenic differentiation of human adipose-derived stem cells (hASCs) (Jin et al, 2017).

Furthermore, MIAT lncRNA shows a significant positive correlation with NEAT1_1 isoform. Such observations could be related to the role of MIAT lncRNA in promoting the expression of Oct4 mRNA. Oct4 is reported to promote and enhance the expression of NEAT1 and MALAT1 lncRNAs via Oct4/NEAT1/MALAT1 pathway in lung cancer (Nobil et al, 2017; Jen et al, 2017). In addition, Ling et al (2012) found a high expression levels of Oct 3/ 4 in MCF7 and MDA-MB-231 breast cancer cells. Therefore, this evidence might explain the positive correlation in NEAT1 and MIAT expression in breast cancer.

In summary, this study indicates that the expression of the two nuclear lncRNAs, NEAT1 and MIAT is dysregulated in breast cancer and suggests that these lncRNAs might be involved in the initiation and progression of breast cancer. The study also highlights a difference in the expression of short and long isoform of NEAT1. The study confirmed an interesting correlation between the expression of nuclear lncRNAs MALAT and MIAT with NEAT1. Both NEAT1 and MALAT1 genes are located on chromosome 11q which is often dysregulated in cancer and the results presented in this chapter highlight the possibility of the presence of a coordinated dysregulation of these nuclear lncRNAs loci in cancer. Further studies are required to investigate the potential use of these as diagnostic and prognostic biomarkers due to the variation in their expression, depending on breast cancer stages and molecular subtypes.

Chapter highlights

1. The expression pattern of NEAT1 _1 was different from that of NEAT1 _2 in breast cancer sample.
2. NEAT1_1 short isoform is significantly up-regulated in breast cancer. Such expression was increased in advance stages of breast cancer and in ER, PR +ve, HER –ve molecular subtype.
3. The results showed a small but significant down-regulation of MIAT lncRNA in stage III-IV of breast cancer and in ER, PR+ve, HER –ve samples. However, MIAT expression levels were significantly increased in TNBC.
4. There was a significant positive correlation in expression between NEAT1_1 and MALAT lncRNAs in breast cancer. Both NEAT1_1 and MALAT1 lncRNAs were up-regulated in ER, PR+ve, HER –ve molecular subtype of breast cancer and significantly down-regulated in TNBC. A significant positive correlation was found between NEAT1_1 and MIAT expression levels.
5. NEAT1, MALAT1 and MIAT lncRNAs have the potential to be used as diagnostic and prognostic biomarkers because of the variation in their expression, according to breast cancer stages and molecular subtypes.

Chapter 4

The role of Nuclear Enriched Abundant Transcript 1 (NEAT1) on breast cancer

4.1 Introduction

Evidence presented in Chapter 3 suggests that NEAT1 is implicated in breast cancer. NEAT1 short isoform was found to be significantly up regulated in breast cancer cells. Its expression was increased in advance stages and in ER, PR +ve, HER –ve molecular subtype of the disease. An increasing number of studies have implicated NEAT1 in the regulation of cell survival (Lo et al, 2016a; Choudhry et al, 2015; Ke et al, 2016). NEAT1 was identified independently as a candidate regulator of apoptosis using an unbiased functional screen to identify genes regulating apoptosis (Williams et al, 2006). Such screen has identified several genes including the long non-coding RNA GAS5, which plays very important role in the control of cell death and survival (Mourtada-Maarabouni et al, 2009; Mourtada-Maarabouni et al, 2010).

NEAT1 was found to be significantly up-regulated during hypoxia in breast cancer cells and was characterised to be a direct transcriptional target of hypoxia-inducible factor in breast cancer cells (Choudhry et al, 2015). The study confirmed the hypoxic induction of NEAT1 expression, which was accompanied by an increase in the formation of nuclear paraspeckles (Choudhry et al, 2015). Furthermore, an interaction between NEAT1 and the RNA binding protein FUS (Fused in sarcoma/translocated in liposarcoma) have been described in breast cancer cells by Ke et al. (2016). In which, FUS has the ability to bind with NEAT1 forming a complex that is important for maintaining the survival of breast cancer cells (Ke et al, 2016). Another important interaction between NEAT1 and miR-548 has been reported, where over-expression of miR-548 leads the suppression of NEAT1 expression resulting in an increase the apoptotic cell death of breast cancer (Lo et al, 2016a; Ke et al, 2016). Thus, NEAT1 overexpression participates in breast

cancer tumorigenesis via enhancing cell proliferation and reduction in apoptosis (Lo et al, 2016a). Therefore, it was of interest to study the functional effects of NEAT1 down-regulation on the survival of breast cancer cells.

The aims of this chapter is to study the functional effects of NEAT1 silencing on the cell survival of two breast cancer cell lines which include oestrogen receptor positive MCF-7 and the TNBC, MDA-MB-231. Therefore, The specific aims are to determine: i) the effects of silencing NEAT1 on breast cancer cell survival, ii) the implications of reduced NEAT1 expression on the breast cancer cell response to chemotherapeutic agents and UV treatment, iii) the effects of NEAT1 silencing on the expression of MALAT1 and BAD, two genes located on the same chromosome

4.2 Material and methods

4.2.1 RNA interference

MCF7 cells were transfected with specific siRNAs targeting NEAT1 long isoform using the HiPerFect transfection reagent, as described in section 2.2.5. Nucleofection was also used to transfect MCF7 and MDA-MB-231 with NEAT1 specific siRNAs or NEAT1 antisense oligonucleotides (ASO) which target both the short and long isoforms, as explained in section 2.2.5 and 2.2.6 respectively. Information about the specific NEAT1 siRNAs and NEAT1 ASO are presented in Table 2.2

4.2.2 Functional analysis: determination of cell survival, apoptosis, cell cycle profile and cell migration

After transfection, cells were harvested by trypsinization then re-plated at 2×10^5 cells into 6 well plates. Cells were cultured for further 24 and 48h before being trypsinized to determine cell survival and apoptosis as described in section 2.2.7. Long-term survival was determined by replating transfected cells in culture medium supplemented with 10% (v/v) cell-conditioned medium in 6-well plates. Cells were cultured for 2-3 weeks before counting the colonies after staining with crystal violet as present in section 2.2.9. Cell cycle analysis was carried out 24h post re-plating as described in section 2.2.8.

Cell migration was performed as detailed in section 2.2.10. Initially, measurement of the wound area was taken from four places using Image J software to document the pre-migration area of the cell-free detection zone. The distance across each wound was then measured in four places at 18 h and 36h or until wounds were completely closed.

4.2.3 Induction of cell death and cell survival assays

At 48 h post transfection with Antisense oligonucleotide (ASO), cells were trypsinised and treated with Ultraviolet-C (UV-C) irradiation prior to plating as present in section 2.2.11. Cell survival and apoptosis were assessed after 24 and 48h as described in section 2.2.7. Long-term survival was assessed by colony forming assay as in section 2.2.9, which was performed following irradiation.

Regarding drug treatment, transfected cells were cultured for 20 h before addition of each of the chemotherapy drug as in section 2.2.11. Cell viability was determined by MTS assay where sample absorbance readings at 490 nm (A490) were corrected for the appropriate medium plus drug blank values. The growth inhibitory effects of chemotherapeutic drugs were determined at 24h and 48h post drug treatment and calculated according to the following equation:

$$\% \text{ of cytotoxicity} = 100 - [\text{OD}_{490} \text{ of treated sample} / \text{OD}_{490} \text{ of untreated sample (vehicle)}] \times 100$$

4.2.4 Real time RT-PCR

Total RNAs were isolated from 1×10^6 of transfected MCF7 and MDA-MB231 cells as in section 2.2.12. Cytosolic and nuclear RNAs were isolated as explained in section 2.2.12. TaqMan gene expression assays (Table 4.1) were used on cDNA prepared by random hexamer priming and Omniscript, as described in section 2.2.13. Reactions (20 μ l) contained 50 ng sample cDNA or 0.1–30 ng of standard cDNA (prepared from MCF7, MDA-MB-231, T47D, MDA-MB 361 and Hs5T cells). Endogenous gene expression levels of samples were calculated from their respective threshold cycle (CT) values using standard curves generated with each assay. Data were expressed relative to 18S rRNA.

Table 4.1 TaqMan gene expression assays. The table contains the details and locations of the different gene specific TaqMan gene expression assays used in this study.

Probe	RefSeq	ID	Exon Boundary	Assay location
NEAT1 (short)	NR_131012.1	Hs03453534_s1	1-1	3310
NEAT1 (long)	NR_131012.1	Hs01008264_s1	1-1	7996
MALAT	NR_002819.3	Hs00273907_s1	1-1	4952
BAD	NM_004322.3	Hs0018893_m1	1-2	524
XIAP	NM_001167.3	Hs00745222_s1	2-2	441

4.3 Results

4.3.1 The effects of NEAT1 siRNAs on the survival of breast cancer cell using cationic lipid-mediated siRNA delivery system

To examine the effects of reduced NEAT1 expression on breast cancer cell survival, NEAT1 siRNAs were employed to silence endogenous NEAT1 expression. Two different siRNAs targeting the long isoform (NEAT1,1 siRNA targets nucleotides at position 12013-12033 and NEAT1,2 siRNA targets nucleotides at 12084-12104) were employed. Initially, HiPerFect transfection reagent was used for siRNA delivery. HiPerFect is a blend of cationic and neutral lipids that enables effective siRNA uptake and efficient release of siRNA inside cells, resulting in high gene knockdown even when using low siRNA concentrations and has minimal cytotoxicity effects (Qiagen, 2010). Such method of transfection is used routinely in the lab for siRNA delivery and was found to be efficient (Maarabouni et al, 2008). Therefore,

it was chosen as a method for transfecting NEAT1 specific siRNA into MCF7 cells. RNA was isolated from MCF7 cells 72h post-transfection and RT-qPCR was carried to assess the level of NEAT1 silencing in these cells. Surprisingly, RT-qPCR results showed that compared to control, endogenous expression levels of NEAT1 long and short isoforms were significantly increased in the cells transfected with each of the two NEAT1 siRNAs (Figure 4.1 A and B) . Since the RT-qPCR primers and the probe are located in the short isoform of NEAT1, the possibility that the siRNAs used might have caused a decrease in the expression levels of the long isoform and such decrease led to the increase in the expression levels of the short isoform was investigated. To this end, another TaqMan gene expression assay targeting the long isoform was used to determine the level of NEAT1 expression in these transfected cells. The results confirmed that both NEAT1 siRNAs also caused significant increase in the levels of NEAT1 long isoform expression as illustrated in (Figure 4.1 B). siRNAs delivered via Cationic lipid polymers are reported to be released in the cytoplasm and do not reach the nucleus. Therefore, a cellular fractionation was performed to isolate the cytoplasmic from nuclear NEAT1 transcripts was carried out to assess the levels of NEAT1 expression in the cytoplasmic and nuclear fraction. The results revealed that although there was a significant up-regulation in the nuclear NEAT1 levels, there was a significant down-regulation in the cytoplasmic NEAT1 levels, as shown in Figure 4.1C and D respectively. Overall, these observations suggest that NEAT1 siRNAs delivered via lipid polymers mediated transfection have silencing effects on the expression levels of cytoplasmic NEAT1 transcripts in MCF7 cells.

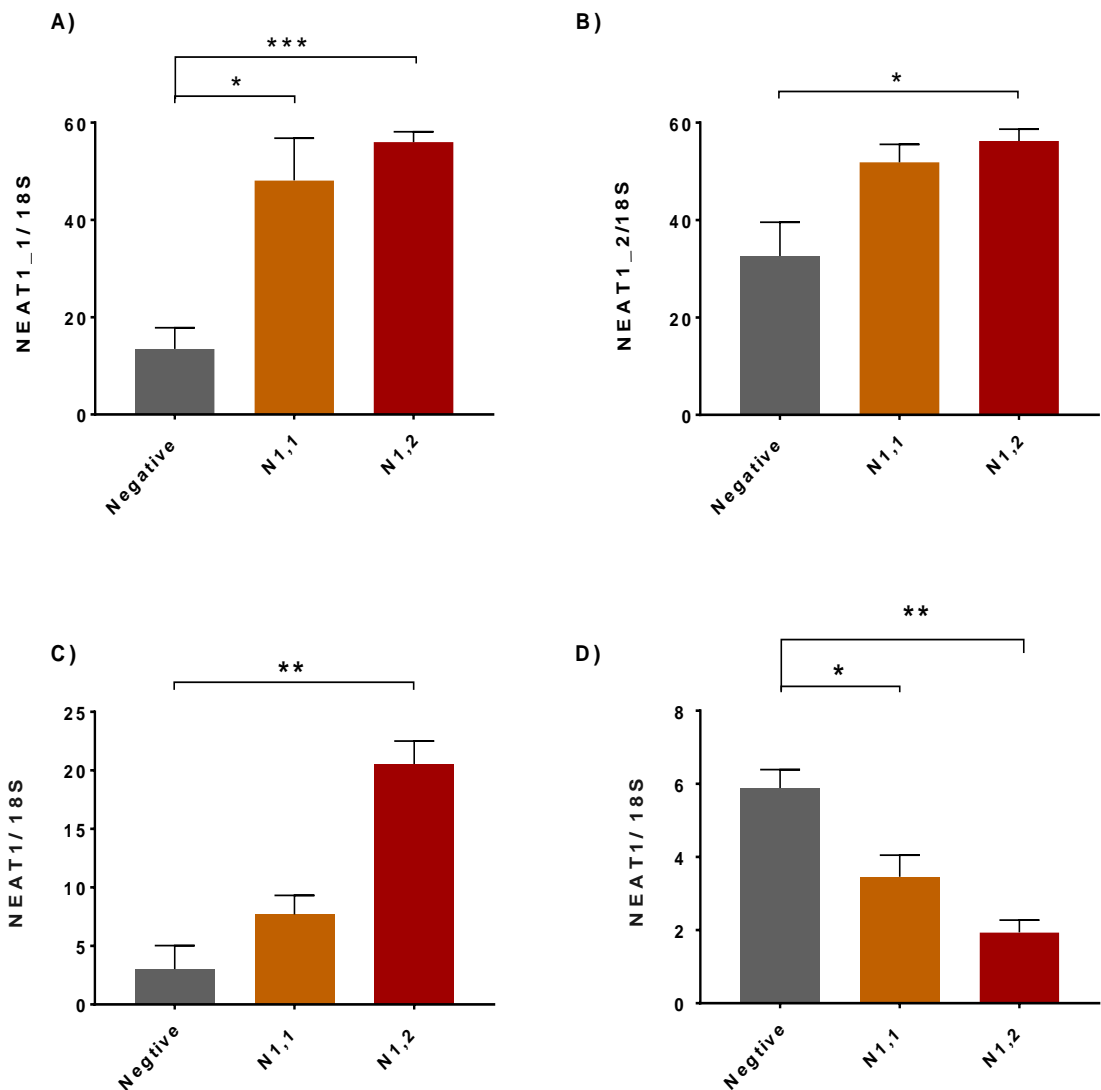


Figure 4.1 The effect of NEAT1 specific siRNAs on NEAT1 expression in MCF7 using cationic-lipid polymers mediated transfection. NEAT1 expression was determined by RT-PCR 72h post transfection with the negative siRNA or one of the NEAT1 siRNAs (N1, 1 and N1, 2). (A) There was a significant ($*P<0.05$ and $***P<0.001$; $n=4$) increase in NEAT1 expression in transfected cells respectively when use NEAT1 assay located on NEAT1_1. (B) Demonstrates the significant ($*P<0.05$; $n=4$) elevation in NEAT1 expression particularly in transfected cells with N1,2 siRNA, after using NEAT1 assay that located on NEAT1_2 only. (C and D) represent the nuclear and cytoplasmic NEAT1 expression respectively. Although, there was a significant ($**P<0.01$; $n=4$) up-regulation in nuclear NEAT1 expression particularly in cells transfected with N1,2 siRNA. There was a significant ($*P<0.05$ and $**P<0.01$; $n=4$) down-regulation in cytoplasmic NEAT1 expression in transfected cells respectively. Unpaired t test.

In the next set of experiments, the effects of elevated expression of nuclear NEAT1 on cell survival were investigated following the transfection of NEAT1 siRNAs using the cationic-lipid mediated transfection. The results showed that the decrease in the cytoplasmic and increase in the nuclear NEAT1 expression levels caused significant increase in the number of total and viable cells as displayed in Figure 4.2 A and B respectively. Such increase was associated with an enhancement in the long-term survival of these cells evidenced by an increase in their colony forming ability (Figure 4.2 C).

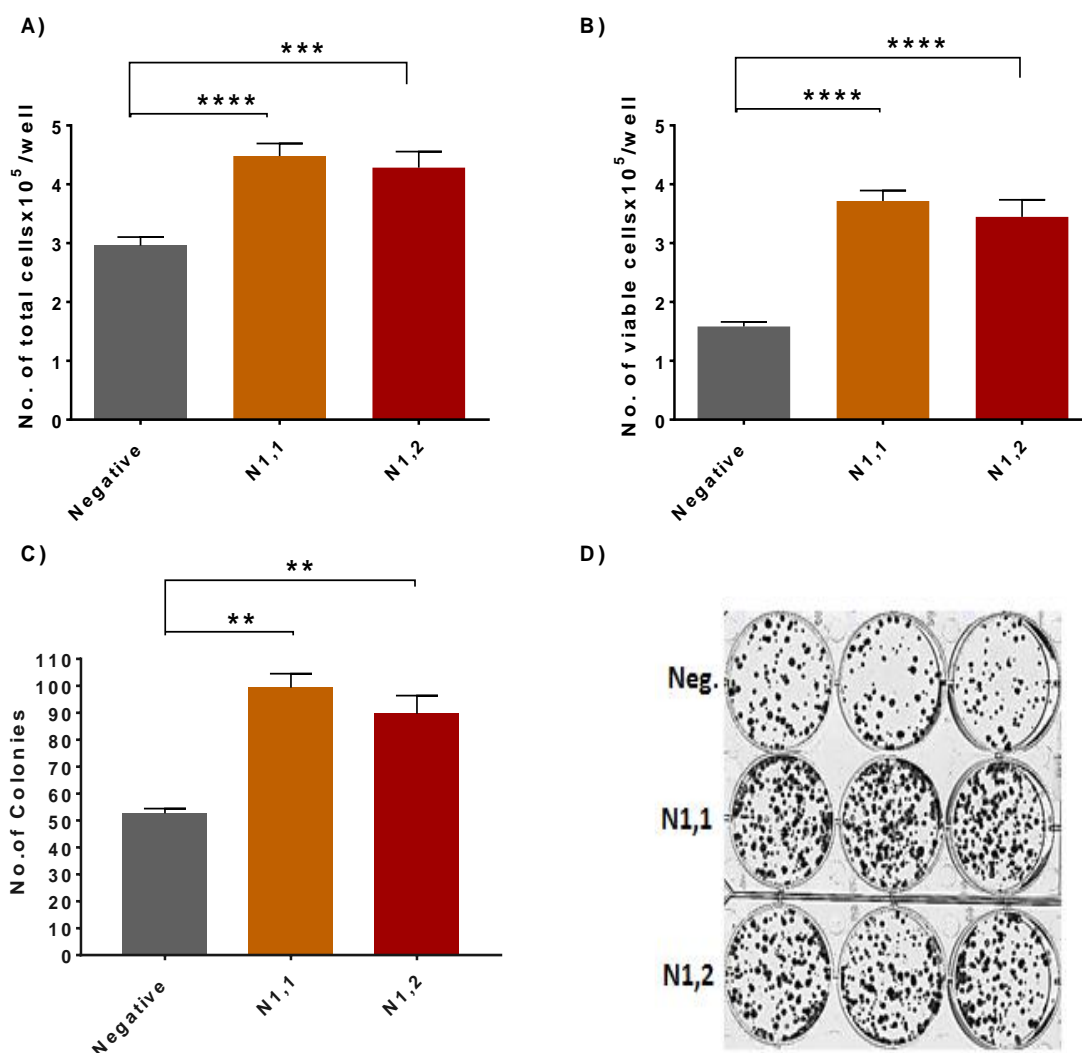


Figure 4.2 The effects of NEAT1 specific siRNAs on the basal cell survival of MCF7 cells using cationic-lipid polymer mediated transfection. MCF7 Cells were transfected with negative siRNA or one of the NEAT1 siRNAs (N1, 1 and N1, 2). Cells were harvested at 72 h post-transfection and re-plated for colony forming assay as well as for cell survival assessment after a further 24 h. Flow cytometry were used for determining the number of total and viable cells. (A and B) represent the number of total and viable MCF7 cells respectively. There was a highly significant ($****P < 0.0001$; $n=4$) increase in number of cells as compared with the negative control. (C) Shows the number of colonies formed in long-term clonogenic assays. A significant ($**P < 0.01$; $n=4$) elevation in the number of colonies compared with the negative control. (D) An example image of clonogenic assay plates after crystal violet staining. (Unpaired t-test)

Besides the elevation in number of total and viable cells, over- expression of nuclear NEAT1 transcripts lead to a significant reduction in the percentage of apoptotic cells in comparison with the negative control as presented in (Figure 4.3 A). Previous studies have shown that down-regulation of paraspeckles associated proteins also negatively affected NEAT1 expression, These proteins include HECT Domain E3 Ubiquitin Protein Ligase 3(HECTD3), RNA Binding Motif Protein 14(RBM14), Zinc Finger Protein 24(ZNF24), Non-POU Domain Containing Octamer Binding (NONO) and X-linked inhibitor of apoptosis protein (XIAP) (Fong et al, 2013). XIAP regulates apoptosis by inhibiting caspases (Eckelman et al, 2006), it was therefore necessary to investigate whether the increase in nuclear NEAT1 expression had an effect on the levels of XIAP leading to the decrease in the basal apoptosis level. Indeed, the results showed that increase in nuclear NEAT1 expression levels was associated with an up regulation of XIAP expression levels as in (Figure 4.3 B).

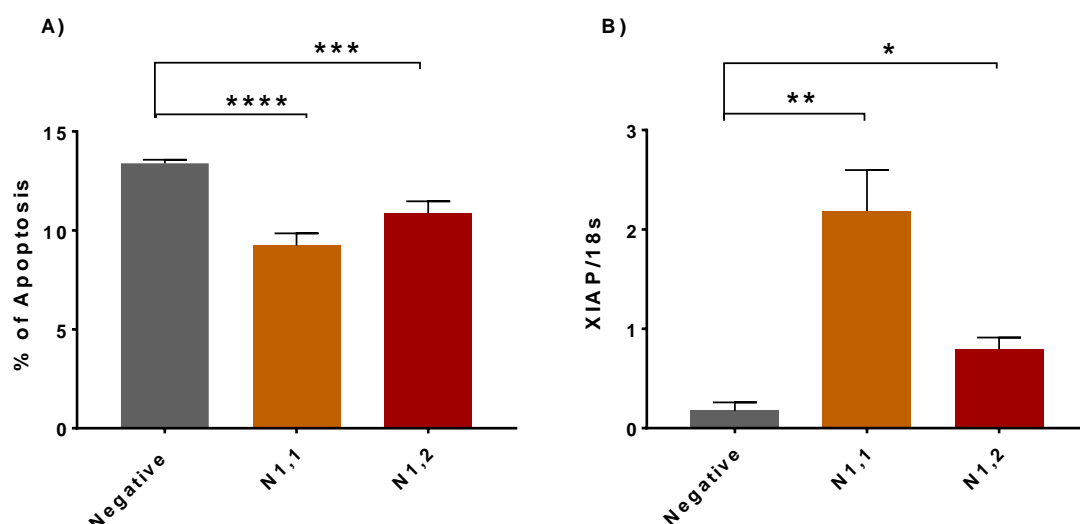


Figure 4.3 The effects of NEAT1 specific siRNAs on basal cellular apoptosis in MCF7 cells using Cationic lipid polymers-mediated transfection. MCF7 cells were transfected by HiPerFect transfection reagent using the negative siRNA or one of the NEAT1 siRNAs (N1, 1 and N1, 2). Cells were harvested at 72 h post-transfection and re-plated 24h for assessment of apoptosis. Muse cell analyser and Muse Annexin V and dead cell kit were used for determining the percentage of apoptosis. (A) Shows the apoptotic ratio, where there was a highly significant (****P<0.0001 and ***P<0.001; n=4) decrease in the percentage of apoptosis as compared with the negative control. (B) Represents the cellular expression levels of XIAP, in which there was a highly significant (**P<0.01, *P<0.05; n=4) increase in XIAP expression for those MCF7 cells transfected with N1, 1 and N1, 2 siRNAs respectively. Unpaired t-test

4.3.2 The effects of NEAT1 specific siRNAs on the survival of MCF7 breast cancer cells using nucleofection mediated delivery

One of the major limiting factors in successful targeted silencing of nuclear transcripts is the direct delivery of the siRNAs into the cell nucleus. Previous studies have shown that siRNA delivered by both liposome and cationic polymers is localised in the cytoplasm (Berezhna et al, 2006). It was therefore important to use another method of transfection in order to investigate whether the results described above were due to the lack of siRNA delivery to the nucleus. Nucleofection, an electroporation-based transfection method, uses a combination of electrical parameters with cell-type specific reagents enabling nucleic acid substrates delivery not only to the cytoplasm, but also through the nuclear membrane and into the nucleus (Aluigi et al, 2006). Therefore, using Nucleofection could result in a direct silencing effect on nuclear NEAT1 transcripts. Two types of NEAT1 siRNAs (N1a and N1c) were used targeting different sites on the short and long isoforms of NEAT1 (Table 2.2). MCF7 cells were nucleofected with NEAT1 specific siRNAs (N1a and N1c). In addition, the scrambled siRNA (negative siRNA) was used as control. The expression level of NEAT1 was determined at 48h post transfection using two different NEAT1 gene expression assays that can detect the short and the long isoforms of NEAT1. Results showed that nucleofection of MCF7 with both types of siRNA resulted in a significant NEAT1 down- regulation compared to control (Figure 4.4 A and B).

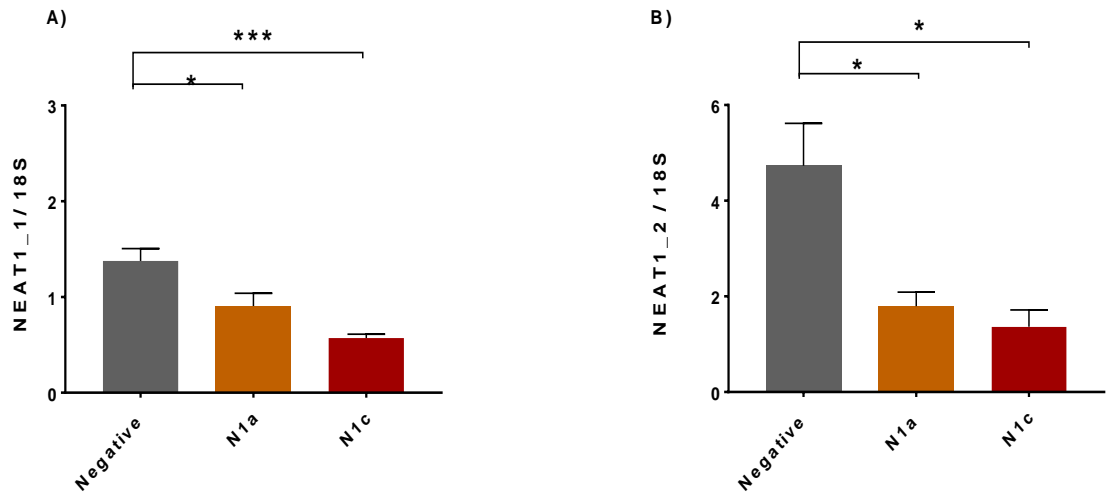


Figure 4.4 The expression levels of NEAT1 in transfected MCF7 cells with NEAT1 siRNAs using Nucleofection. Cellular NEAT1 levels were determined by RT-qPCR 48h post transfection with the negative siRNA or one of the NEAT1 siRNAs (N1a and N1c). (A) Represents the expression levels of cellular NEAT1 after using NEAT1 assay that located at the position 3310 (short isoform), where there was a significant (* $P < 0.05$ and *** $P < 0.001$; $n = 5$) downregulation in cells transfected with N1a and N1c siRNAs, respectively. (B) A graph showing the NEAT1 expression levels using NEAT1 assay that located at the position 7996 (long isoform). There was a significant (* $P < 0.05$; $n = 5$) down-regulation in cells transfected with N1a and N1c siRNAs. Unpaired t-test

NEAT1 silencing led to a significant reduction in the number of total and viable cells at 24h and cell viability at 48h of re-plating versus negative control (Figure 4.5 A, B and C). Down-regulation of NEAT1 expression levels also caused a decrease in long-term cell survival, which is illustrated by a significant reduction in the number of colonies formed as presented in Figure 4.5 D.

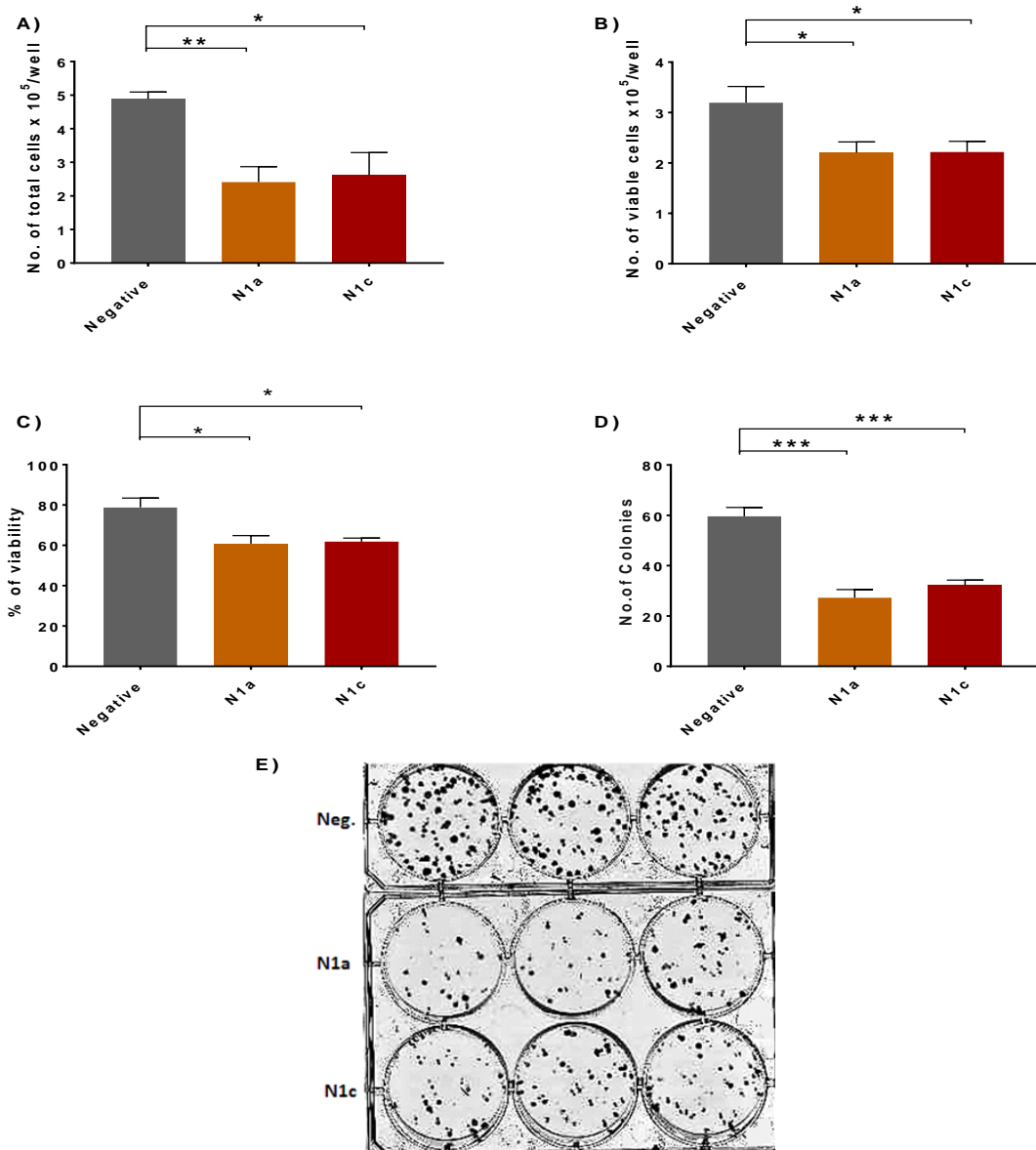


Figure 4.5 The effects of NEAT1 silencing on the short and long-term survival of MCF7 cells. MCF7 Cells were transfected with negative siRNA or one of the NEAT1 siRNAs (N1a and N1c). Cells were harvested at 48 h post-transfection, re-plated for colony forming assay and assessment of cell survival after a further 24 and 48h. Flow cytometry was used to determine the number of total and viable cells and the percentage of viability. (A and B) represent the number of total and viable MCF7 cells respectively at 24h re-plating, which showed a statistically significant decrease (** $P < 0.01$ and * $P < 0.05$; $n = 5$). (C) Shows a significant reduction in the percentage of viability at 48h of cell re-plating (* $P < 0.05$; $n = 5$). (D) The percentage of colonies formed in long-term Clonogenic assay, which is significantly decreased as compared to the negative control (*** $P < 0.001$ $n = 5$). (E) An example image of Clonogenic assay plates after crystal violet staining. Unpaired t-test

basal level of apoptosis was also assessed and it was found that NEAT1 down-regulation caused statistically insignificant elevation in the percentage of basal apoptosis after 48h of re- plating (Figure 4.6).

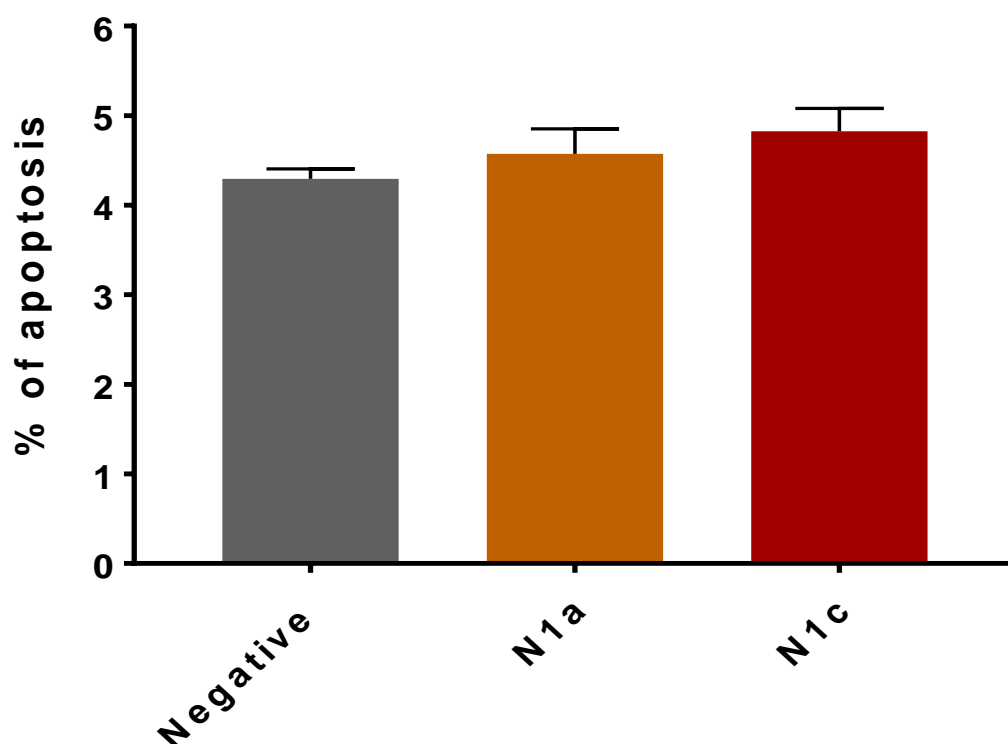


Figure 4.6 The effect of NEAT1 down-regulation on basal cell apoptosis in MCF7 cells. MCF7 Cells were transfected by nucleofection using the negative siRNA or one of the NEAT1 siRNAs (N1a and N1c). Cells were harvested 48 h post-transfection and re-plated for assessment of apoptosis after a further 24h (the data not present) and 48h. Muse cell analyser using the Muse Annexin V and dead cell kit measured the apoptosis level. There was insignificant elevation in the percentage of apoptosis after 48h of replating. Unpaired t-test.

Further experiments were carried out to investigate whether the growth suppression produced by NEAT1 down-regulation was due to apoptosis, to cell cycle arrest, or to both. A cell cycle analysis was performed using propidium iodide staining and flow cytometry. The results revealed that the proportion of cells in NEAT1 down-regulated cultures is consistently higher in G1 phase than that in the controls and the percentage of cells in S phase is consistently lower, suggesting that NEAT1 down-regulation might cause arrest the cells in G1 phase (Figure 4.7).

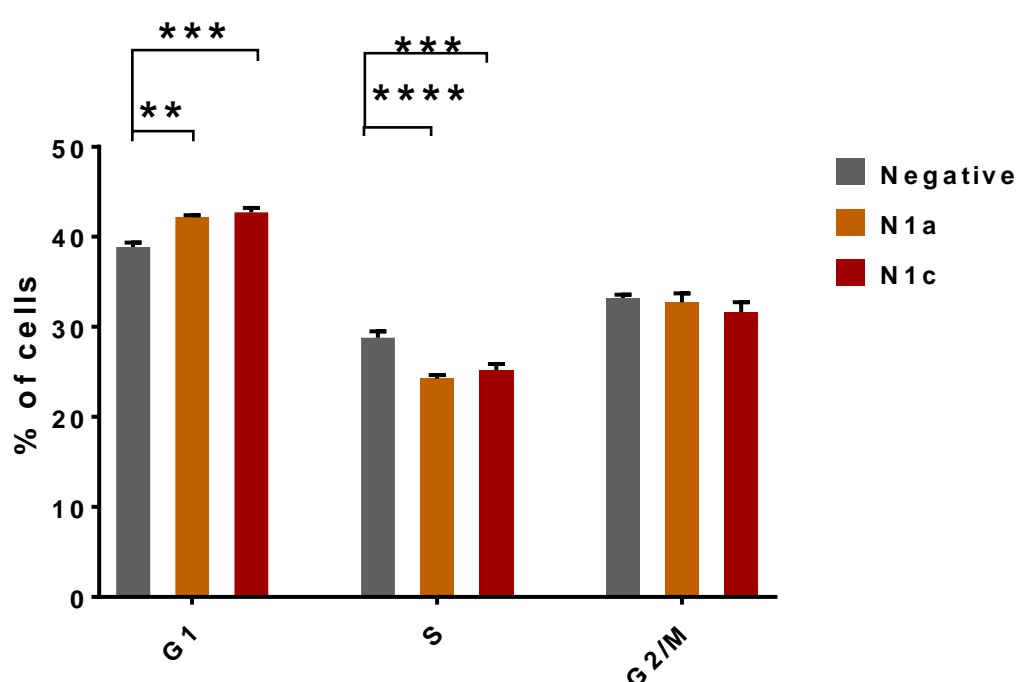


Figure 4.7 Effects of NEAT1 silencing on the cell cycle profile of MCF7 breast cancer cells. MCF7 cells were transfected with Negative siRNA or one of the NEAT1 siRNAs (N1a and N1c) that target the short and long NEAT1 isoforms. Cells were harvested 48h post transfection and re-plated for further 24h for cell cycle analysis. Cell cycle analysis involved quantifying DNA content by propidium iodide staining of fixed cells and fluorescence flow cytometry. There was a significant elevation in percentage of cells in G1 phase (** $P < 0.01$ $n=5$) and a highly significant reduction in the ratio of cells in S phase (** $P < 0.001$; $n=5$). Unpaired t-test

4.3.3 The effects of NEAT1 specific siRNAs on the triple negative MDA-MB-231 breast cancer cells

To examine the effects of NEAT1 silencing on the survival of the triple negative breast cancer cell line MDA-MB-231. MDA-MB-231 cells were nucleofected with NEAT1 specific siRNA (N1a and N1c). NEAT1 siRNAs significantly reduced NEAT1 transcript levels by up to one-third of control as in (figure 4.8). This decrease in NEAT1 levels was associated with a significant reduction in the number of total and viable cells (Figure 4.9) at 24 and 48h of re-plating respectively. Furthermore, the reduction in NEAT1 transcript levels was also associated with a decrease in the cellular long-term survival as shown by a significant reduction in the number of colonies formed (Figure 4.9E).

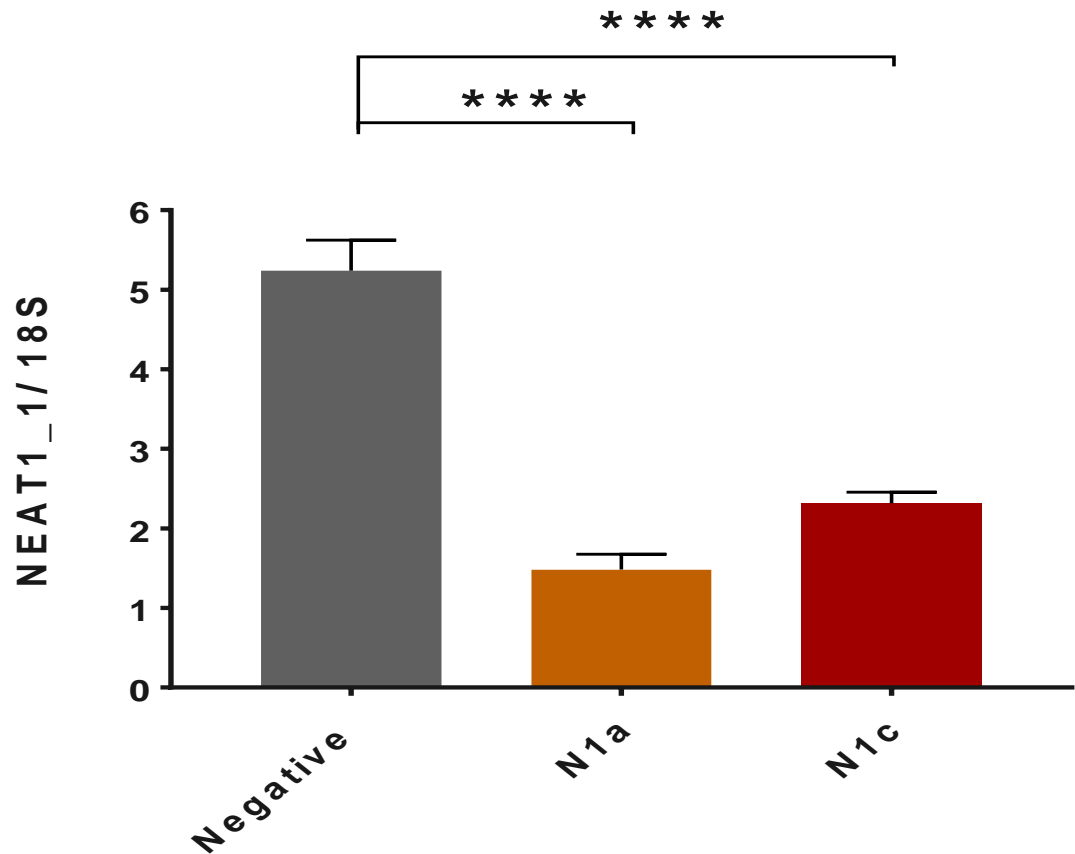


Figure 4.8 The effects of NEAT1 specific siRNAs on NEAT1 expression levels in MDA-MB-231 cells using Nucleofection as transfection method. Cellular NEAT1 levels were determined by RT-qPCR 48h post transfection with the negative siRNA or one of the NEAT1 siRNAs (N1a and N1c). The results of cellular NEAT1 expression after using NEAT1 assay that located at the position 3310 showed a highly significant down-regulation in the cells transfected with N1a and N1c siRNAs (**** $P < 0.0001$; $n = 4$). Unpaired t-test

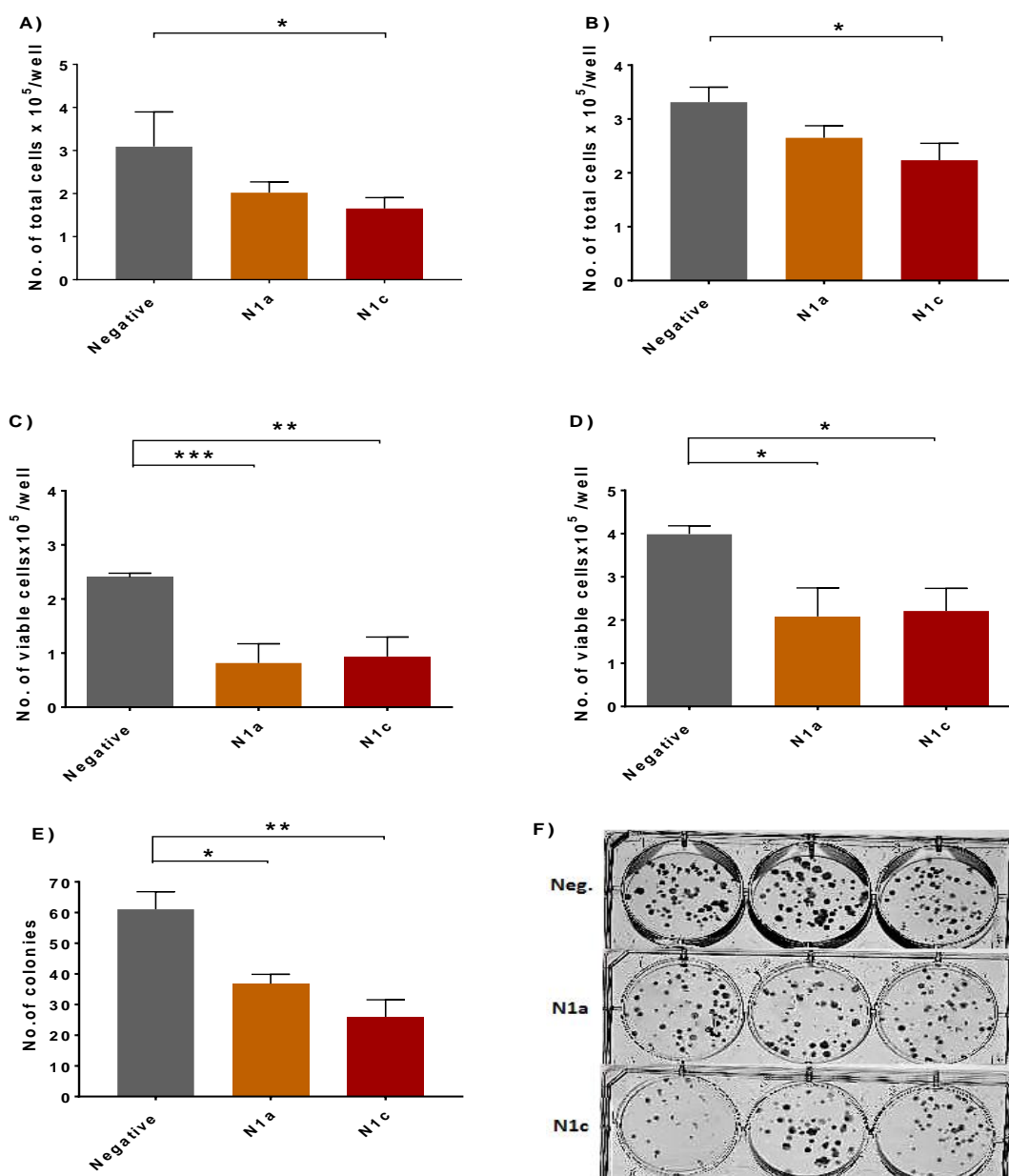


Figure 4.9 The effects of NEAT1 silencing on the basal survival of triple negative breast cancer cells MDA-MB-231. MDA-MB-231 Cells were transfected with negative siRNA or one of the NEAT1 siRNAs (N1a and N1c). The cells were harvested 48 h post-transfection and re-plated for colony forming assay and assessment of cell survival after a further 24 and 48h by flow cytometry. (A and B) represent the number of total MDA-MB-231 cells at 24 and 48h of re-plating respectively, in which there was a significant (* $P < 0.05$; $n = 4$) reduction in total cells. (C and D) reveal the significant (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ $n = 4$) reduction in number of viable MDA-MB-231 cells at 24 and 48h of re-plating respectively. (E) Number of colonies formed in long-term clonogenic assays (* $P < 0.05$ and ** $P < 0.01$ $n = 4$). (F) An example image of Clonogenic assay plates after crystal violet staining. Unpaired t-test

In spite of its effects on short term and long-term viability, silencing of NEAT1 expression levels had no effect on the basal apoptosis levels (results not shown). However, the reduction of NEAT1 expression levels affected the cell cycle profile of MDA-MB-231 and resulted in a significant increase in the percentage of cells in G1 phase and a concomitant decrease in cells in S and G2/M phases (Figure 4.10)

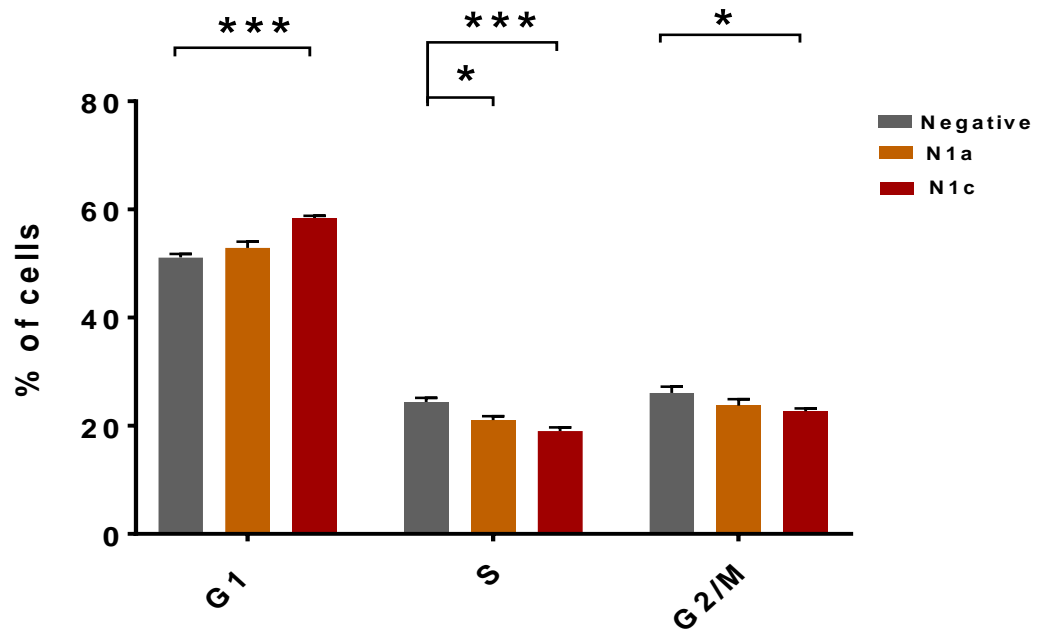


Figure 4.10 Effects of NEAT1 silencing on the cell cycle profile of MDA-MB 231 breast cancer cells. Cell cycle analysis was performed 48h post transfection and 24h post replating. Cell cycle analysis was performed by the propidium iodide staining of fixed cells and fluorescence flow cytometry. There was a highly significant (***) elevation in the percentage of cells in G₁ phase and reduction in cells in S phase (*P<0.05 and ***P<0.001 n=4) and G₂/M (*P<0.05). Unpaired t-test.

4.3.4 The effects of NEAT1 specific antisense oligonucleotides (ASOs) on the survival of breast cancer cells

In order to confirm the silencing effects on cell survival observed using NEAT1 specific siRNAs, further experiments were carried out using NEAT1 specific antisense oligonucleotides (ASOs). The mechanisms of ASO action in silencing of target RNA differ than the siRNA-mediated silencing (Watts & Corey, 2012). ASO is a single stranded that binds the target RNA to start its effect. While, siRNA is double stranded that needs further processing after association with RNA-induced silencing complex (RISC) to eliminate the passenger strand and keep the guide strand to bind to the complementary RNA target leading to its silencing (Figure 4.11) (Watts & Corey, 2012). In these experiments, the effects of NEAT1 specific ASOs on the silencing of NEAT1 and cell survival were investigated.

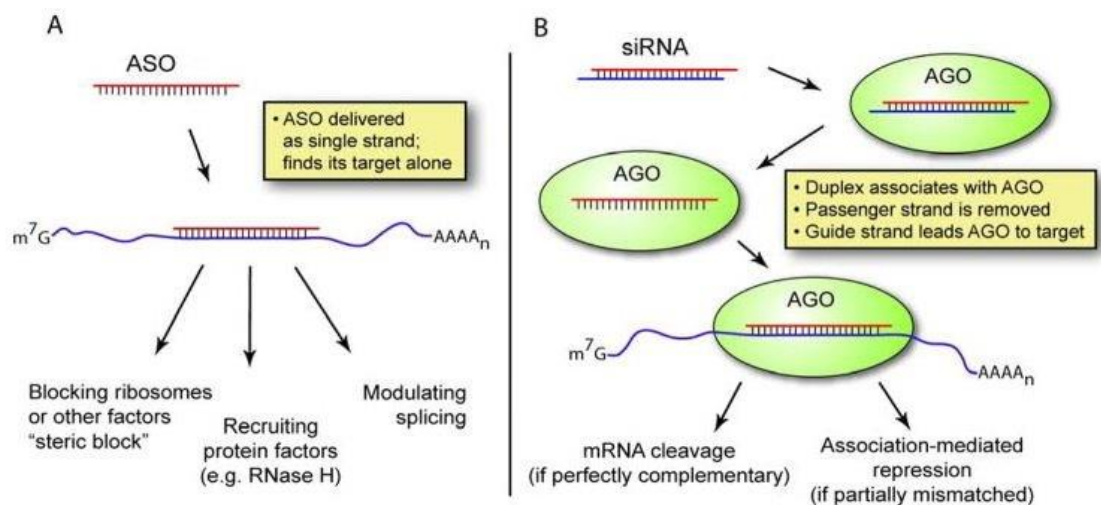


Figure 4.11 A diagram illustrating the comparison between ASO and siRNA mechanisms of action. (A) Represents the mechanisms of ASOs action delivered as a single-stranded oligonucleotide to bind to complimentary strand of their target RNA. (B) Shows the mechanism of siRNAs effects, which is delivered as duplex and taken up by Argonaute (AGO) part of the RNA-induced silencing complex (RISC). The diagram was adapted from Watts and Corey (2012).

MCF7 and MDA-MB231 cells were nucleofected with NEAT1 specific ASOs or a control ASO. NEAT1 transcript levels were determined using RT-PCR 48h post Nucleofection. Both NEAT1 specific ASOs caused significant down-regulation of NEAT1 transcript in MCF7 (Figure 4.12 A) and MDA-MB-231 (Figure 4.12 B) compared to the control. The expression of NEAT1 neighbouring gene, MALAT1 was also determined to find out if there is a correlation between the expressions of both genes. Indeed the results showed that NEAT1 down-regulation was associated with a decrease in MALAT1 expression in MCF7 and MDA-MB-231 cells (Figure 4.12 C and D). Such down-regulation in MCF7 cells was associated with a significant decrease in the number of total and viable cells at 24h of re-plating, but had no effects on long term survival (Figure 4.13). In MDA-MB-231, ASO mediated down-regulation of NEAT1 caused a significant decrease in the number of viable cells detected after 24h (Figure 4.14 A), an increase in the basal apoptosis level (Figure 4.14 B) and a decrease in the long term survival shown by a low number of colonies (Figure 4.14 C). The effects of NEAT1 silencing on breast cancer cell migration were also investigated. In comparison with the negative control, only MCF7 cells transfected with N1,2 ASO showed a significant inhibition of cell migration at 36h only (Figure 4.15 A). In MDA-MB231 cells, ASO mediated silencing caused a highly significant reduction in the rate of cell migration at 18h (Figure 4.15 B), no significant changes in this ratio after 36h of re-plating (results are not shown)

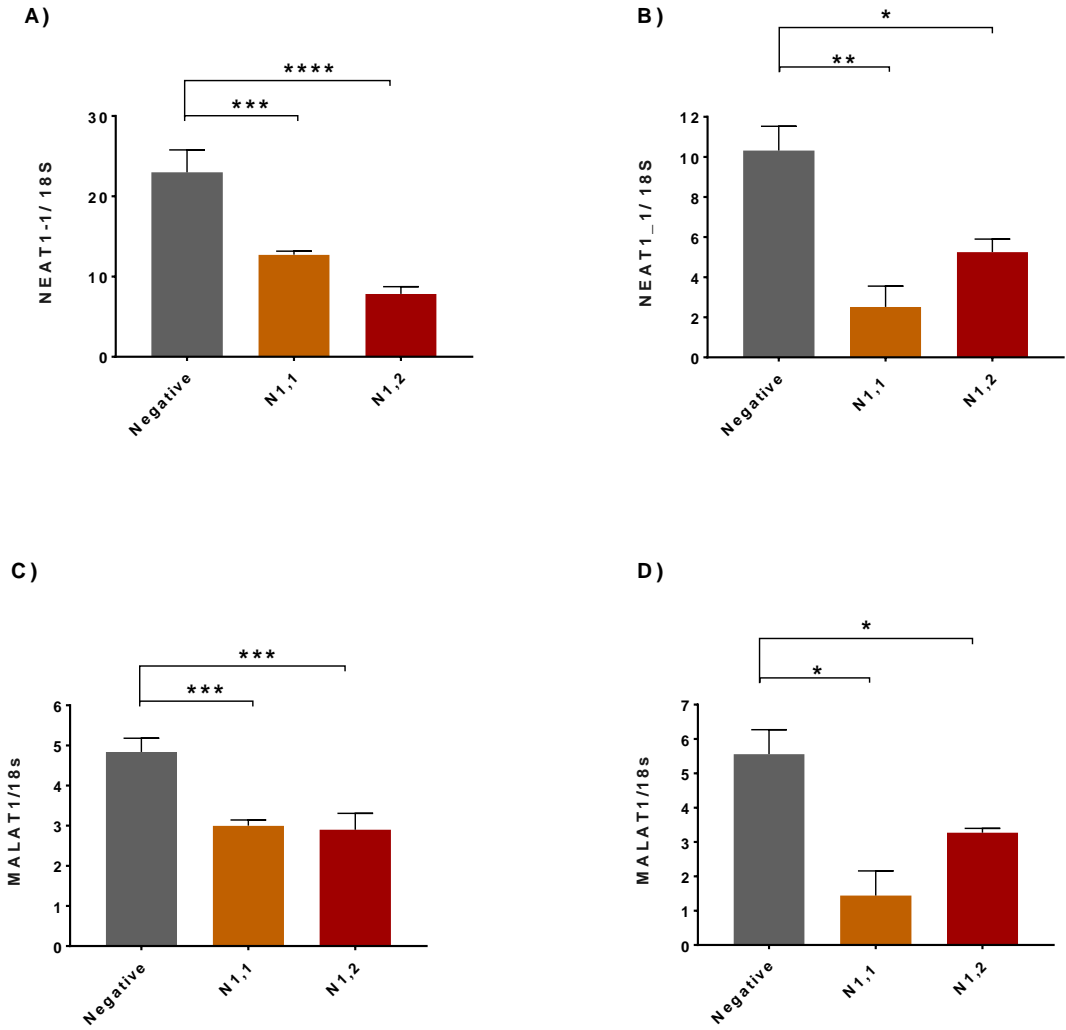


Figure 4.12 The effect of NEAT1 specific ASOs on NEAT1 and MALAT1 cellular expression in MCF7 and MDA-MB-231 cells. The expression levels were determined by RT-qPCR 48h post transfection with negative ASO or one of the NEAT1 ASO (N1,1 and N1,2). (A and B) The results of cellular NEAT1 expression after using NEAT1 assay that located at the position 3310 showed a highly significant and significant ($***P<0.001$ and $**P<0.01$; $n=4$) down-regulation in both MCF7 and MDA-MB-231 cells as compared with negative control. (C and D) represents the cellular MALAT1 expression in MCF7 and MDA-MB-231 transfect cells respectively. In which, there was highly significant ($**P<0.01$ and $*P<0.05$; $n=4$) down-regulation in their expression respectively as compared with negative control, this observation indicates a positive correlation in the expression of NEAT1 and MALAT1 transcripts. Unpaired t-test

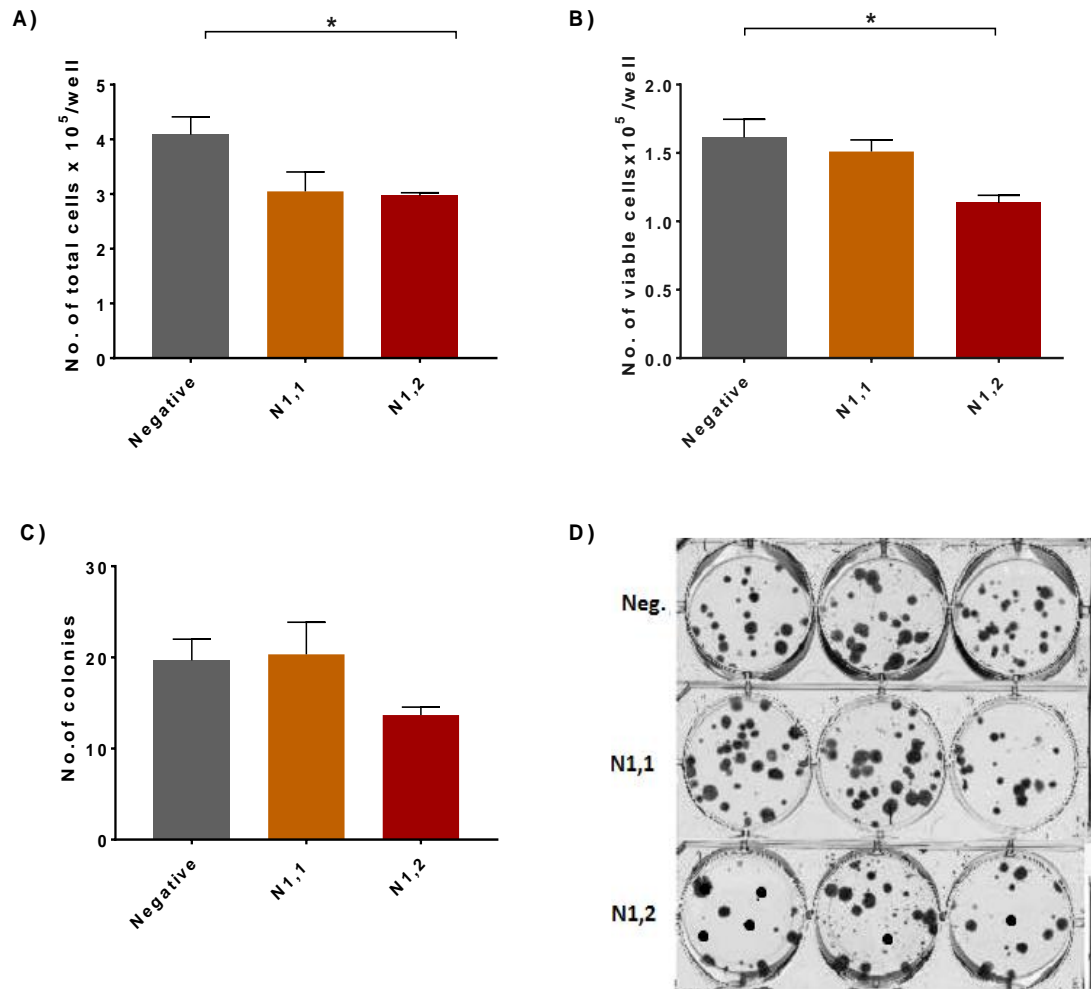


Figure 4.13 The effects of NEAT1 specific ASOs on the basal survival of MCF7 cells. MCF7 Cells were transfected with either control ASO (negative) or one of the NEAT1 ASOs (N1,1 and N 1,2). The cells were harvested 48 h post-transfection and re-plated for colony forming assay and assessment for cell survival after a further 24h. Muse cell analyser using Muse Count & Viability Assay Kit was used to determine the number of total and viable cells. (A and B) represent the number of total and viable MCF7 cells at 24h of re-plating respectively. There was significant (*P<0.05 n=4) decrease in the cell number. (C) Shows the number of colonies formed in long-term clonogenic assays, which revealed no significant changes in number of colonies formed as compared to the negative control. (E) An example image of clonogenic assay plates after crystal violet staining. Unpaired t-test.

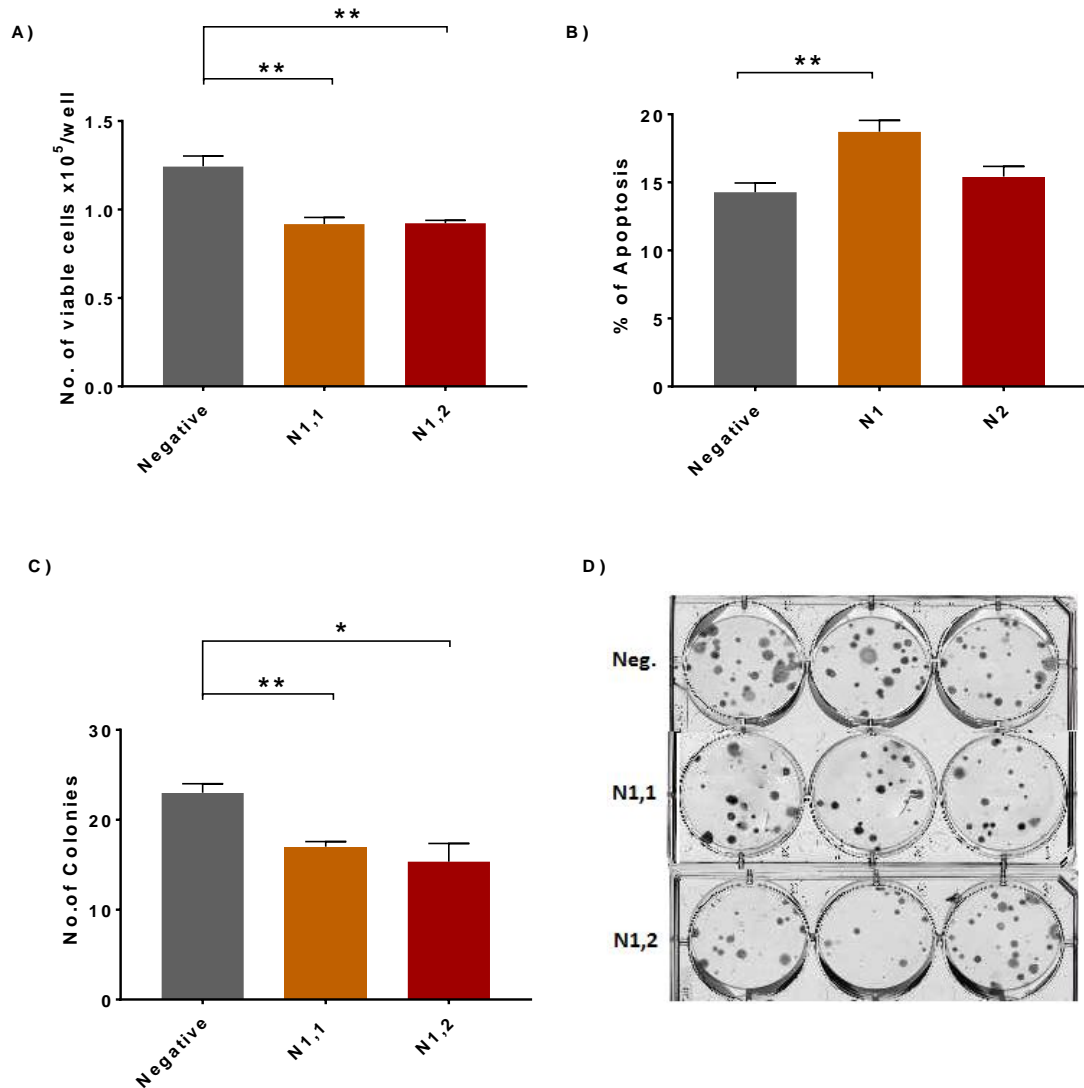


Figure 4.14 The effects of ASOs-mediated silencing of NEAT1 on the basal survival of MDA-MB-231 cells. MDA-MB231 cells were transfected with negative ASO or one of the NEAT1 ASOs (N1,1 and N1, 2). The cells were harvested 48 h post-transfection and re-plated for colony forming assay and assessment of cell survival after 24 and 48h. Viable cell number and the percentage of apoptosis were measured by flow cytometry. (A) Reveals the number of viable MDA-MB231 cells at 24h which shows a highly significant (** $P < 0.01$ $n=4$) decrease in the number of viable cells as compared with the negative control. (B) Shows the basal apoptosis levels. There was a highly significant (* $P < 0.05$; $n=4$) increase in the percentage of apoptosis particularly in N1,1 ASO transfect cells after 24h of cell re-plating. (C) Number of colonies formed in long-term clonogenic assay, which revealed a highly significant (** $P < 0.01$) decrease in number of colonies formed as compared with the negative control. (D) An example image of clonogenic assay plates after crystal violet staining. (Unpaired t-test).

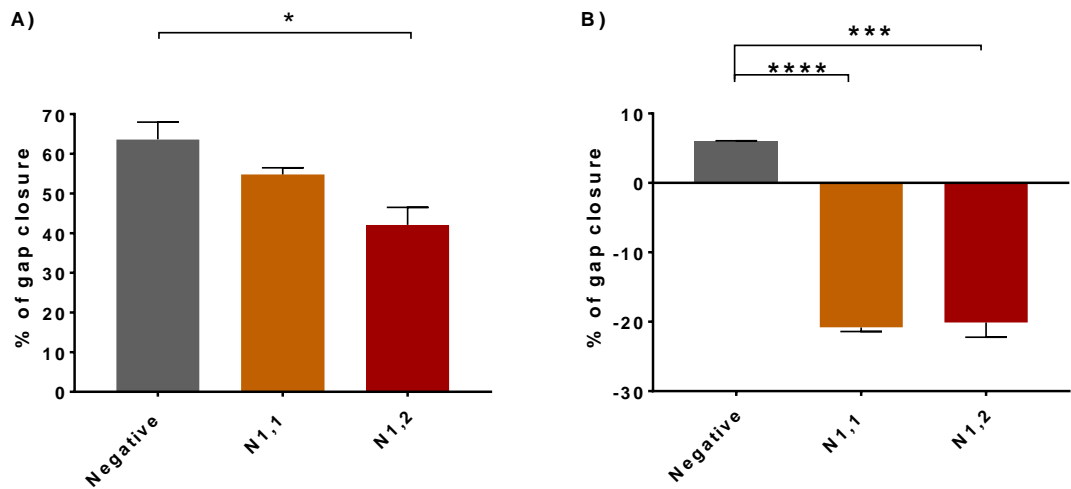


Figure 4.15 The effects of NEAT1 specific ASO on the migration ability of MCF7 and MDA-MB-231 breast cancer cells. The cells were transfected with negative ASO or one of the NEAT1 ASO (N1, 1 and N1, 2). Cells were harvested 48h post transfection and re-plated for further 24h to be 80% -90% confluence. Then a scratch line was done by using 10 μ l pipette tips. The cell migration was determined by measuring the gap distance of the scratch line under the light microscope at 0 time, 18 and 36h intervals. (A) Represents the gap closure ratio of MCF7 cells. In which, there was a significant (* $P < 0.05$; $n = 3$) decrease at 36h of cell re-plating as compared to the negative control. while (B) shows the gap closure ratio of MDA-MB-231 cells after 18h of cell re-plating, which was significantly (**** $P < 0.0001$ $n = 4$) decrease as compared to the negative control. Unpaired t-test

4.3.5 The effects of NEAT1 down-regulation on breast cancer cells response to UV and chemotherapeutic drugs

The influence of NEAT1 silencing on MCF7 and MDA-MB-231 survival was examined under the effect of cell death stimuli. Ultraviolet (UV) light exposure has been one of the major inducers of apoptosis. UV exposure causes pyrimidine dimers and DNA fragmentation leading to cell cycle arrest and induction of apoptosis (Dunkern& Kaina, 2002). The effects of NEAT1 silencing on UV-induced cell death in breast cancer cells were investigated In MCF7 and MDA-MB-231 cells. Reduced NEAT1 transcript levels enhanced UV-induced apoptosis (Figure 4.16 B, Figure 4.17 B) respectively and increase the rate of growth inhibition as well as decrease in long term survival (Figure 4.16 A and C, Figure 4.17 A and C) respectively. LncRNAs has been reported to affect gene expression and their effects can occur in cis (on neighbouring genes) or in trans (on distantly located genes) (Vance and Ponting, 2014). One of the genes located on Chromosome 11 and on the same cytogenetic band, q13.1 is BCL2 Associated Agonist of Cell Death (BAD). BAD is a pro-apoptotic member of the Bcl-2 family. Its effects are mediated by its ability to heterodimerize with survival proteins such as Bcl-XL leading to the promotion of cell death. The possibility that the increase in cell death and inhibition of cell survival induced by UV in NEAT1 silenced cells might be due to the change of BAD expression levels, which was investigated. NEAT1 silencing enhanced UV- induced cell death and this response was associated with a significant increase in the expression levels of BAD (BCL2-Associated Agonist of Cell Death). The results showed that the enhancement of UV-induced cells in NEAT1 silenced cells was associated with a significant increase in the expression levels of BAD (Figure 4.18 A and B) respectively.

Further experiments were carried out to investigate the effects of NEAT1 silencing on the response of breast cancer cells to chemotherapeutic drugs. Accordingly, the effects of NEAT1 silencing on the response of MCF7 and MDA-MB-231 to Docetaxel, 5Fluorouracil (5-FU), Nutlin-3a and Mitoxantrone were examined at 48h of treatment. Control cells contained 0.25% dimethyl sulphoxide as a vehicle. In MCF7 cells, decreased NEAT1 transcript levels enhanced the growth inhibition induced by Docetaxel , 5-FU and Nutlin-3a (Figure 4.19) and had no effects on Mitoxantrone induced cell death (result not shown). In MDA-MB 231 breast cancer cells, NEAT1 silencing enhanced the cells response to Docetaxel, 5-FU, Nutlin-3a and Mitoxantrone (Figure 4.20)

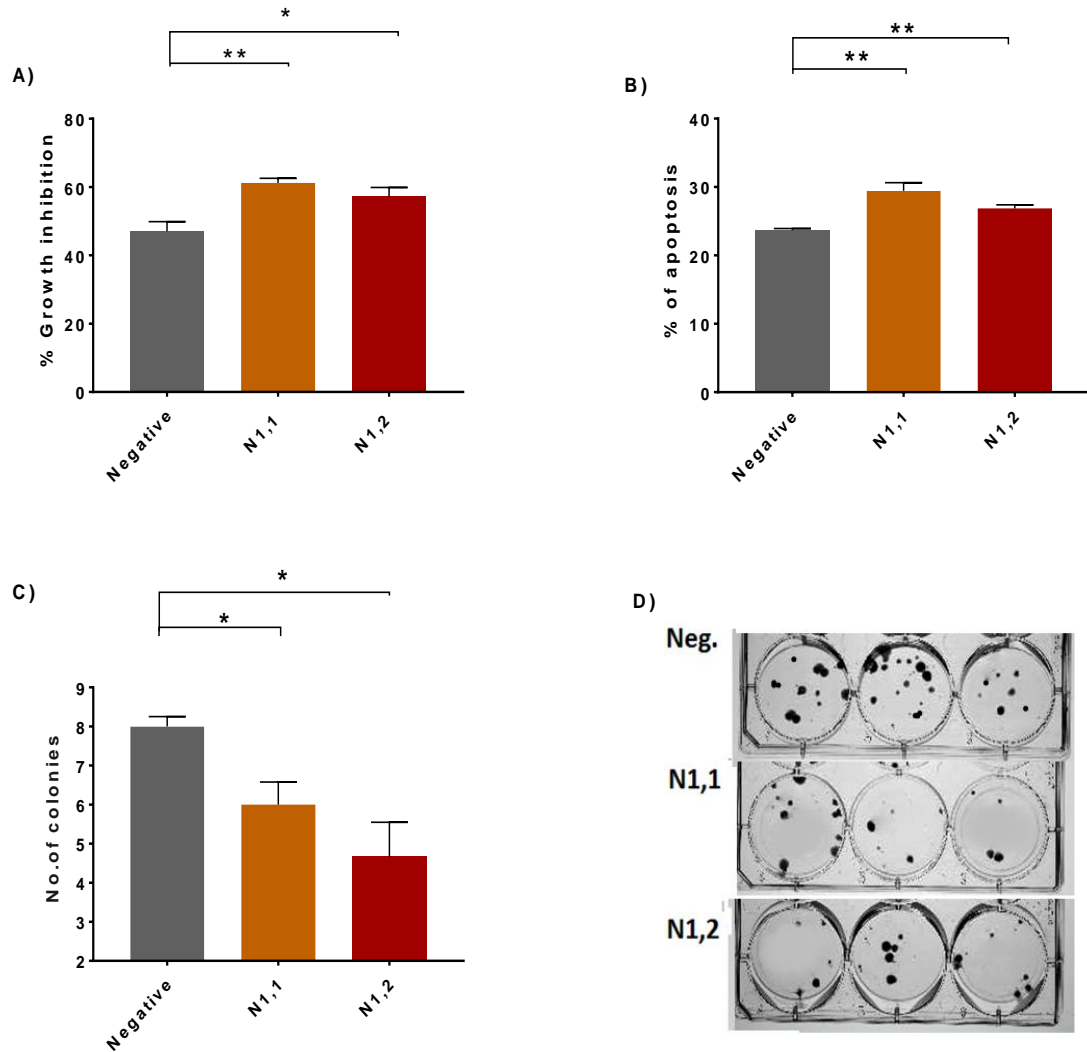


Figure 4.16 The effects of NEAT1 specific ASOs on UV-induced cell death MCF7 cells. Cells were transfected by nucleofection using the negative ASO or one of the NEAT1 ASOs (N1,1 and N1,2). Cells were harvested 48 h post-transfection and irradiated with UV-C light for 20s, then re-plated at density of 2×10^5 cell/well for cell function assessment. The growth inhibition ratio calculated according to an equation $\% \text{ Decrease} = (\text{basal state} - \text{after exposing to UV-C}) \div \text{basal state} \times 100$ (A) Represents the ratio of growth inhibition after exposing to UV irradiation, which was highly significant (** $P < 0.01$ and * $P < 0.05$; $n = 4$). (B) shows the highly significant (** $P < 0.01$; $n = 4$) increase in ratio of apoptosis level using Muse cell analyser and Muse cell Annexin V and dead cell kit. (C) Reveals the effects of UV-C irradiation on the long-term cell survival. There was significant (* $P < 0.05$; $n = 4$) decrease in number of colonies. (D) An example image of clonogenic assay plates after crystal violet staining. Unpaired t-test

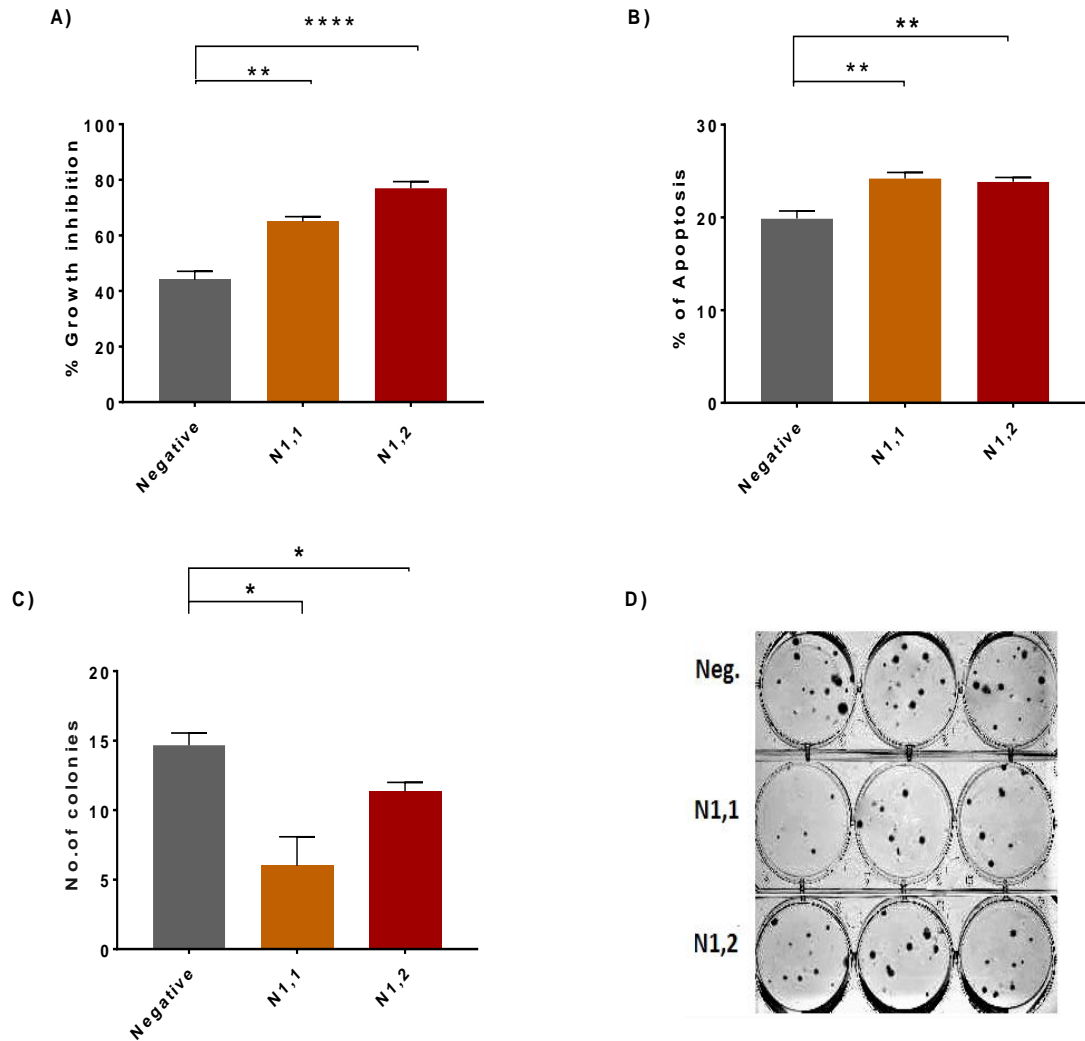


Figure 4.17 The effects of NEAT1 specific ASOs on UV-induced cell death of MDA-MB-231. Cells were transfected by nucleofection using the negative ASO or one of the NEAT1 ASOs (N1,1 and N1, 2). Cells were harvested 48 h post-transfection and irradiated with UV-C light for 20 s, then re-plated at density of 2×10^5 cell/well for cell function assessment. The growth inhibition ratio calculated according to an equation $\% \text{ Decrease} = (\text{basal state} - \text{after exposing to UV-C}) \div \text{basal state} \times 100$. (A) Represents the ratio of growth inhibition after exposing to UV-C irradiation, which was highly significant (** $P < 0.01$ and *** $P < 0.001$; $n=4$). (B) Shows the highly significant (** $P < 0.01$; $n=4$) increase in ratio of apoptosis levels using Muse cell analyser and Muse cell analyser specific kit for Annexin V and dead cell. (C) Revealed the effects of UV-C irradiation on the long-term cell survival, there was significant (* $P < 0.05$; $n=4$) decrease in number of colonies. (D) An example image of clonogenic assay plates after crystal violet staining. Unpaired t-test

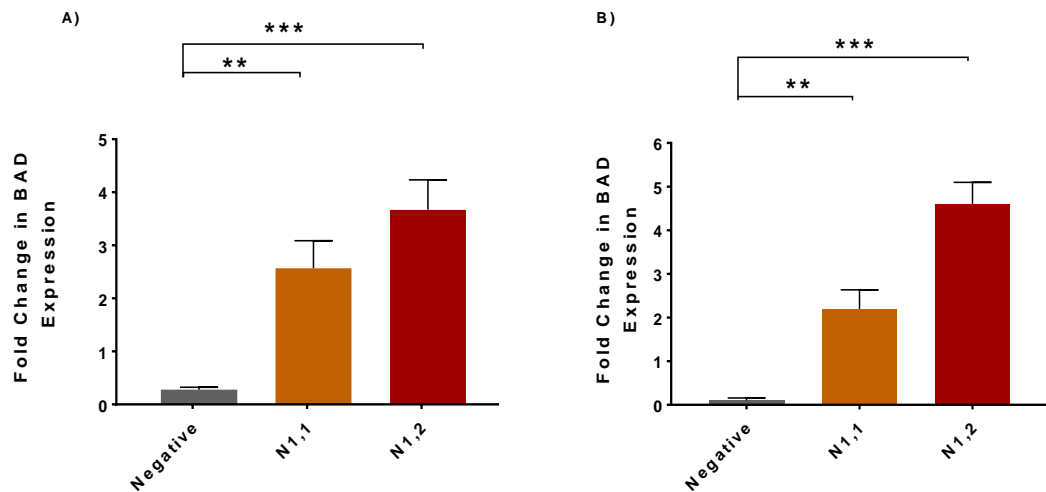


Figure 4.18 Effects of NEAT1 down-regulation on BAD expression levels following UV-C irradiation. Cells were transfected with either negative ASO or one of the NEAT1 ASOs (N1,1 and N1, 2) . 48h post transfection, the cells were exposed to UV-C irradiation for 20s and incubated at 37°C and 5% CO₂ for 48h. Bad expression levels were determined by RT-qPCR. Log of base 2 were used to assess the fold of expression change in comparison to the expression in the basal status. Meanwhile, the significance in the fold change of the expression was determined according to the negative control. (A) Shows the fold increase in BAD expression following UV exposure in MCF7 cells transfected with NEAT1 ASOs , which was highly significant (**P<0.01 and ***P<0.001; n=4) in comparison to the negative control. (B) Revealed the fold increase in BAD expression in MDA-MB-231 cells transfected with NEAT1 ASOs following UV exposure, which was highly significant (**P<0.01 and ***P<0.001;n=4) increase in comparison to negative control. Unpaired t-test

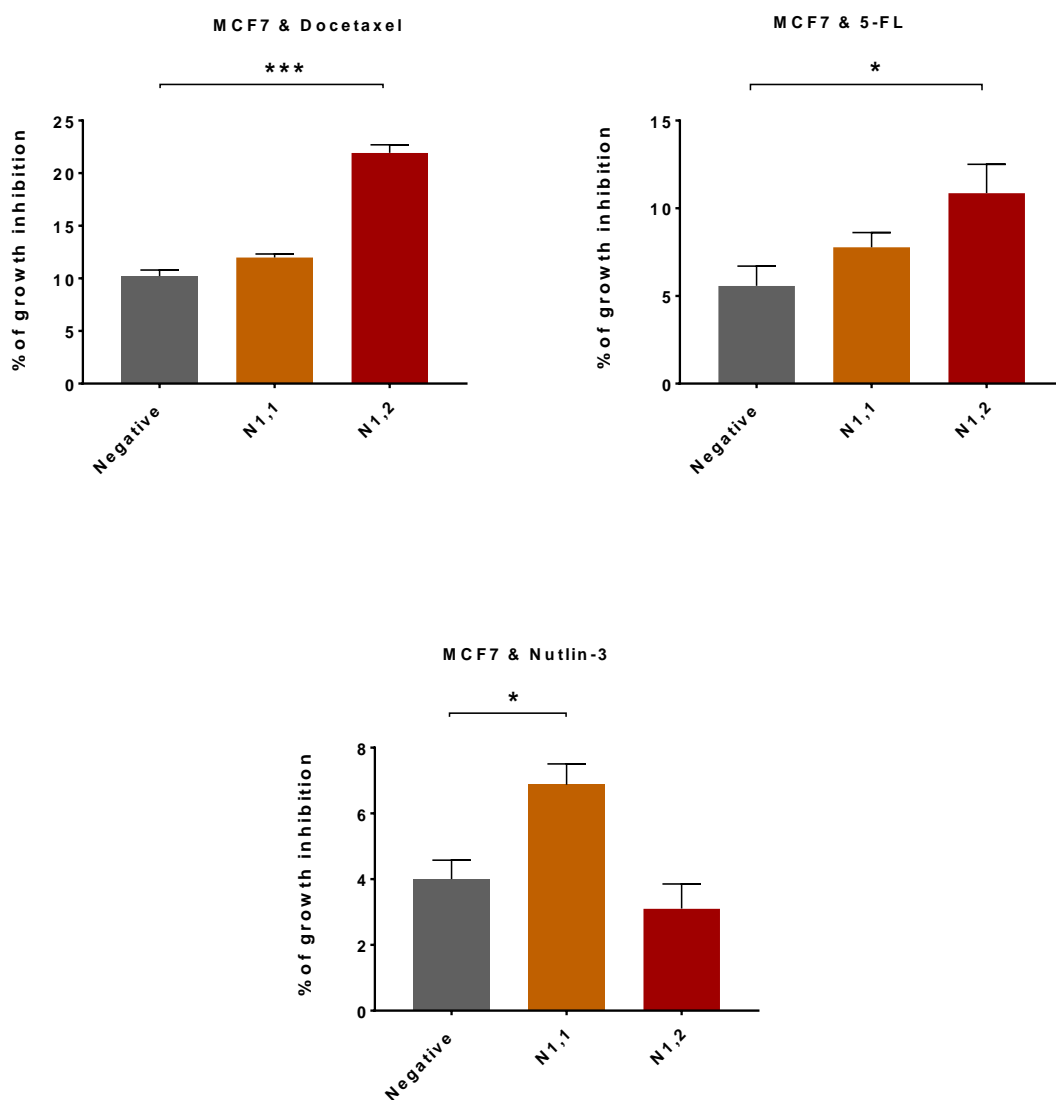


Figure 4.19 Effects of NEAT1 silencing on chemotherapeutic drug-induced death of MCF7 breast cancer cells. Cells were transfected with either negative ASO or one of the NEAT1 ASOs (N1,1 and N1, 2). Cells were harvested 48 h post-transfection and a density of 0.8×10^5 cell/ml were cultured for minimum 20h in 96 well plate before treating them with Docetaxel ($10\mu\text{M}$), 5-FU ($175\mu\text{M}$), Nutlin-3a ($2.5\mu\text{M}$) and Mitoxantrone ($50\mu\text{M}$) or vehicle (0.25% DMSO). Cell viability was assessed by MTS assay after 48h of incubation. The results were represented as the percentage of cell growth inhibition versus to control. There was significant cytotoxic effect of chemotherapeutic drugs in transfected cells (** $P < 0.001$, * $P < 0.05$, $n=4$). Unpaired t-test.

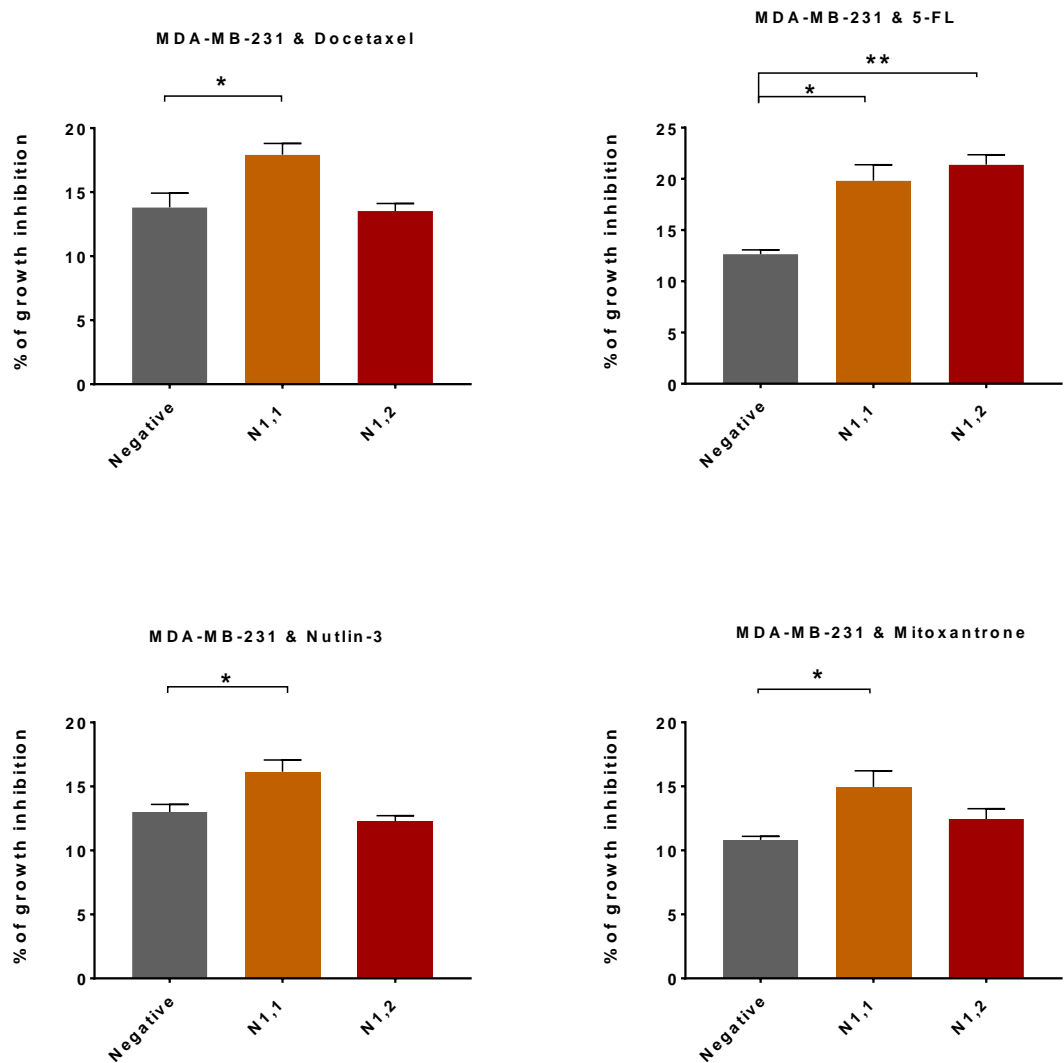


Figure 4.20 Effects of NEAT1 silencing on chemotherapeutic drug-induced death of MDA-MB-231 breast cancer cells. Cells were transfected with either negative ASO or one of the NEAT1 ASOs (N1,1 and N1, 2). Cells were harvested 48 h post-transfection and a density of 0.8×10^5 cell/ml were cultured for minimum 20h in 96 well plate before treating them with Docetaxel (5 μ M), 5- FU (100 μ M), Nutlin-3a (5 μ M) and Mitoxantrone (50 μ M) or vehicle (25% dimethyl sulphoxide). Cell viability was assessed by MTS assay after 48h of incubation. The results were represented as the percentage of cell growth inhibition versus to control. There was significant cytotoxic effect of chemotherapeutic drugs in transfected cells (**P<0.01, *P<0.05, n=4). Unpaired t-test.

4.4 Discussion

Targeted therapies development has transformed the treatment of some breast cancer subtypes mainly the hormone receptor-positive and human epidermal growth factor receptor 2 (HER2)-positive breast cancer. However, the problems of resistance to these therapies still exist (Barrios et al, 2009; Brown et al, 2004, Chumakova et al, 2006). Besides, approved targeted therapy for TNBC subtype, which lacks oestrogen receptor, progesterone receptor and HER2 amplification does not currently exist. This subtype is often highly malignant and shows a strong metastatic behaviour and high risk of relapse. Consequently, finding the best chemotherapeutic drugs, which leads to longer metastasis free and increase the overall survival is a real necessity (O'Reilly et al, 2015). It is therefore important to identify novel therapeutic targets for multiple breast cancer subtypes. An increasing number of evidence suggests that lncRNAs regulate many fundamental biological processes and therefore may offer new opportunities in developing new diagnostic tools and therapeutic approaches to treat the different subtypes of breast cancer (Paralkar and Weiss, 2013; Rasool et al, 2016). Indeed, NEAT1 is already of particular interest in relation to breast cancer, since its expression is up-regulated in tumour tissues (Qian et al, 2016; Choudhry et al, 2015). Current findings in the hormone receptor-positive and HER2-positive breast cancer cells (MCF7) and TNBC cells (MDA-MB-231) demonstrates here that NEAT1 plays an important role in regulation the survival of both types of breast cancer cells. Up-regulation of NEAT1 promoted short and long-term survival and inhibited apoptosis in MCF7 cells, whereas the decrease in its expression levels was associated with the decrease of cell survival and migration in both types of cells. The present study also confirms that a decrease in NEAT1 expression levels is associated with a loss of

short term and long term viability. The study also demonstrates, for the first time, the presence of a cytoplasmic NEAT1 transcript beside to the expression of NEAT1 nuclear transcript.

In this study, siRNA mediated down-regulation of NEAT1 revealed that NEAT1 is required for the survival of breast cancer cells. These results are in agreement with other studies which demonstrated the role of NEAT1 lncRNA in promoting cell proliferation in different types of cancer such as gastric, colorectal, lung, oesophageal, hepatocellular and breast cancer (Ma et al, 2016; Xiong et al, 2017; Yu et al, 2017; Ke et al, 2016; Peng et al, 2016). siRNA mediated silencing of NEAT1 also affected the cell cycle via promoting cell arrest in G0/G1 phase and preventing their progression to the S phase. Therefore, this is resulted in a noticeable regression in cell proliferation, which was interpreted by a decrease in both short and long term cell survival. These results are in agreement with other studies which reported the role of NEAT1 lncRNA in regulation of cell cycle particularly at G0/G1 and S-phase cells (Li et al, 2016; Wang et al, 2016). For instance, Wang et al. (2016) reported a regulatory loop of NEAT1/miR-107/CDK6 that stimulates cell proliferation in laryngeal squamous cell carcinoma (Wang et al, 2016). miR-107 related to the miR-103/107 family that act as a tumour suppressor in different cancer types (Datta et al, 2012), and CDK6 is a cell cycle regulator protein that responsible for the transition phase at G1/S. Therefore, NEAT1 down-regulation might result in an up-regulation in miR-107, which lead to a decrease in the levels of CDK6 protein and hence increase in the number of arresting cells in G1 phase (Wang et al, 2016). Ke et al. (2016) have also reported similar results, where down-regulation of NEAT1 expression in the same cell lines (MCF-7 and MDA-MB-231), inhibited cell growth and induced cell apoptosis. The study reported an interaction between NEAT1, miR-548ar-3p and the RNA-binding protein fused in sarcoma/translocated in

liposarcoma (FUS/TLS). They found that FUS/TLS physically interacts with NEAT1 forming a complex, which is important for maintaining the survival of breast cancer cells (Ke et al, 2016). The study also reported a regulatory access between miR-548ar and NEAT1. The increase in miR-548ar-3p expression was able to decrease NEAT1 expression and promote apoptosis. Recent studies have also demonstrated the role of NEAT1 in hepatocellular carcinoma (HCC) (Mang et al, 2017). The study reported an elevation of NEAT1 transcript levels in HCC tissues compared with non-cancerous liver tissues. Silencing of NEAT1 reduced HCC cell proliferation, invasion and migration (Mang et al, 2017). The study also indicated that NEAT1 regulated Heterogeneous Nuclear Ribonucleoprotein A2 (hnRNP A2) expression. Down-regulation of NEAT1 resulted decrease in the expression levels of hnRNP A2 and over-expression of hnRNP A2 rescued the proliferation and invasion of HCC cells that expressing low levels of NEAT1 (Mang et al, 2017).

An increasing number of evidence confirms the effects of NEAT1 down-regulation on promoting apoptosis and inhibition the cell growth (Lo et al, 2016, Ke et al, 2016, Mang et al, 2017). The study presented here showed that reduced expression levels of NEAT1 leads to a decrease in cell survival and insignificant increase in the basal apoptosis levels. It is highly likely that the time point used to determine the apoptosis levels was not appropriate where all the apoptotic cells were disintegrated. Apoptosis levels were measured by Annexin V staining. This method depends on the interaction of Annexin V, a calcium-dependent phospholipid binding protein, with a phosphatidylserine (PS) translocated from the internal to the external cell membrane during the early stages of apoptosis (Muse TM user's guide, # MCH100105; Walton et al, 1997). Accordingly, there might be certain factors affecting the translocation of phosphatidylserine in the outer part of the cell membrane such as the incubation period of transfected cells (Schuffner et al, 2002).

The variation in the levels of flippase activity, an aminophospholipid translocases responsible for translocate PS from the exoplasmic to the cytoplasmic faces of the plasma membrane and the concentration of intracellular calcium, might have also affected the results. The flippase and calcium levels are responsible for regulating the PS externalization in relation to the type of cancer cells where cell lines that show high PS in the outer membrane are characterised by low flippase activity and high intracellular calcium and vice versa (Vallabhapurapu et al, 2015). MDA-MB-231 breast cancer cells are characterised by low PS in the outer membrane because of high flippase activity and low concentration of intracellular calcium (Vallabhapurapu et al, 2015) and therefore this might explain why the apoptosis level was not affected by the down-regulation of NEAT1 expression levels.

The effects of siRNA mediated silencing of NEAT1 were also confirmed using NEAT1 specific modified ASOs. Specific NEAT1 antisense DNA and RNA phosphorothioate-modified oligonucleotides containing selective phosphorothioate backbone modifications and 2' O-methyl RNA bases were transfected into breast cancer cells. Studies have shown that chemical modifications of ASOs improve potency and selectivity by increasing binding affinity of oligonucleotides for their complementary sequences. NEAT1 specific ASOs were effective in silencing the endogenous levels of NEAT1 in both MCF7 and MDA-MB-231 cells. In MCF7 cells, ASO-mediated silencing inhibited short-term survival and cell migration but had no significant effects on long-term survival. ASO-mediated silencing of NEAT1 in MDA-MB-231 resulted in an increase in basal apoptosis levels and inhibited both short and long-term viability. Although both ASOs reduced endogenous levels of NEAT1, the functional effects of both ASOs were not always consistent and were short-lived. It is possible that higher concentration of ASOs is required in order to produce the effects for a longer period (Watts and Corey et al, 2012; Bertrand et al, 2002). The

effects of NEAT1 silencing on cell migration have also been reported by Song et al. (2017), who described the effects of NEAT1 on promoting cell invasion and migration in colorectal cancer. Studies have also shown that overexpression of NEAT1 lncRNA leads to suppression of miR-662 resulting in over-expression of ZEB2 (Zinc finger E-box-binding homeobox 2), a transcription protein involved in epithelial-mesenchymal transition process (Song et al, 2017). The effects of NEAT1 silencing on cell migration might be also related to the low levels of MALAT1 in these cells. MALAT1 has been reported to be involved in promoting the migration of lung cancer cells (Gutschner et al, 2013).

Using cationic lipid polymer mediated siRNA transfection resulted in an increase in NEAT1 expression levels. Further experiments revealed that delivery of NEAT1 specific siRNAs via lipid polymers resulted in a decrease in the cytoplasmic NEAT1 expression levels and an increase in the expression levels of nuclear NEAT1. It is well known that using a lipid polymer-mediated transfection method depends on a chemical concept characterised by forming a complex between negatively charged nucleic acid and cationic lipid reagent to facilitate their cellular uptake (Brazas and Hagstrom, 2005). siRNAs delivered via this method remained in the cytoplasm, where it is incorporated with the functional RNA -induced silencing complexes (RISC) to start their silencing effects (Brazas and Hagstrom, 2005). Therefore, the obtained results using this method of transfection in MCF7 cells were unexpected and suggest that the siRNAs delivered via this method remained in the cytoplasm and acted on the cytoplasmic NEAT1 transcript causing a reduction of its expression. These results demonstrate that in addition to its nuclear location, NEAT1 is also located in cytoplasm and suggest that the cytoplasmic transcript might exert inhibitory effects on the expression of nuclear NEAT1. The decrease in the levels of cytoplasmic NEAT1 transcript levels was associated with an over-

expression of nuclear NEAT1 and an increase in the number of total and viable cells as well as an increase in long-term survival. Overexpression of NEAT1 also resulted in a decrease in basal apoptosis levels, which appear to be related to the increase in X-inhibitory of apoptosis (XIAP) expression levels, a potent enzymatic inhibitor of mammalian caspase (including both the extrinsic and the intrinsic caspase pathway) (Eckelman et al, 2006). The inhibition of basal apoptosis levels observed in cells overexpressing NEAT1 could be due the activation of PI3K/AKT signalling pathway which inhibits the activity of the tumour suppressor Phosphatase and tensin homolog (PTEN) in breast cancer (Weng et al, 2001). The other possible cause could be due to the activation of ataxia telangiectasia and Rad3-related protein (ATR) kinase pathway by NEAT1 and hence the contribution of this activated pathway in inhibiting P53 (Adriaens et al, 2016).

Overall the results confirmed the oncogenic role of NEAT1 in breast cancer. The cell survival effects resulted from elevated NEAT1 expression levels in MCF7 cells may be due to correlation between NEAT1 and ER α expression in ER- positive tumours. ER α is reported to have positive effects on NEAT1 expression particularly in ER- positive tumours where oestrogen plays an important role in enhancing the growth and differentiation of mammary epithelium (Girdler and Brotherick , 2000). Accordingly, an alteration in ER α mRNA expression might lead to the breast cancer formation. The correlation between NEAT1 and ER α was demonstrated by Chakravarty et al (2014) in prostate cancer. The study demonstrated the effects of ER α on the expression of NEAT1, which contributes to the epigenetic changes of other genes leading to their aberrant expression in prostate cancer (Chakravarty et al, 2014). The survival effects of NEAT1 may be related to the positive correlation of NEAT1 lncRNA with the activation of PI3K/AKT (phosphoinositide 3-kinase / serine/threonine kinase) pathway, a signalling pathway activated in basal-like breast

tumours and responsible for regulation of cell proliferation, apoptosis and migration (Cancer Genome Atlas Network, 2012; Chin et al, 2013). In addition, a direct relation between AKT kinase pathway and NEAT1 expression was also confirmed in colorectal cancer where down-regulation of NEAT1 leads to inactivation of AKT kinase pathway causing an inhibitory effects on cell cycle progression and cell survival (Peng et al, 2016; Chin et al.,2013).

Although the effects of NEAT1 expression on breast cancer cell survival has been addressed before in breast cancer cells and other cell types (Choudhry et al, 2015; Lo et al, 2016a; Ke et al, 2016; Peng et al, 2016; Mang et al,2017). The consequences of its reduced levels on breast cancer cell response to apoptosis inducing agents have not been addressed. Here, the results demonstrate that reduction in NEAT1 expression levels are associated with increase cell death in response to a range of apoptosis-inducing agents (UV-C irradiation, 5-FU, docetaxel, and Mitoxantrone (only in MDA-MB-231). The enhancement of UV-induced inhibition of cell growth by NEAT1 silencing was associated with an increase in BCL2 Associated Agonist Of Cell Death (BAD) expression levels. These results suggest that NEAT1 inhibits the expression of BAD and UV irradiation of these cells with NEAT1 silencing relieves such inhibition leading to an increase in UV-induced BAD expression resulting in an increase in cell death. The results also indicate that NEAT1 affects gene expression and its effects can occur in cis as shown by its effects on MALAT1 and BAD. NEAT1 appears to selectively modulate the action of chemotherapeutic agents Docetaxel, 5-FU, Nutlin-3a and Mitoxantrone, this perhaps related to their differing mechanisms of engagement of the apoptotic machinery. Docetaxel is an anti-mitotic drug, 5-FL is an antimetabolite that prevent DNA synthesis, Nutlin-3a is Mdm2 antagonist and Mitoxantrone is an antitumour antibiotic that affects cell cycle (Longley et al, 2003; Herbst and Khuri,

2003; Tabe et al, 2009; Fox, 2004). From a therapeutic perspective, the results suggest that reducing cellular NEAT1 levels might improve the cytotoxic activities of conventional chemotherapies drugs. However, further experiments are required using NEAT1 specific siRNAs and a higher dose of ASOs to investigate in details the consequences of NEAT1 down-regulation for breast cancer cell survival following treatment with chemotherapeutic agents.

Chapter highlights

1. The results suggest that in MCF7 breast cancer cells, NEAT1 is distributed in both nuclear and cytoplasm compartments. Down-regulation of cytoplasmic NEAT1 levels leads to an increase in the expression of nuclear NEAT1, which was associated with an increase in short and long-term survival and a decrease in apoptosis.
2. Silencing of NEAT1 decreased short and long-term viability, altered the cell cycle and inhibited cell migration of both triple-negative and estrogen receptor-positive cells.
3. Down regulation of NEAT 1 reduced the expression level of its neighbouring gene, MALAT1.
4. NEAT1 silencing enhanced UV- induced cell death and this response was associated with a significant increase in the expression levels of BAD.
5. NEAT1 silencing enhanced growth inhibition induced by some classical chemotherapeutic ag
6. Both ASOs and siRNAs were effective in silencing NEAT1. However, functional effects of siRNAs lasted longer than those of ASOs

Chapter 5

The influence of NEAT1 down-regulation on gene expression in breast cancer cells

5.1 Introduction

Various studies confirm the importance of lncRNAs in their contribution via many different pathways for regulating the cellular function in health and disease (Yang et al, 2012a; Bernard et al, 2010, Batista and Chang, 2013; Han et al, 2017).

lncRNAs play important roles in regulating multiple aspects of gene transcription, often through regulation of transcription factor expression or by recruiting regulatory complexes through RNA–protein interactions to influence the expression of nearby or distant genes (Batista and Chang, 2013). Many lncRNAs have been demonstrated to interact with chromatin at several thousand different locations across multiple chromosomes and to modulate large-scale gene expression programs (Wang et al, 2011; Vance and Ponting, 2014)

For instance, Bernard et al. (2010) has revealed the role of MALAT1 lncRNA in regulating the expression of genes that are involved in synapse formation and maintenance.

Furthermore, lncRNAs can be involved in transcriptional interference, a mechanism that regulates gene expression through its regulatory sequences like the activating or blocking of the promoter sites (Batista and Chang, 2013). They can exert their effects in integrated manner with a set of non-coding and coding RNAs rather than individually such as AK123657, BX649059 and BX648207 lncRNAs, which are down-regulated in colorectal cancer in versus to normal colorectal tissues, suggesting their protective role in colorectal cancer pathology (Hu et al, 2014, Han et al, 2017).

NEAT1 up-regulation promotes glioma cell proliferation, invasion and migration via activating the expression of c-Met gene, which encodes a receptor tyrosine kinase involved in cell proliferation and migration via c-Met signalling pathway (Yan et al, 2017, Organ and Tsao, 2011). The effects of NEAT1 on MET gene expression were attributed to the ability of NEAT1

to act as a sponge to miR-449b-5p, which regulates the expression of c-Met gene (Yan et al, 2017). Additionally, NEAT1 regulates the expression of paraspeckles target genes through sequestration of NEAT1 binding transcription factors in the paraspeckles (Hirose et al, 2014). NEAT1 knockdown leads to the repression of the transcription of several genes including the RNA-specific adenosine deaminase B2 (ADARB2), such effects were dependent on the sequestration of the paraspeckle protein SFPQ (Splicing factor proline/glutamine rich) (Hirose et al, 2014).

Therefore, the aims of this study are to determine the effects of NEAT1 down-regulation on the expression of genes involved in breast cancer and cell cycle, using Breast Cancer and Cell Cycle RT2 Profiler™ PCR Arrays. The study also investigated the effects of NEAT1 down-regulation on global gene expression in breast cancer cells using RNA sequencing.

5.2 Material and methods

5.2.1 RT2 profiler PCR array

RT2 profiler PCR array is a combination of using the qRT-PCR and microarray analysis. Quantification of gene expression was performed using ready to use Human Breast Cancer and Human Cell Cycle RT2 Profiler PCR Arrays (QIAGEN). Each array contains primers for 84 tested and 5 housekeeping genes, and controls for RT2 and PCR reactions (QIAGEN, 2013).

RNAs were isolated for MCF7 48h following transfection with NEAT1 ASOs (Section 2.2.12). cDNA was synthesised as discussed in Section 2.2.13 and used in RT2 profiler PCR array as indicated in Section 2.2.14. After determining the Ct value for each well by using the real time cycler software, the results were analysed using the Web-based PCR Array Data Analysis software at www.SABioscience.com/pcrarraydataanalysis.php.

5.2.2 RNA Sequencing

MDA-MB-231 cells were re-plated for 6 h after 48h post-transfection with NEAT1 ASOs before isolation of RNAs, according to the protocol described in Section 2.2.12. The quality of RNA samples was assessed using gel electrophoresis (Section 2.2.12.1). The RNA purity and the concentration were measured by a NanoDrop. RNA with 260/280 nm ratio of ~ 2 was considered as pure and good quality (Desjardins and Conklin, 2010).

For RNA-sequencing, samples at a concentration of 1-5µg of total RNA (20 ng/µl in 60 µl) were prepared according to the Earlham Institute (Norwich Research Park, Norwich, UK) and sent to Earlham Institute on dry ice. The obtained results were analysed using the Galaxy Web based platform for bioinformatics analysis, IPathway Guide for Next-gen pathway analysis and Reactome pathway database

5.3 Results

5.3.1 Effects of NEAT1 down-regulation on the expression of cell cycle and breast cancer genes

Two RT2 profiler PCR arrays were used to investigate the effects of NEAT1 down-regulation on the expression of genes involved in the regulation of genes involved in cell cycle and breast cancer. One of the arrays used was the Human Cell Cycle RT2 Profiler PCR Array, which profiles the expression of 84 genes key to cell cycle regulation. This array contains genes that involved in: 1) positive and negative regulation of cell cycle, 2) the cell cycle process, 3) the transitions between the cell cycle phases, 4) DNA replication, and 5) checkpoints and arrest. The other array was the Human Breast Cancer RT2 Profiler PCR Array, which profiles the expression of 84 key genes involved in the dysregulation of signal transduction and

other biological processes involved in breast carcinogenesis. This array includes genes involved in: 1) tumour classification, 2) signal transduction, 3) angiogenesis, 4) adhesion, 5) proteolysis, 6) cell cycle, and 7) apoptosis.

Using the Human Cell Cycle RT2 Profiler PCR Array, revealed the fold changes in expression of 48 genes as detected in various stages of cell cycle process (Figure 5.1). Furthermore, the RT2 profiler PCR data analysis illustrated the effects of NEAT1 down-regulation on the expression of specific genes that are responsible for regulating each phase of the cell cycle. In which, there was a down-regulation in cyclin dependent kinase (CDK4/6) and cyclin dependent kinase regulator (CCND1 and CCNE1) genes, which are responsible for regulation of G1, S and G2/M phases as present in Figure 5.2. Additionally, NEAT1 down-regulation resulted in increase in the expression of most cell cycle regulatory checkpoint genes which includes ATM, ATR, BRCA1, BRCA2, CASP3, CCNA2, CCNG2, CDC25A, CDC25C, , CDKN1A (p21CIP1, WAF1), CDKN1B (P27KIP1), CDKN2A (p16INK4a), CDKN2B (p15INK4b), CDKN3, CHEK1 Using the Human Cell Cycle RT2 Profiler PCR Array, revealed the fold changes in expression of 48 genes as detected in various stages of cell cycle process (Figure 5.1). Furthermore, the RT2 profiler PCR data analysis illustrated the effects of NEAT1 down-regulation on the expression of specific genes that are responsible for regulating each phase of the cell cycle. In which, there was a down-regulation in cyclin dependent kinase (CDK4/6) and cyclin dependent kinase regulator (CCND1 and CCNE1) genes, which are responsible for regulation of G1, S and G2/M phases as present in Figure 5.2. Additionally, NEAT1 down-regulation resulted in increase in the expression of most cell cycle regulatory checkpoint genes which includes ATM, ATR, BRCA1, BRCA2, CASP3, CCNA2, CCNG2, CDC25A, CDC25C, , CDKN1A (p21CIP1, WAF1), CDKN1B (P27KIP1), CDKN2A (p16INK4a), CDKN2B (p15INK4b), CDKN3, CHEK1 CHEK2 (RAD53),

CUL1, CUL2, CUL3, GADD45A, KNTC1, MAD2L1, MDM2, RAD1, RAD17, RAD9A, RB1, RBBP8, TP53 (p53) and WEE1 (Figure 5.3 A). Four of these cell cycle regulatory checkpoint genes (ATM, BRCA1, CDKN2B and TP53) function as negative regulators of the cell cycle. Reduced NEAT1 levels also resulted in the up-regulation of cell cycle negative regulatory genes (Figure 5.3 B). Two other genes involved in the negative regulation of the cell cycle, RBL1 and RBL2, were also up-regulated as a result of reduced expression levels of NEAT1.

Moreover, NEAT1 down-regulation has a positive and negative influence on the expression of human breast cancer focused genes as illustrated in Figure 5.4. Genes that were found to be up-regulated are RAD9A, RB1, RBBP8, RBL1, RBL2, RPA3, SERTAD1, SUMO1, TFDP1, TFDP2, TP53, UBA1, CUL2, CUL3, DNM2, GADD45A, GTF2H1 and GTSE1 (Figure 5.5 A). On the other hand, genes that were found to be down-regulated by the reduction of NEAT1 expression levels include HUS1, MAD2L1, MCM3, MCM4, MKI67, MNAT1, SKP2, CDK4, CDK6, CDH1, GATA3, IGF1R, KRT18, KRT19, KRT8, MAPK3, MLH1, MMP9, PYCARD, SLC39A6, TFF3, XBP1 and E2F4 (Figure 5.5 B).

At the same time, the RT2 profiler PCR array results revealed list of communal genes between human breast cancer and cell cycle that had their expression affected by NEAT1 down-regulation, as illustrated in Figure 5.6. These genes include BRCA1, BRCA2, CCND2, CCNE1, CDK2, CDKN1A, TP53, CCND1, CDKN2A and MKI67. There was an up-regulation in the levels of BRCA1, BRCA2, CDKN1A, CDKN2A and TP53. Genes that showed down-regulation in both arrays include CCND1, CCND2, CCNE1, CDK2 and MKI67 (Figure 5.6).

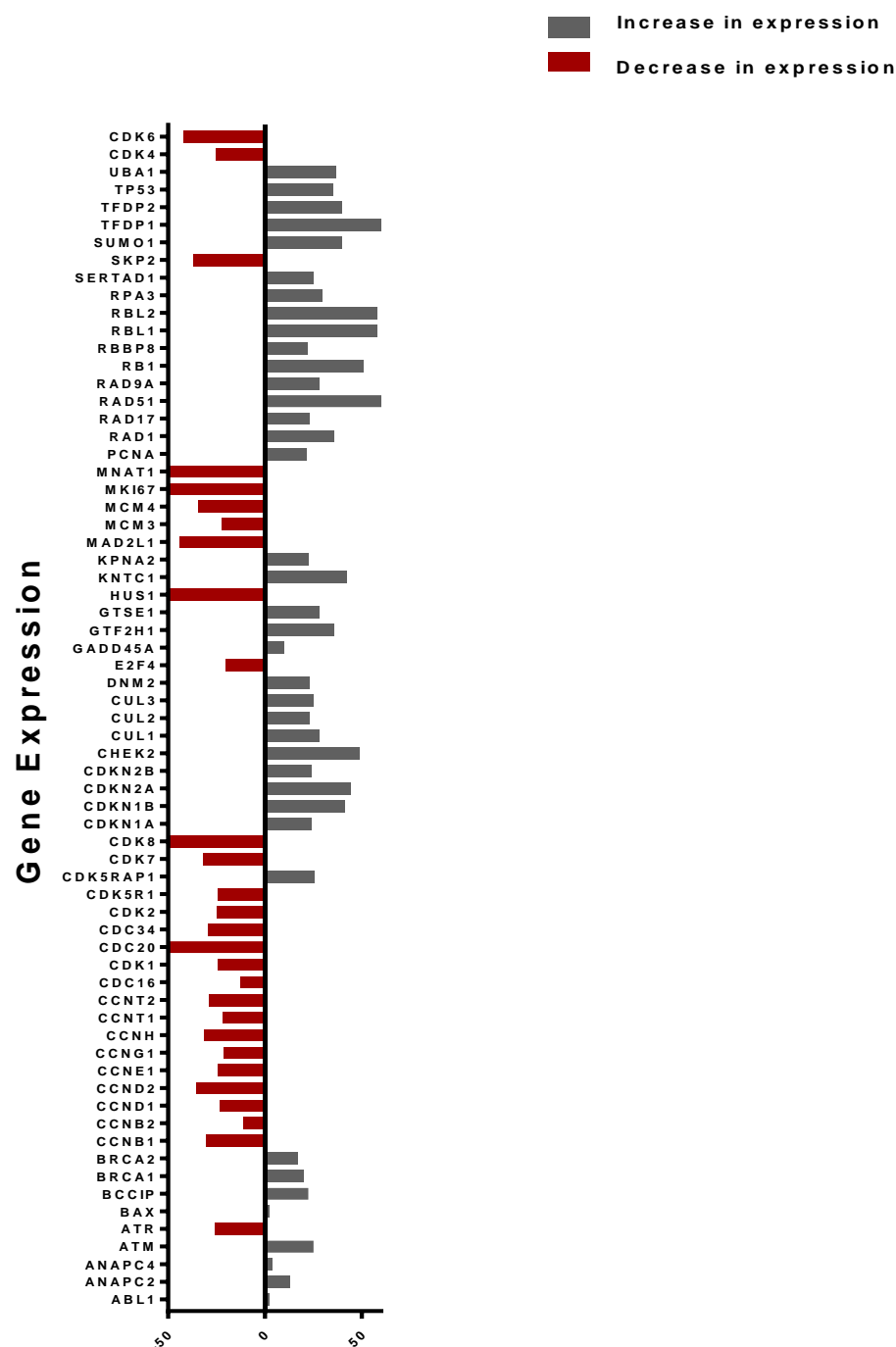


Figure 5.1 Cell cycle genes affected by NEAT1 down-regulation. RT 2 profiler PCR array bar graph showing the expression profile of 84 focused- cell cycle genes. The average Ct value was normalised to a set of 5 own internal housekeeping genes at cut-off was 35. The fold change in gene expression was organised according to a cut-off 2. The analysis of the results was performed using a Web-based PCR Array Data Analysis software at www.SABioscience.com/pcrarraydataanalysis.php.

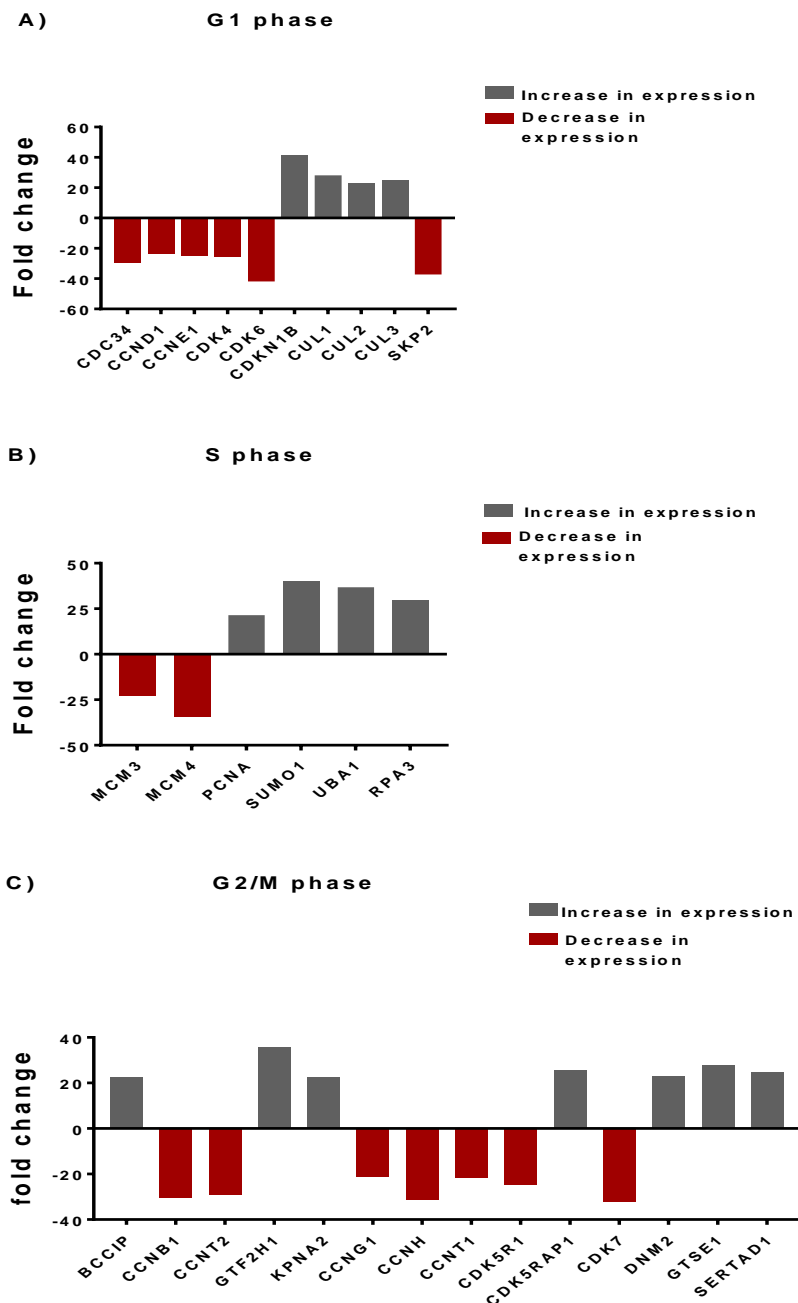


Figure 5.2 NEAT1 down-regulation effects on the expression of genes involved in each stage of the cell cycle. RT2 profiler PCR array bar graphs show the expression profile of 84 cell cycle focused- genes. The average Ct value was normalised to a set of 5 own internal housekeeping genes at cut-off was 35. The fold regulation in gene expression was organised according to a cut-off 2. (A) Represents the list of genes expression relevant to G1 phase of cell cycle. (B) Shows the list of genes expression relevant to S phase of cell cycle. (C) Revealed the expression of genes responsible for G2/M phase of cell cycle. The analysis of the results was carried out through a Web-based PCR Array Data Analysis software at www.SABioscience.com/pcrarraydataanalysis.php.

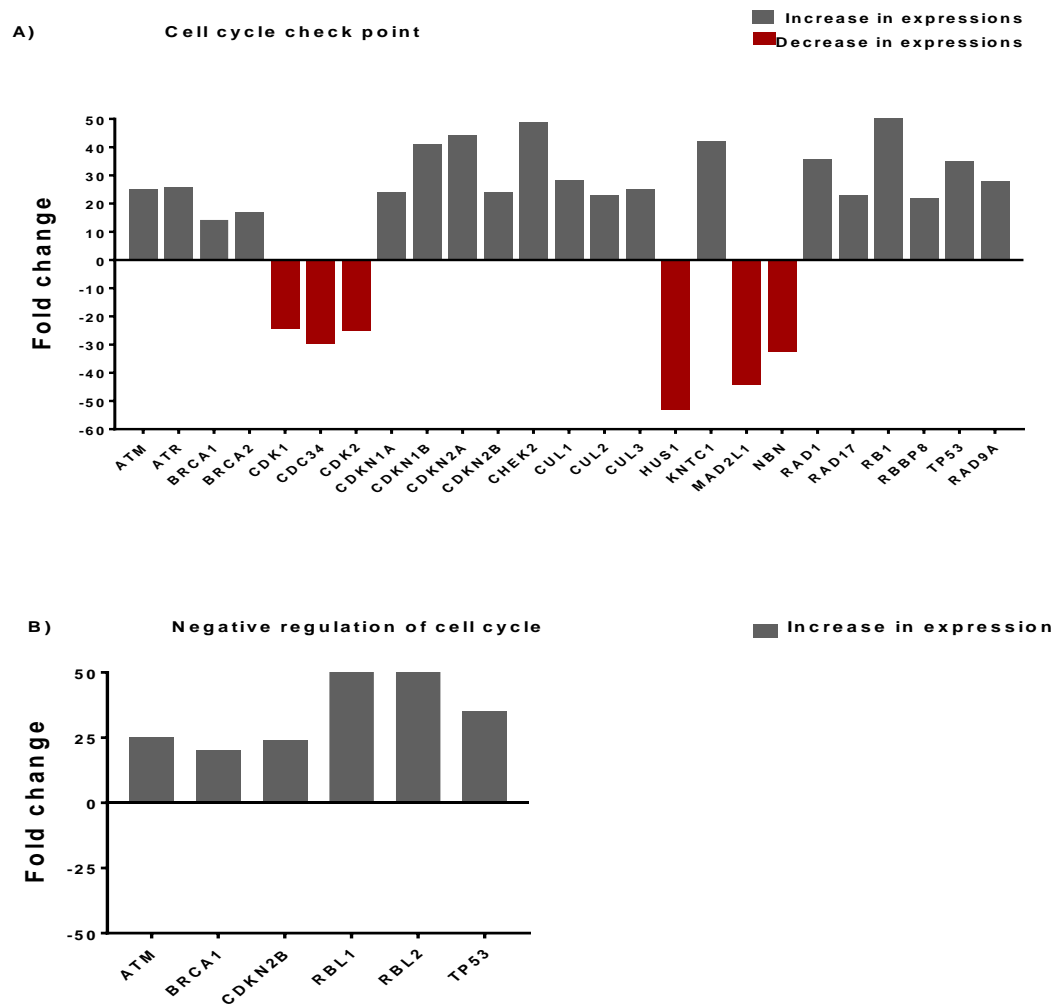


Figure 5.3 Cell cycle checkpoint genes affected by the decrease in NEAT1 transcript levels. RT2 profiler PCR array bar graphs show the expression profile of cell cycle focused- genes. The fold regulation in gene expression was organised according to a cut-off value was 2. (A) Represents the list of genes responsible for regulating the cell cycle checkpoints. (B) Shows the expression of genes expression considered as a negative regulator of cell cycle. The analysis of the results through a Web-based PCR Array Data Analysis software at www.SABioscience.com/pcrarraydataanalysis.php.

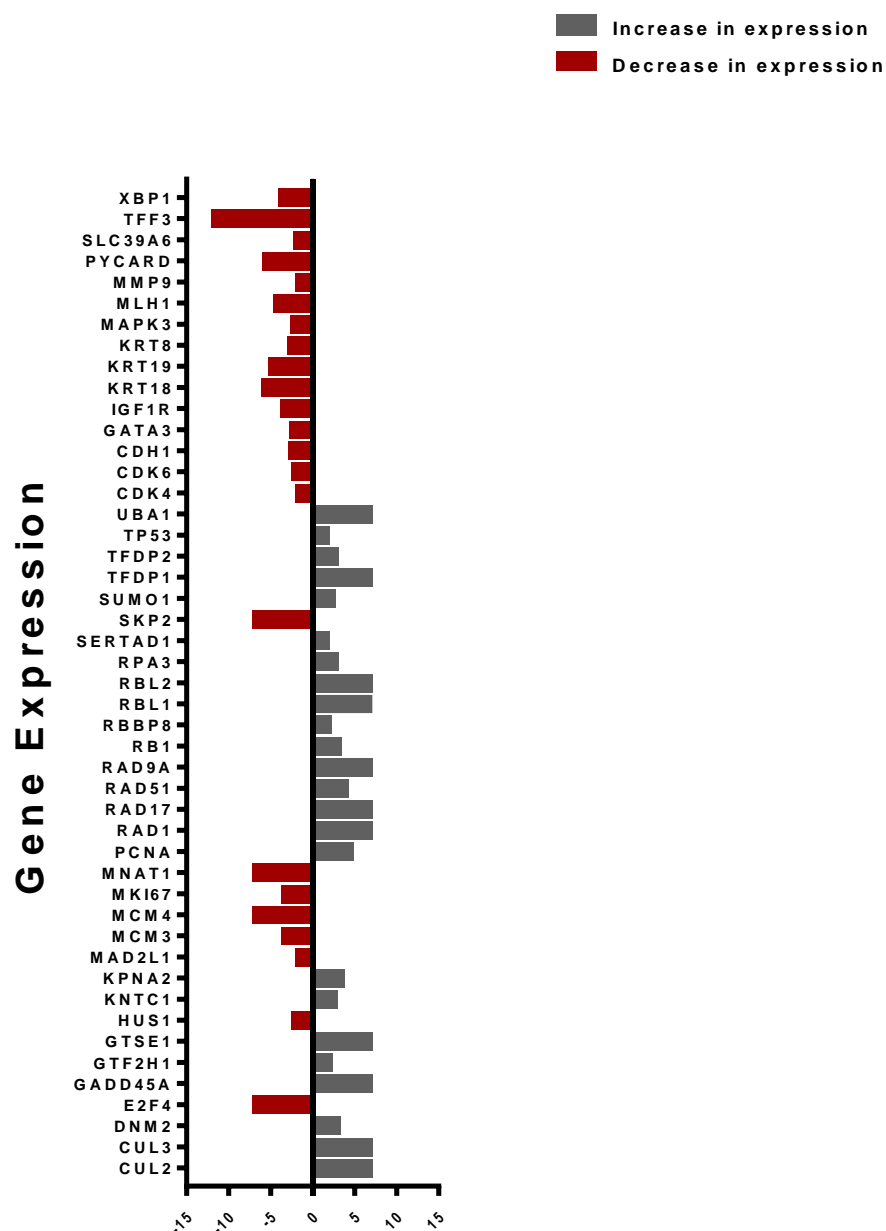


Figure 5.4 The effects of NEAT1 down-regulation on the expression of breast cancer genes. RT2 profiler PCR array bar graphs show the expression profile of 84 focused-human breast cancer cells. The average Ct value was normalised to a set of 5 own internal housekeeping genes at cut-off was 35. The fold change in gene expression was organised according to a cut-off 2. The analysis of the results through a Web-based PCR Array Data Analysis software at www.SABioscience.com/pcrarraydataanalysis.php

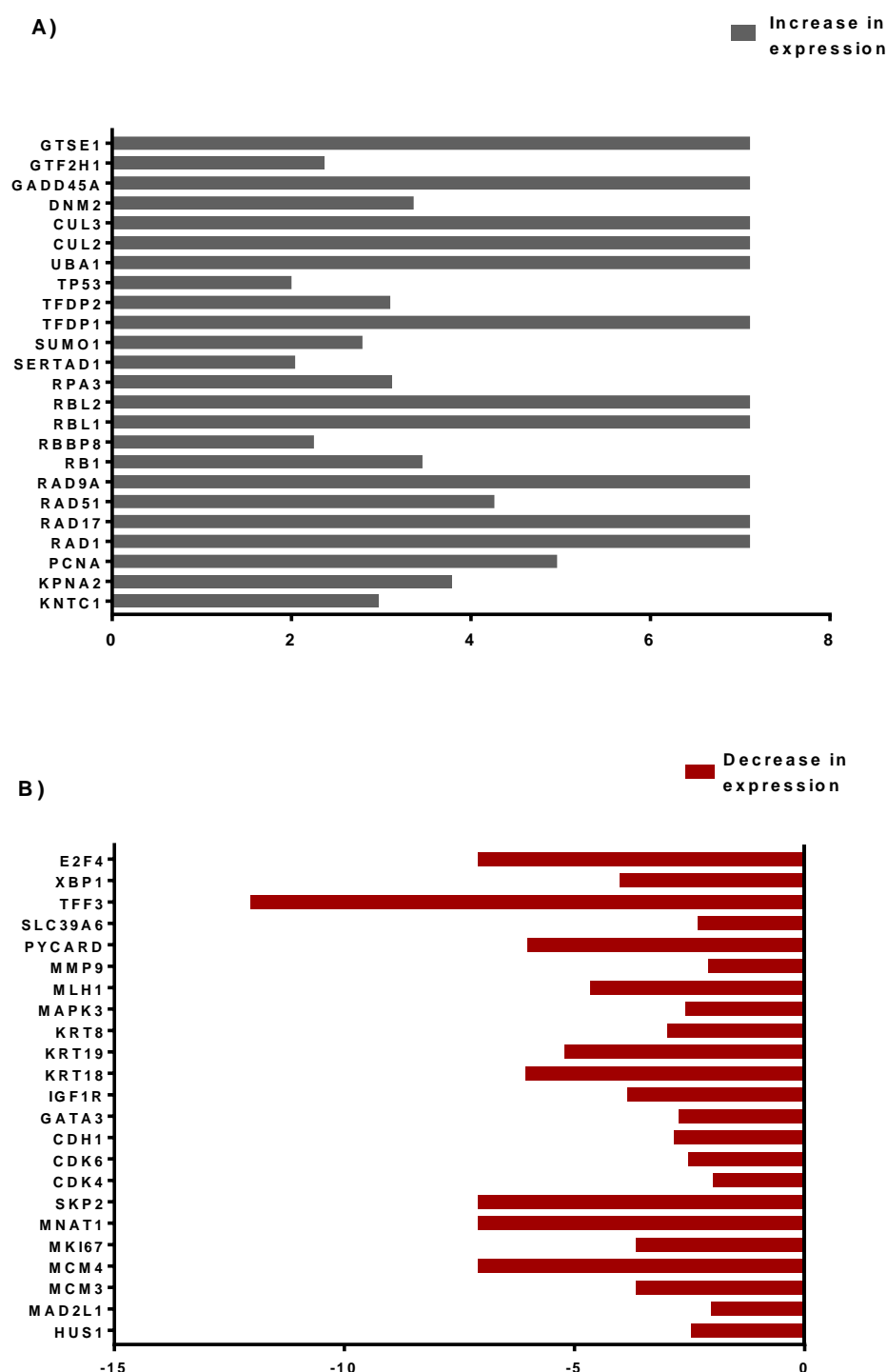


Figure 5.5 Breast cancer genes affected by the silencing of NEAT1 expression levels. RT2 profiler PCR array bar graphs show the expression profile of 84 focused- human breast cancer cells in relevance to NEAT1 down-regulation. The average Ct value was normalised to a set of 5 own internal housekeeping genes at cut-off was 35. The fold change in gene expression was organised according to a cut-off 2. A) Revealed the genes that show an increase in the level of expression. B) Showed the genes which were down-regulated as a result of NEAT1 down-regulation. The analysis of the results using a Web-based PCR Array Data Analysis software at: www.SABioscience.com/pcrarraydataanalysis.php.

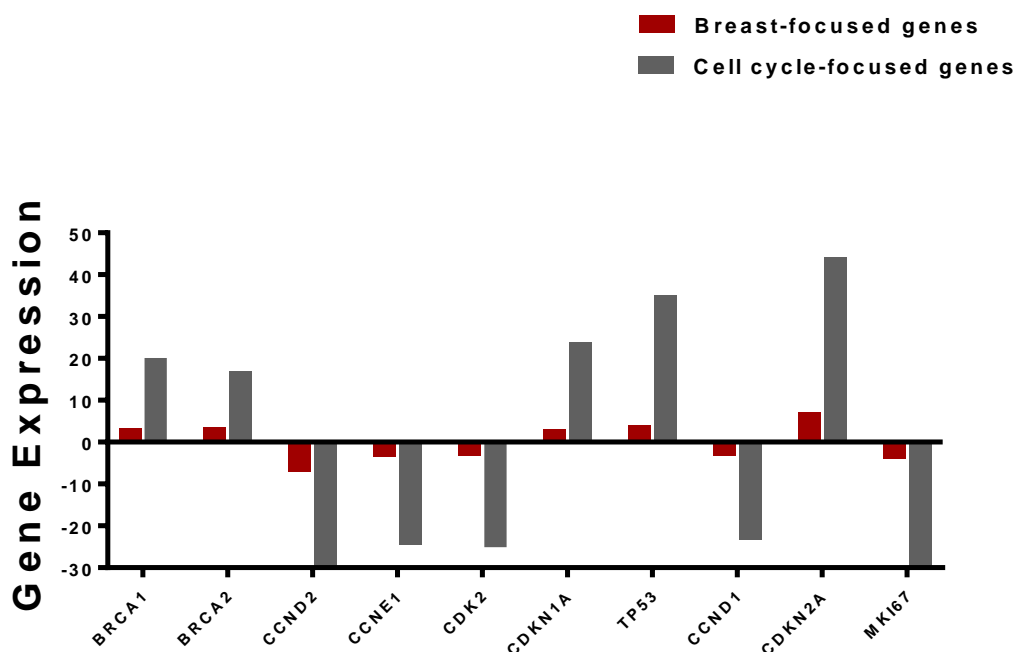


Figure 5.6 Bar graph showing the effects of NEAT1 down-regulation on the expression profile of genes that are shared between human breast cancer and cell cycle. The average Ct value was normalised to a set of 5 own internal housekeeping genes at cut-off was 35. The fold change in gene expression was organised according to a cut-off 2. The analysis of the results through a Web-based PCR Array Data Analysis software at: www.SABioscience.com/pcrarraydataanalysis.php.

5.3.2 Effects of NEAT1 down-regulation RNA sequencing following the transfection of MDA-MB-231 with NEAT1 ASOs

MDA-MB-231 was transfected with negative ASO, NEAT1,1 ASO or NEAT1,2 ASO and cultured for further 48 h before being trypsinised and re-plated for further 6 h. Total RNA was isolated from transfected cells following the 6h incubation. The RNA sequencing data analysis showed NEAT1 expression (Figure 5.7,Appendix I). NEAT1 levels were reduced by 35% compared to control only in the cells transfected with NEAT1,1 ASO (Figure 5.8). Cells transfected with NEAT1, 2 showed no significant decrease in the expression levels of NEAT1 (Figure 5.8). These results

support the previous observation in Chapter 4 that the effects of NEAT1 ASOs are short lived and suggest that the levels of NEAT1 have returned to the normal endogenous levels at the time of RNA collection. Therefore, only the results of RNA sequencing from the cells transfected with NEAT1,1 ASO will be described here. The 35% decrease on NEAT1 expression levels resulted in the significant ($P < 0.05$ and $Q < 0.05$) up-regulation of 67 genes ($P < 0.05$) (Figure 5.8 and Table 5.1). There were changes in the fold expression of other genes detected by the RT- Profiler, however these changes were not significant ($P > 0.05$). These genes includes the negative regulators of the cell cycle ATM, BRCA1, CDKN2B and TP53 and the genes affected in both cell cycle and breast cancer arrays (BRCA1, BRCA2, CCND2, CCNE1, CDK2, CDKN1A, TP53, CCND1, CDKN2A and MKI67). The changes were in the same direction as found in the RT profiler arrays. These results could be due to the fact that the expression levels of NEAT1 have started to recover at the time point chosen for the RNA collection and this has caused a loss of some of the effects of NEAT1 on the gene expression.

The RNA sequencing data analysis showed a significant ($P < 0.05$) up-regulation of 67 genes as presented in Table 5.2, Appendix I DEGS2, RNF223, HSPB1 and ISG15 showed the highest up-regulation levels (Table 5.2, Appendix I). DEGS2 gene encodes an enzyme involved in the key reaction of the biosynthesis of phytosphingolipids including sphingomyelin. Sphingomyelin (SM) and its metabolic products ceramide and sphingosine-1-phosphate, are known to have second messenger functions in a variety of cellular signalling pathways including apoptosis. RNF223 is a ring finger protein and HSPB1 is heat-shock protein of 27 kDa (HSP27) which is reported to be expressed at higher levels during oxidative stress in renal tubular cells in acute kidney injury (Matsumoto et al, 2015).

ISG15 gene encodes an ubiquitin-like modifier protein and involved in cellular communication and in defence against viral infection (Desai, 2015). Interestingly, 7 genes located on chromosome 11 were found to be up-regulated. Two of these genes (Coiled-Coil Domain Containing 85B and Sjogren Syndrome/Scleroderma Autoantigen 1) are located on 11q13.1 the same chromosomal band as NEAT1 and another one, Nudix Hydrolase 8, on 11q13.2. Another four of these genes are located on 11p15.5. These include Tumour Suppressing Subtransferable Candidate 4, Plakophilin 3, Ras Association Domain Family Member 7 and Ribonuclease / Angiogenin Inhibitor 1. It is also worth noting that Tumour Protein P53 Inducible Protein 13 (TP53I13) and BRCA1 Associated ATM Activator 1 (BRAT1) were found to be up-regulated. The expression of BRAT1 and TP53I13 are regulated by BARC1 and TP53, respectively.

Further analysis using IPathway Guide for Next-gen pathway analysis showed that the genes affected are implicated in a number of pathways with the most significant ones include galactose catabolism, tight junction and Fatty acid (Table 5.1). Other pathways affected include systemic lupus erythematosus, viral carcinogenesis.

AMPK and MAPK signalling pathways, RNA degradation, VEGF signalling pathway, DNA repair, Central carbon metabolism in cancer, Sphingolipid metabolism, HIF-1 signalling pathway, and cell cycle. Reactome database has identified 11 of the genes to be involved in regulation the immune system, 13 in the regulation of metabolism, 5 in signal transduction and 4 in the cellular response to stress (the list of pathways affected by NEAT1 down-regulation illustrated in Table 5.3, Appendix I)

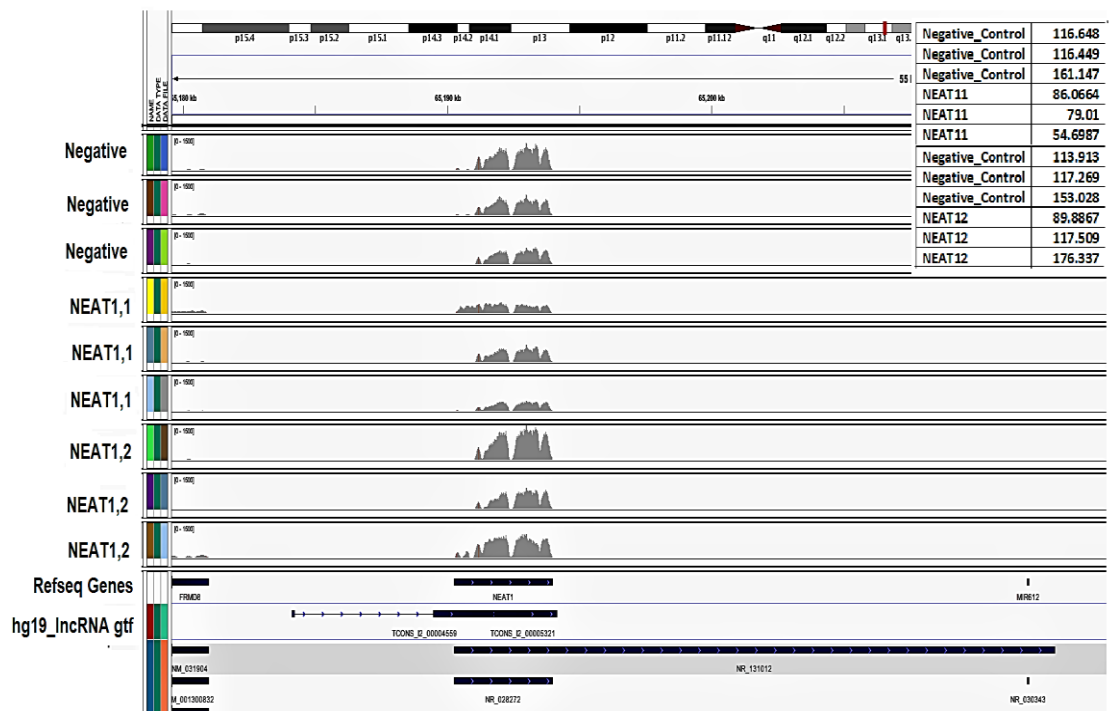


Figure 5.8 NEAT1 raw reads in the linear scale of NEAT1. Raw reads obtained from the RNA sequencing data. Levels of NEAT1 expression in transfected cells with NEAT1,1 ASO are lower than those in the negative control (n=3). While, there was no change in NEAT1 expression in transfected cells with NEAT1,2 ASO. The table presents NEAT1 number of reads in each sample. The diagram was obtained from Integrative Genomics Viewer (IGV).

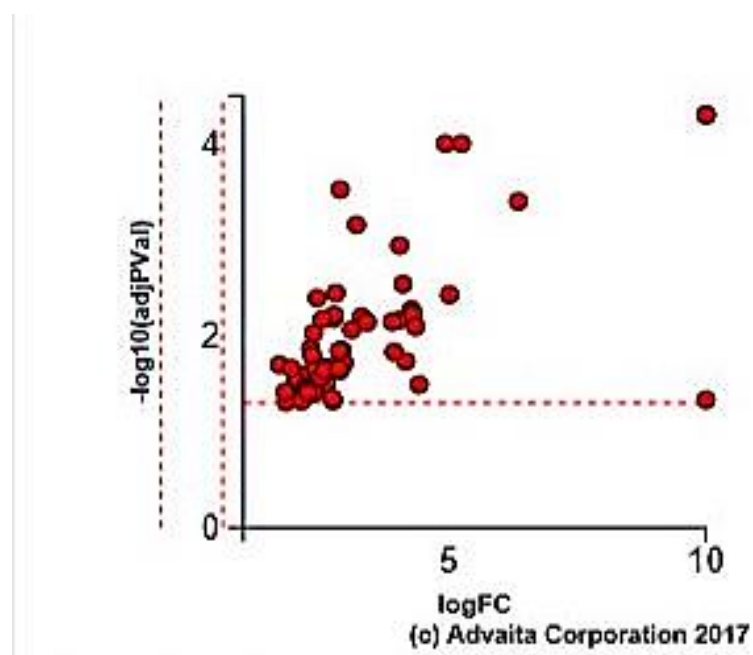


Figure 5.9 Volcano plot revealed 67 significantly differentially expressed (DE) genes are represented in terms of their measured expression change (x-axis) and the significance of the change (y-axis). The significance is represented in terms of the negative log (base 10) of the p-value, so that more significant genes are plotted higher on the y-axis. The dotted lines represent the thresholds used to select the DE genes: 0.6 for expression change and 0.05 for significance. <https://ipathwayguide.advaitabio.com/report/24273/contrast/294>

Table 5.1: Top pathways that have been affected by NEAT1 down-regulation and their associated p-values

Pathway name	The involved Genes	p-value
Galactose catabolism	Galactokinase 1(GALK1); Phosphofructo-1-Kinase Isozyme B (PFKL); GAA (producing an enzyme acid alpha-glucosidase);	1.567e-4
Tight junction	Scribbled Planar Cell Polarity Protein (SCRIB)	0.02
Fatty acid Biosynthesis	Fatty Acid Synthase (FASN)	0.05

5.4 Discussion

The work described in this chapter aimed at studying the effects of silencing NEAT1 on the expression of genes involved in cell cycle and human breast cancer were investigated. RNA sequencing was also carried out to analyse the effects of reduced NEAT1 transcript levels on global gene expression in MDA-MB231 breast cancer cells. The results revealed that down-regulation of NEAT1 affected the expression of genes, which regulate cell cycle and are involved in breast cancer. RNA sequencing identified 67 differentially expressed genes in the RNA isolated from cells that had up to 35% reduction in the expression levels of NEAT1. These genes are involved in a number of important cellular functions. Reduction in the NEAT1 expression levels down-regulated six genes involved in G1 phase and G1 transition. These include CCND1 (cyclin D1), CCNE1 (cyclin E1), CDK4 (cyclin dependent kinase 4), CDK6 (cyclin dependent kinase 6), CDKN3 (cyclin dependent kinase inhibitor 3), SKP2 (S-phase kinase associated protein 2). Both CDK4 and CDKN3 are involved in the regulation and progression of G1. G1 Phase and G1/S transition genes over-expressed as a results of NEAT1 down-regulation include CUL1, CUL2 and CUL3 (Cullin 1, 2 and 3). Cullins are family of NEDD8 targets important in the stabilization and degradation of proteins, such as hypoxia-inducible factor (Curtis et al, 2015). Genes involved in S phase and DNA replication were also affected by the silencing of NEAT1 including two genes involved in DNA replication, MCM3 and MCM4 (Minichromosomal maintenance deficient 3 and 4). Seven genes involved in G2 phase and G2/M transition were down-regulated including CCNB1 (Cyclin B1) and CCNH (Cyclin H), and eight of these genes were found to be up-regulated. Interestingly, expression of six genes involved in cell-cycle checkpoint and arrest were up-regulated. These genes include ataxia telangiectasia mutated (ATM),

breast cancer 1 (BRCA1), cyclin dependent kinase inhibitor 2B (CDKN2B), RB transcriptional corepressor like 1(RBL1), RB transcriptional corepressor like 2 (RBL2) and tumour protein P53 (TP53). It is likely that up-regulated expression of these genes is the proximal cause for the inhibition of cell-cycle progression shown by reducing NEAT1 expression levels. ATM/ATR kinases are responsible for maintaining the DNA integrity via their contribution in DNA repair and controlling the cell cycle checkpoints (Maréchal and Zou, 2013). Therefore, activation of these pathways leads to the inhibition in cell proliferation and increase in the rate of apoptosis (Taylor and Lindsay, 2016). In addition to the above, the importance of BRCA1 in regulating cell cycle depends on its ability to encode multiple tumour suppressor genes, which are involved in all stages of cell cycle (Anderson et al, 1998; Zhang et al, 2016a). The effects of NEAT1 down-regulation on the enhancement of the expression of cell cycle regulator gene such BRCA1 has been demonstrated in by Lo et al. (2016). This study reported a signalling axis involving BRCA1/NEAT1/miR-129-5p/WNT4 which plays a role in breast cancer initiation (Lo et al, 2016b). BRCA1 deficient cells were shown to have high expression of NEAT1 and this suppressed the activity of miR-129-5p by DNA methylation at the CpG Island in miR-129 genes. Thus, down-regulation of miR-129-5p leads to up-regulation of WNT4 that stimulate the oncogenic WNT pathway (Lo et al, 2016b).

The other cell cycle inhibitor up-regulated as a result of reduction in NEAT1 expression levels is CDKN2B. CDKN2B is a cell cycle inhibitor that forms a complex with CDK4 and 6 preventing their effects and hence controlling cell proliferation (Suzuki et al, 1995; Kitagawa et al, 2013). Therefore, inactive CDKN2B protein might enhance malignant cell proliferation a fact has been proved in different studies (Li et al, 2014a

Omura et al, 2000; Hu et al, 2017). Expression of CDKN2B was induced by suppression of AKT kinase in oral squamous cell carcinoma (Nakashiro et al, 2015). RBL1 and RBL2 (Retinoblastoma transcriptional corepressor like 1, 2) code for the p107 and p130 proteins, respectively (Henley and Dick, 2012). These proteins are called pocket proteins because they contain a conserved domain referred to as the 'pocket' that interacts with the LXCXE motif found in viral proteins such as TAg (Henley and Dick, 2012). Pocket proteins are thought to be central to the regulation of proliferation in many diverse organisms and deregulation of cell cycle control in cancer (Henley and Dick, 2012).

TP53 was also found to be up-regulated as a consequence of NEAT1 down-regulation. TP53, a tumour suppressor protein, plays an important role in regulating the cell cycle, DNA replication and cell division (Vogelstein et al, 2000). Therefore, any abnormal expression of TP53 because of gene mutation leads to a disturbance in normal cell function, and hence increases the incidence of different pathological problems like cancer (Vogelstein et al, 2000). Usually, TP53 presents as inactive protein that become active after phosphorylation by one of the 3 distinct pathways, which are ATM/CHK2 pathway, ATR kinase pathway, and the last one as a consequence to p14 activation due to oncogene stimulation like Ras or Myc (Vogelstein et al, 2000). Therefore, activation of these kinases ATM, CHK2, ATR, CHK1 protein as a response to DNA damage will promote the phosphorylation processes of TP53 (Vogelstein et al, 2000; Schwartz and Rotter, 1998). Phosphorylated TP53 plays an important role in regulating the cell cycle by arresting the cells in G1 phase and preventing them to progress into S phase, an action to keep the genome integrity via increasing the possibility of repairing the damaged DNA or enhance the rate of apoptosis (Vogelstein et al, 2000). TP53 promotes the expression of non-coding genes beside to protein coding genes that are responsible

for cell cycle arrest, DNA repair and apoptosis (Botcheva et al, 2011; Blume et al, 2015; Riley et al, 2008). TP53 is reported to induce NEAT1 expression in CLL (chronic lymphocytic leukaemia) (Botcheva et al, 2011; Blume et al, 2015). On the other hand, NEAT1 expression contribute in regulating the TP53- dependent gene expression via increase the formation of paraspeckles, which are involved in controlling gene expression and function in editing A-I mRNA, transcription and splicing (Idogawa et al, 2017, Adriaens et al, 2016). Idogawa et al. (2017) revealed the importance of NEAT1_2 (long isoform) in regulating the TP53- induced gene expression and tumour suppression function. Activation of P53 by oncogene stress leads to up-regulation of NEAT1 and increase in paraspeckles formation, which in turn attenuate the oncogene-induced p53 activation (Adriaens and Marine, 2017), suggesting the important role of NEAT1 in regulating p53 protein by evolution a negative feedback loop that is involved in attenuating the oncogene- dependent p53 activation.

NEAT1 down-regulation has also resulted in the over-expression of 24 genes involved in breast cancer and the down-regulation of 24 genes. Genes affected include genes involved in tumour classification, signal transduction, angiogenesis, adhesion, proteolysis, cell cycle, and apoptosis. While further experiments are required to validate the results obtained with the RT2 Profiler™ PCR Arrays, both cell cycle and breast cancer arrays results confirmed NEAT1 effects on genes shared by both arrays. The fact that the same genes were changed in both arrays confirms the effects of NEAT1 down-regulation on the expression of these genes. In both arrays, reduction of NEAT1 expression levels resulted in the increase in levels of BRCA1, BRCA2, CDKN1A, CDKN2A and TP53. The decrease in NEAT1 transcript levels led to the down-regulation of the expression of CCND2, CCND1, CCNE1 and MKI67. Over- expression of BRCA1 leads to co-activation of the p53-

mediated gene expression like p21 and GADD45, which are involved in suppressing cell cycle progression. This elucidates the importance of BRCA1 over-expression in activation and stabilization of P53 signalling pathway (MacLachlan et al, 2002; Yoshida and Miki, 2004). In addition, BRCA1 and BRCA2 play a significant role in repairing of DNA double strand break by homologous recombination (HR) pathway via activation of certain proteins, such as ATM, CHK2, ATR, BRCA2, RAD51, RAD50 (Yoshida and Miki, 2004; Powell and Kachnic, 2003).

Shen et al. (2017) have previously reported the positive correlation of NEAT1 lncRNA associated proteins P54nrb and PSF protein and the level of c-Myc mRNA translation in stress condition, which is a transcription factor involved in the activation of the expression of BRCA1 (Chen et al 2011; Shen et al, 2017). CDKN1A (p21cip1) belongs to the cyclin dependent kinase inhibitor (CDKI) family and inhibits cell-cycle progression by inhibiting CDK2 and CDK4 and by blocking DNA replication and repair by binding to PCNA (Harper et al., 1993; Cayrol et al., 1998). This inhibitor of cell-cycle progression causes arrest at G1, S, and G2 phases (Harper et al., 1993; Cayrol et al., 1998). CDKN2A also belongs to the CDKI family and known as cyclin-dependent kinase Inhibitor 2A (Soto et al, 2005). Its role as a tumour suppressor gene is well established. The down-regulation of MKI67 observed suggests that down-regulation of NEAT1 lead to an increase in MDA-MB-231 cells entering the resting state (G0) since the gene product for MKI67 (Ki-67) encodes KI67, a cell marker linked to proliferation and is present in all stages of the cell cycle with the exception of the G0 stage (Gerdes et al, 1984). Both CCND2 (Cyclin D2) and CCND1 (Cyclin D1) are frequently deregulated in cancer and are biomarkers of cancer phenotype and disease progression. The protein product of CCND2 and CCND1 are able to activate the cyclin- dependent kinases CDK4 and CDK6 mediating their oncogenic actions (Musgrove et al., 2011).

The results of RNA sequencing revealed changes in the same direction in some genes identified using the RT2 profiler arrays; however, the changes were not statistically significant due to the fact that the level of expression of NEAT1 was returning to its basal levels at the time of RNA collection. However, the RNA sequencing results have provided important information and showed that even a small decrease in the levels of NEAT1 expression results in changes in the expression of a number of genes involved in many cellular function. The reduction in NEAT1 expression levels resulted in the up-regulation of 67 genes. These differentially expressed genes might be direct targets of NEAT1 or targets of genes affected by NEAT1. For example, TP53I13 and were found to be up-regulated. The expression of BRAT1 and TP53I13 are regulated by BARC1 and TP53, respectively. It is therefore possible that NEAT1 down-regulation causes the increase in TP53 and BRCA1 levels, which in turn leads to an increase in the expression levels of their target genes. TP53I13 is reported to act as a tumour suppressor and its over-expression is known to inhibit tumour cell growth (Hata et al, 2004). The protein encoded by BRAT1 interacts with the BRCA1 and ATM (ataxia telangiectasia mutated) protein. ATM, BRCA1 and BRAT1 complex (BRAC1-associated genome surveillance complex) plays an important role in the cellular responses to DNA damage (Low et al, 2015; Aglipay et al, 2006).

RNA sequencing results confirmed that NEAT1 functions as important cis and trans-acting modulator for the expression of protein-coding genes. Seven genes located on chromosome 11 were found to be up-regulated as a result of decrease in NEAT1 transcript levels. Two of these genes are located on 11q13.1 the same chromosomal band as NEAT1; one gene is located on 11q13.2. Another four of these genes are

located on 11p15.5. The other genes affected by NEAT1 are located on different genomic locations across the genome. These results confirm that NEAT1 controls gene expression and has an impact on its target genes in *cis* and in *trans*. Genes affected by NEAT1 down-regulation are implicated in a number of pathways with the most significant ones include galactose catabolism, tight junction and Fatty acid metabolism. Other pathways affected include systemic lupus erythematosus, viral carcinogenesis, AMPK and MAPK signalling, RNA degradation, VEGF signalling, DNA repair, central carbon metabolism in cancer , sphingolipid metabolism, HIF-1 signalling pathway, Sphingolipid signalling pathway and cell cycle

Overall, the results presented in this chapter confirm the modulatory effects of NEAT1 on gene expression. The current study supports a role for NEAT1 in the regulation the expression of genes involved in regulating the cell cycle, proliferation and functions as tumour suppressors. The results also confirm that NEAT1 can act in *cis* to regulate the expression of nearby genes or in *trans* to regulate genes at other genomic locations across the genome. Since no changes were seen in the long isoform. It is therefore possible that NEAT1 short isoform is the transcript involved in the regulation of gene expression. Further experiments are required to validate the effects of NEAT1 on the expression of the genes identified in the present work.

Chapter highlights

1. NEAT1 down-regulation affects the expression of genes involved in the regulation of cell cycle checkpoints and promotes the expression of genes involved in the negative regulation of the cell cycle.
2. NEAT1 down-regulation leads to increase in the expression of BRCA1, BRCA2, CDKN1A, CDKN2A and TP53.
3. Decrease of the NEAT1 transcript levels resulted in the down-regulation of the expression of CCND2, CCND1, CCNE1 and MKI67
4. NEAT1 down-regulation has a significant impact on the regulation of a number of pathways including galactose catabolism, tight junction and fatty acid synthesis
5. The short isoform of NEAT1 may act in *cis* to regulate the expression of nearby genes or in *trans* to regulate genes at other genomic locations across the genome.

Chapter 6

The role of the long non-coding RNA

MIAT in breast cancer

6.1 Introduction

In addition to NEAT1 and MALAT1, myocardial infarction associated transcript (MIAT) is another nuclear lncRNA localised to nuclear bodies. Many studies revealed the importance of MIAT in biological cell function and its crucial role in certain pathological conditions (Liao et al 2015; Vausort et al 2014; Yan et al. 2015; Sattari et al.2016). For instance, Ishii et al. (2006) has reported a positive relationship between abnormal expression of MIAT and the incidence of myocardial infarction. The study has also found a single nucleotide polymorphism (SNP) in MIAT gene that leads to its abnormal transcription in patients with myocardial infarction (Ishii et al. 2006). Furthermore, MIAT is reported to be involved in microvascular regulation and in the control of pathways that are responsible for cell proliferation, migration and apoptosis of endothelial cells (Yan et al. 2015). MIAT is reported to act as a competing endogenous RNA (ceRNA) which is involved in a feedback loop with vascular endothelial growth factor (VEGF) and miR-150-5p to regulate endothelial cell function (Yan et al. 2015). Accordingly, in normal circumstances, MIAT binds to miRNA150-5p and suppress its posttranscriptional effects on VEGF mRNA and hence regulating the process of endothelial angiogenesis (Yan et al. 2015).

Recent studies have also implicated MIAT in cancer initiation and progression (Crea et al, 2016). MIAT was found to be upregulated in neuroendocrine prostate cancer (NEPC) and its up-regulation was associated with Polycomb genes, which play a key role in NEPC initiation and progression (Crea et al, 2016). Moreover, MIAT was suggested to act as a new biomarker for detecting the advance stages of chronic lymphocytic leukemia (CLL) (Sattari et al, 2016). MIAT was found to be up-regulated in aggressive forms of CLL and was shown to form a regulatory loop with

Oct4 in malignant mature B cell where both molecules are essential for cell survival (Sattari et al., 2016). Overall, these studies suggest an important role for MIAT in regulating the survival of some cancer cells. The evidence presented in Chapter 3 shows that MIAT is overexpressed in TNBC and suggests that MIAT could be implicated in breast cancer. Since the role of MIAT in breast cancer has not been investigated yet, the present study aimed to assess the functional effects of MIAT down-regulation on the survival of breast cancer cells.

6.2 Materials and Methods

6.2.1 RNA interference by siRNA

MCF7 and MDA-MB-231 breast cancer cells were nucleofected with MIAT specific siRNAs as described in section 2.2.5. Details of MIAT specific siRNAs are presented in Table 2.3.

6.2.2 Assessment of cell survival, apoptosis and cell cycle

At 48h post transfection, cells were trypsinised and re-plated at 2×10^5 cells/well in 6 well plates. Cell survival and apoptosis were determined at 24 h and 48 h post re-plating, as described in section 2.2.7.2 and 2.2.7.4. Long-term survival of MCF7 and MDA-MB 231 was determined by counting the violet stained colonies that formed after 2-3 weeks of incubation (section 2.2.9). Cell cycle profile was determined 24 h post re-plating according to the protocol described in section 2.2.8.

6.2.3 Induction of cell death and cell survival assays

The effect of UV-C irradiation was determined in MCF7 and MDA-MB231 cells as explained in section 2.2.11. Long term survival was assessed by colony forming assay and the percentage of apoptosis was determined by flow cytometry and measurement of Annexin V (Section 2.2.7.4) at 24h and 48h post-UV irradiation.

For drug treatments, siRNA transfected MDA-MB231 cells were re-plated at a density of 0.8×10^5 cell/ml in 96 well plates and cultured for 20 h before being treated with different types of chemotherapy drugs as described in section 2.2.11. The growth inhibitory effect of chemotherapeutic drugs (the cytotoxicity effect) was determined at 24h and 48h of drug treatment using the MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay) and calculated according to this equation:

$$\% \text{ of cytotoxicity} = 100 - [\text{OD}_{490} \text{ of treated sample} / \text{OD}_{490} \text{ of untreated sample (vehicle)}] \times 100$$

6.2.4 Real time RT-PCR (RT-qPCR)

The expression levels of MIAT and Oct4 genes were determined using RT-qPCR. TaqMan gene expression assays (assay codes Hs99999901_m1 for 18S, Hs00402814_m1_for MIAT and Hs0004260367_gH for Oct4 (POU5F1) were employed with cDNA prepared by random hexamer priming, as described previously in Section 2.2.13. Input amounts of samples were calculated from their respective threshold cycle (CT) values, using standard curves generated with each assay. Data were expressed relative to 18S rRNA

6.2.5 Statistical analysis

Data are presented as mean \pm standard error of the mean (S.E.), where (n) represent the number of experiments. Statistical analysis was determined by Student's t-test using the GraphPad 7 software. Two-sided P-values were calculated, and a probability level of less than 0.05 was chosen for statistical significance.

6.3 Results

6.3.1 The effects of MIAT silencing on the survival of MCF 7 breast cancer cells

To examine the effects of reduced MIAT expression on breast cancer cell survival, MIAT siRNAs were employed to silence endogenous MIAT expression in MCF7 cell line; two different siRNAs were employed to reduce the likelihood of 'off-target' effects. The influence of MIAT silencing on MCF7 cell survival was examined under basal conditions and after apoptosis induction by UV.

In MCF7 cells, siRNAs reduced MIAT transcript levels by up to 70-85% compared to control levels (Figure 6.1A). Oct4 is a transcriptional factor reported to promote MIAT expression. A positive correlation between MIAT and Oct4 expression levels have been reported (Sattari et al, 2016). Therefore, the effects of reduced MIAT expression levels on the expression of Oct4 were also determined. Down-regulation of MIAT was found to be associated with a decrease in the expression levels Oct4. In MCF7 transfected cells, Oct4 transcript levels were found to be 70-80% less than that of control (Figure 6.1B), indicating the positive relationship between MIAT and Oct4 expression.

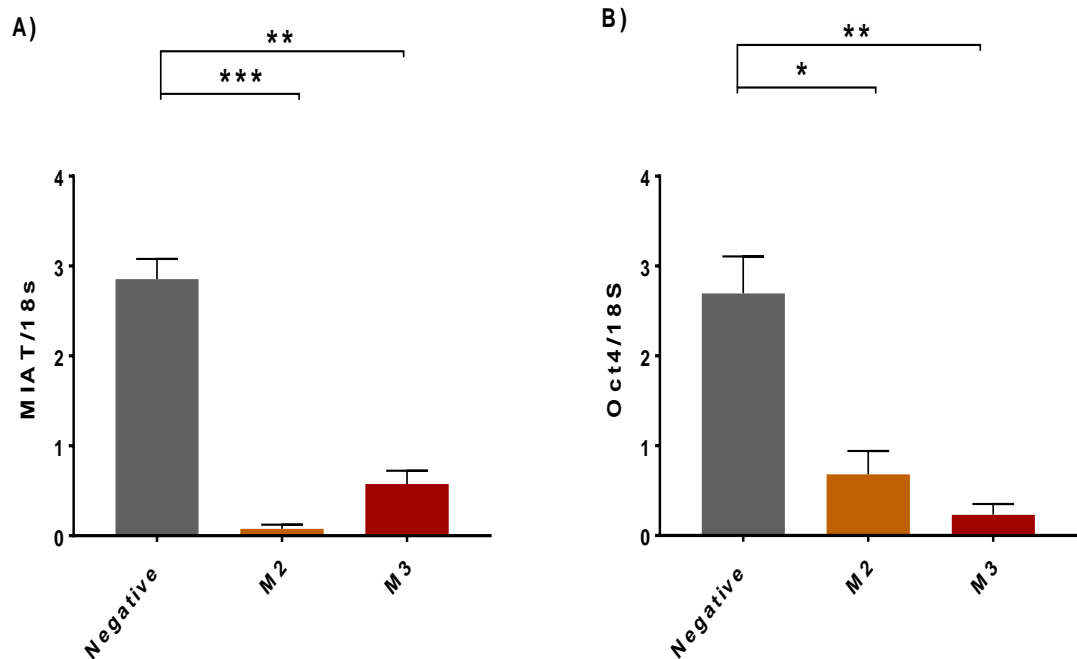


Figure 6.1 The effects of MIAT specific siRNAs on MIAT and OCT4 expression levels in MCF7 cells. MIAT specific siRNAs (M2 and M3) and negative siRNA were nucleofected into MCF7 cells. RNA was isolated 72h post-transfection and qRT-PCR was performed. (A) Cellular MIAT expression using MIAT assay located at the position 1864. There was a highly significant ($***P<0.01$ and $**P<0.01$; $n=4$) down-regulation cells transfected with M2 and M3 siRNAs. (B) Expression profile of Oct4 in cells transfected with MIAT siRNAs. There was a significant ($*P<0.05$ and $**P<0.01$; $n=4$) decrease in expression level as compared to the negative control. (Unpaired t-test)

MIAT down-regulation caused a small but significant elevation in the total cell number of MCF7 cells (Figure 6.2A) and a significant reduction in the number of viable cells as shown in Figure 6.2B. Furthermore, the silencing effects of MIAT siRNAs was prominent in long term cell survival, in which there was a highly significant decrease in number of colonies formed as detected by long term Clonogenic assay in Figure 6.2 C and 6.2 D.

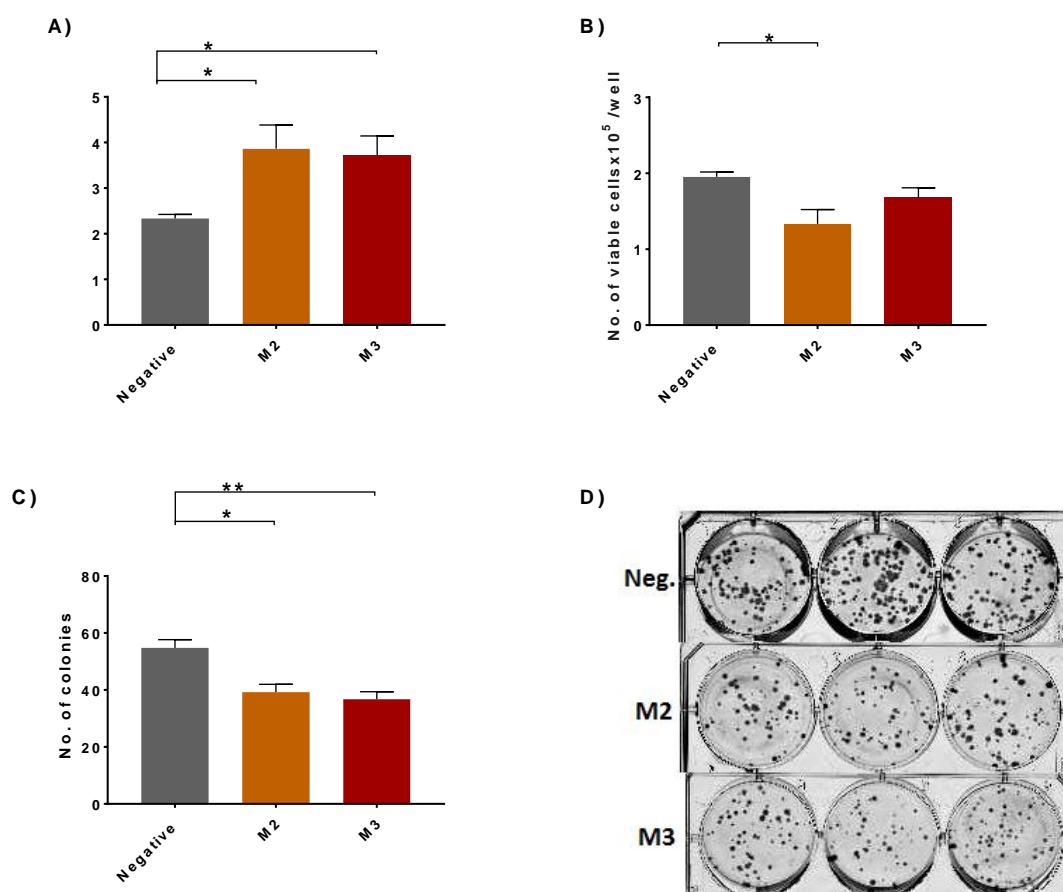


Figure 6.2 The effects of MIAT silencing on the survival of MCF7 cells. MCF7 Cells were transfected with negative siRNA or one of the MIAT siRNAs (M2 and M3 siRNAs) using nucleofection. Cell survival was assessed 48 h post re-plating. Flow cytometry was used to determine cell number using Muse cell analyser and Muse Count & Viability Assay Kit. (A) Number of total MCF7 cells. There was a significant ($*P<0.05$; $n=4$) elevation in the total number of cells. (B) Number of viable MCF7 cells. There was a significant ($*P<0.05$; $n=4$) decrease in number of viable cells particularly in those cells transfected with M2 siRNA as compared with the negative control. (C) Long term survival. Number of colonies formed in long-term clonogenic assays, which showed a significant ($*P<0.05$ and $**P<0.01$ $n=4$) decrease for those cells transfected with M2 and M3 siRNAs respectively as compared with the negative control. (D) An example images of the clonogenic assay after crystal violet staining. (Unpaired t-test)

MIAT down-regulation was also associated in a small but significant increase in the levels of basal apoptosis as in Figure 5.3. Together, these observations provide an evidence that MIAT regulate cell survival and its down-regulation produces growth inhibitory and pro-apoptotic effects on MCF7 breast cancer cells (Figure 6.3).

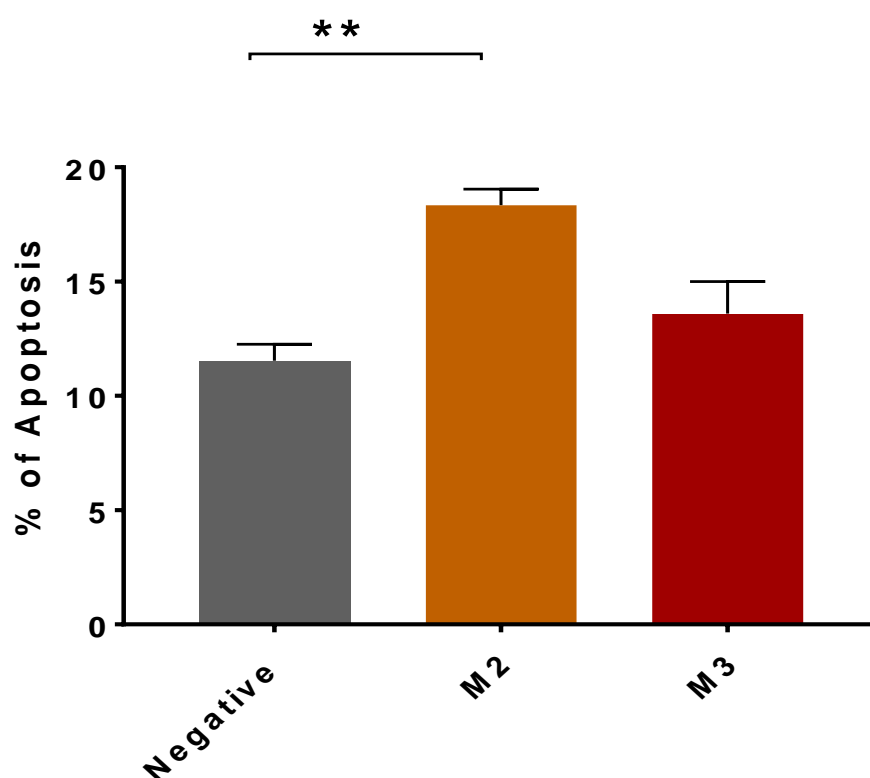


Figure 6.3 Effects of MIAT silencing on basal apoptosis in MCF7 cells. MCF7 Cells were transfected with the negative siRNA or one of the MIAT siRNAs (M2 and M3 siRNAs) using nucleofection. 72h post-transfection, cells were harvested and re-plated in 6 well plates for a further assessment of apoptosis after 24 h. Muse cell analyser and the Muse Annexin V and dead cell kit were used to measure basal apoptosis levels. The results were compared with the negative control. There was a highly significant (* $P < 0.01$; $n = 4$) increase in the percentage of apoptosis in cells transfected with M2 siRNA. (Unpaired t-test)

Further experiments were carried out to investigate the effects of MIAT silencing on the cell cycle profile. MIAT silencing was associated with an increase in the percentage of cells in G1 phase and a concomitant decrease in the percentage of cells in S and G2 phases, as illustrated in Figure 6.4

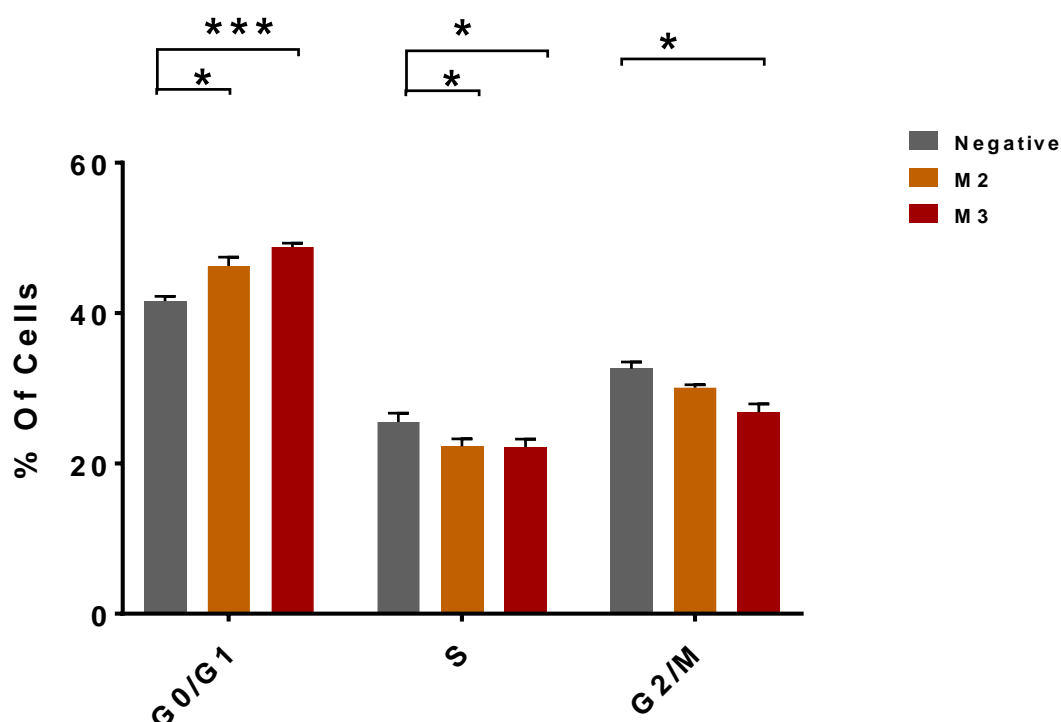


Figure 6.4 Effects of MIAT silencing on the cell cycle profile of MCF7 breast cancer cells. MCF7 cells were transfected with negative siRNA or one of the MIAT siRNAs (M2 and M3). Cell cycle analysis was performed using the Muse cell analyser and the Muse cell cycle kit, which involves quantifying DNA content by propidium iodide staining of fixed cells and fluorescence flow cytometry. There was a significant (* $P < 0.05$ and *** $P < 0.001$; $n = 4$) elevation in the percentage of cells in G0/G1 phase and a significant decrease in percentage of cells in S phase. G2/M phase shows a significant (* $P < 0.05$; $n = 4$) reduction in cell ratio particularly those cells transfected by M3. (Unpaired t-test)

6.3.2 The effects of MIAT silencing on the survival of MDA-MB-231 breast cancer cells

The effects of MIAT silencing on the TNBC cells MDA-MB-231 were also investigated. Transfection of MDA-MB-231 with both MIAT specific siRNAs caused up to 6 fold decrease in the endogenous levels of MIAT compared to the cells transfected with negative siRNA (Figure 6.5 A). Similar to MCF7 cells, this decrease in MIAT expression levels was associated with a decrease in the transcript levels of Oct4 (Figure 6.5 B).

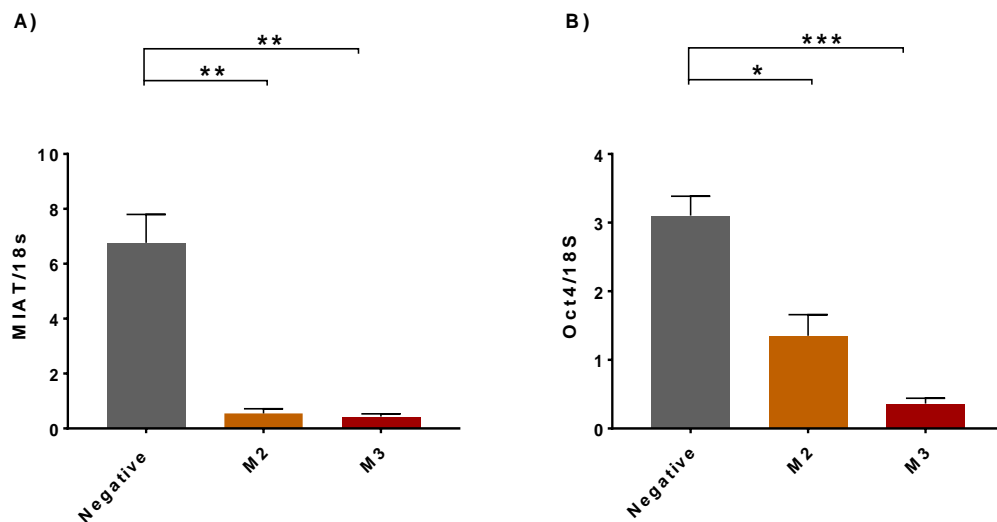


Figure 6.5 Effects of MIAT specific siRNAs on MIAT and Oct4 gene expression in MDA-MB-231 cells. MDA-MB-231 cells were nucleofected with the negative siRNA or one of the MIAT siRNAs (M2 and M3). RNA was isolated 72 h following transfection. (A) Cellular MIAT expression levels using MIAT assay that located at the position 1864. There was a significant (** $P < 0.01$ $n=4$) down-regulation in cells transfected with M2 and M3 siRNAs. (B) Expression levels of Oct4. There was a significant (* $P < 0.05$ and *** $P < 0.001$; $n=4$) respectively decrease in expression level compared to the negative control. (Unpaired t-test)

The decrease in MIAT expression levels led to a small increase in total cell number which was significant with the cells transfected with M2 siRNA and a decrease in the number of viable cells (Figure 5.6 A and B) respectively. MIAT silencing also caused a significant reduction in long term survival reflected by the decrease in the number of colonies in Figure 6.6 C. MIAT silencing also caused a slight but significant increase in basal apoptosis levels (Figure 6.7).

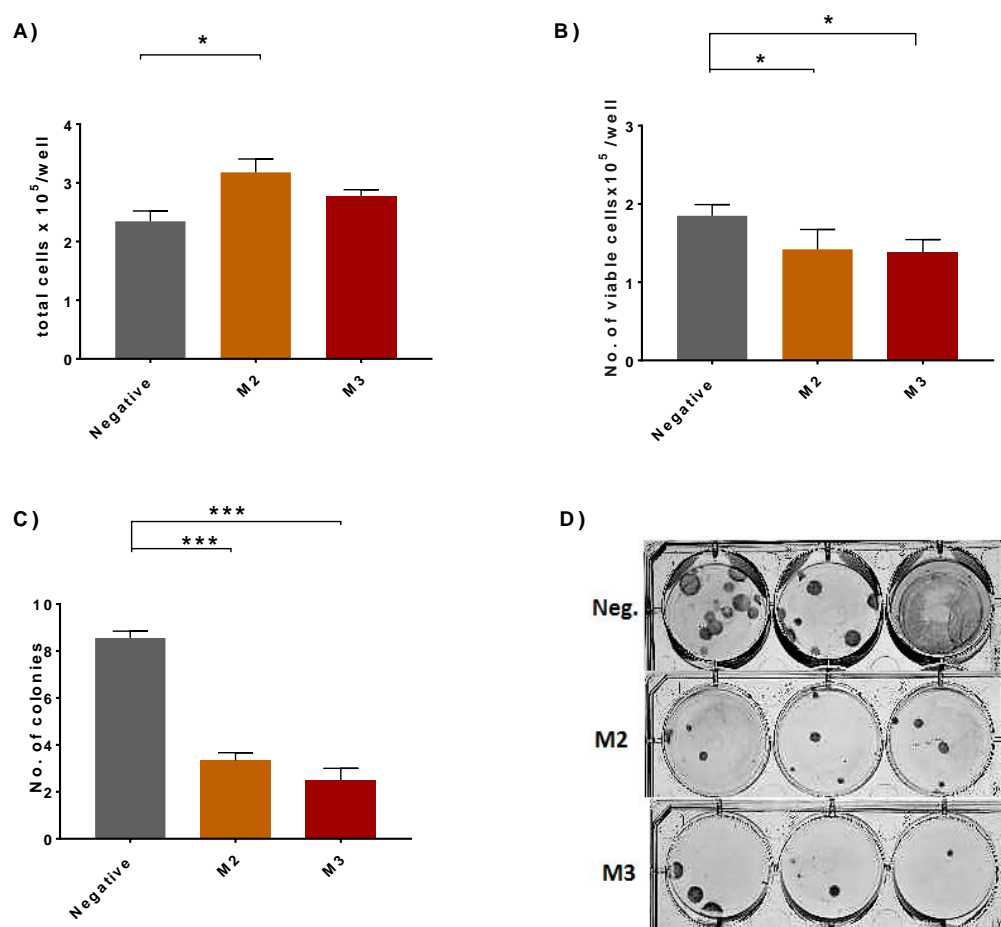


Figure 6.6 The effects of MIAT silencing on the survival of MDA-MB-231 cells. Cells were transfected with negative siRNA or one of the MIAT siRNAs. Total and viable cell numbers were determined using the Muse cell analyser with Muse Count & Viability Assay Kit. (A) The number of total MDA-MB 231 cells in which there was a slight but significant (*P<0.05; n=4) elevation in total cells. (B) The number of viable MDA-MB-231 cells where a significant (*P<0.05; n=4) decrease in number of viable cells in cells transfected with M2 and M3 compared with the negative control. (C) Colony forming assay. There was a significant (***P<0.001 n=4) decrease in the number of colonies for cells transfected with M2 and M3 siRNAs compared with the negative control. (D) An example image of clonogenic assay plates after crystal violet staining. (Unpaired t-test)

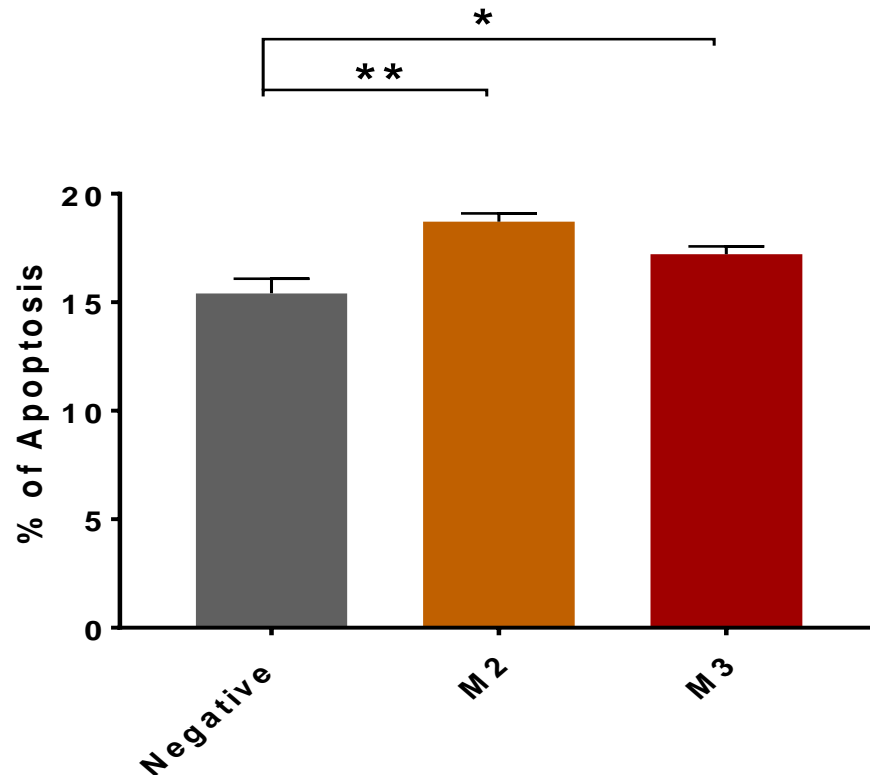


Figure 6.7 The effects of MIAT down-regulation on basal apoptosis levels in MDA-MB-231 cells. MDA-MB-231 Cells were transfected with the negative siRNA or one of the MIAT siRNAs (M2 and M3 siRNAs). Muse cell analyser using the Muse Annexin V and dead cell kit were used to measure the apoptosis levels. The results were compared with the negative control. There was a highly significant (* $P < 0.01$ and * $P < 0.05$; $n = 4$) increase in the percentage of apoptosis. (Unpaired t-test)

Analysing the cell cycle profile revealed that MIAT silencing caused an increase in the percentage of cells in G0/G1, which was not statistically significant (Figure 6.8). However, a significant decrease in the cells in S and G2/M phase was observed in the cells transfected with MIAT siRNAs (Figure 6.8).

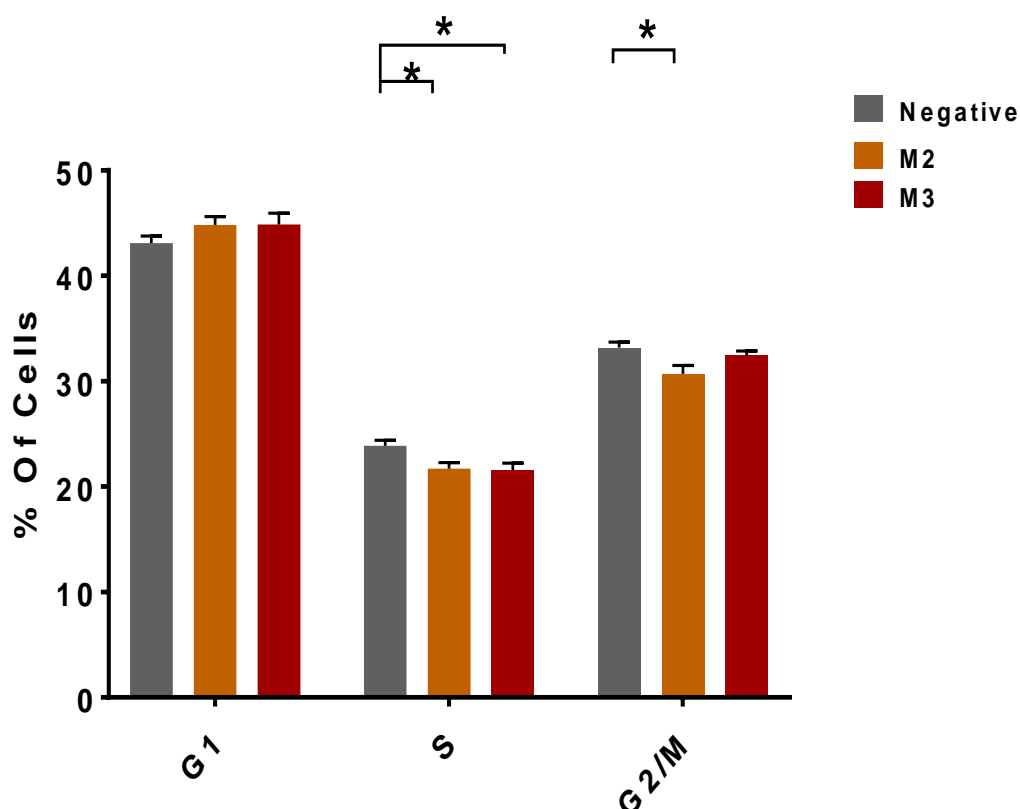


Figure 6.8 Effects of MIAT silencing on the cell cycle profile of MDA-MB-231 breast cancer cells. MDA-MB-231 cells were transfected with negative siRNA or one of the MIAT siRNAs (M2 and M3 siRNAs). Cells were harvested and re-plated for a further 24h for cell cycle analysis. Cell cycle analysis were performed by flow cytometry. There was a significant (* $P < 0.05$ $n = 4$) reduction in the percentage of cells in S phase and small but significant decrease in the percentage of cells in G2/M phase (* $P < 0.05$; $n = 4$). (Unpaired t-test)

6.3.3 The effects of MIAT silencing on UV- and chemotherapeutic drugs- induced cell death in breast cancer cells

The previous results confirmed the importance of MIAT transcript levels in the control of cell fate. MIAT down-regulation is associated with a decrease in cell survival. The next set of experiments examined the effects of MIAT silencing on the cell death induced by a number of apoptotic stimuli. Transfected cells were exposed

to UV-C before being assessed for their ability to form colonies, which represents the most stringent test of cell viability. MIAT silencing enhanced the loss of long term survival induced by UV-C irradiation in MCF7 cells, as determined by long-term clonogenic assay (Figure 6.9 A and B). Similar results were obtained with MDA-MB-231. MIAT silencing in these cells enhanced the loss of long term survival induced by UV irradiation (Figure 6.10 A and B) respectively.

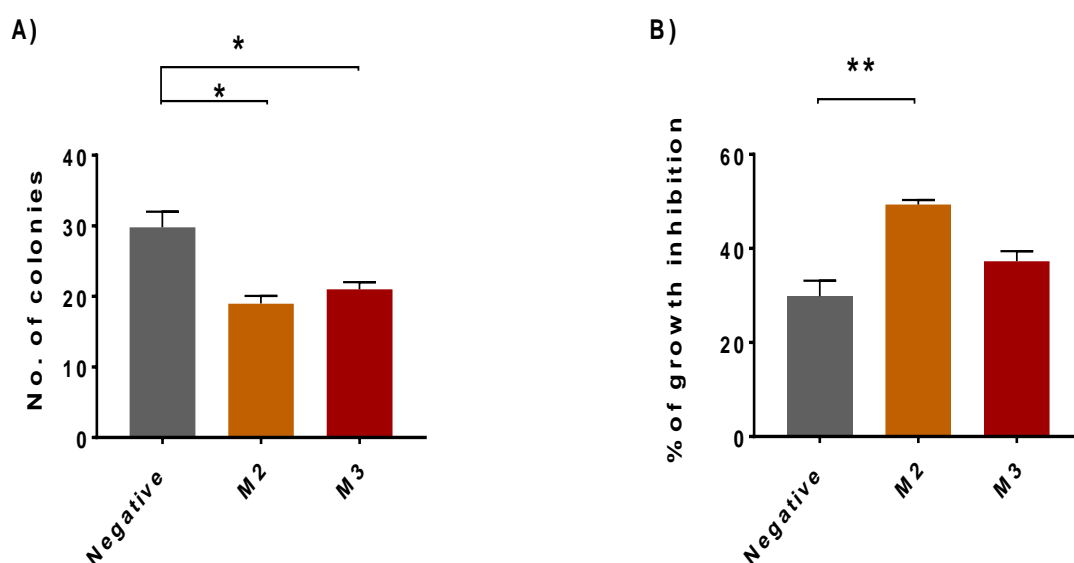


Figure 6.9 The effects of MIAT silencing on UV-induced cell death in MCF7 cells. MCF7 Cells were transfected with negative siRNA or one of the MIAT siRNAs (M2 and M3). Transfected cells were exposed to UV-C irradiation for 20s at dose of 40J/M², before being plated for clonogenic assay and incubated at 37°C and 5% CO₂ for a further 20 days. The effect of UV-C irradiation was determined by calculating the percentage of growth inhibition in cell colony. (A) Shows the number of colonies, where UV irradiation causes a significant (*P<0.05) decrease as compared to the negative control. (B) Revealed the ratio of growth inhibition because of irradiation, in which UV irradiation causes a highly significant (**P<0.01) elevation in growth inhibition ratio as compared to the negative control.. (Unpaired t-test)

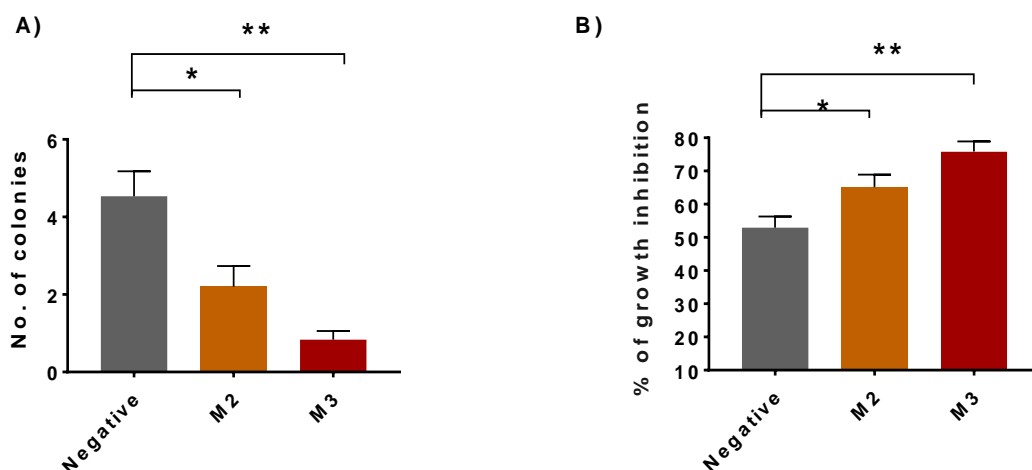


Figure 6.10 The effects of MIAT silencing on UV-induced cell death in MDA-MB-231 cells. MDA-MB-231 Cells were transfected with negative siRNA or one of the MIAT siRNAs (M2 and M3). Transfected cells were exposed to UV-C irradiation for 20s at dose of 40J/M², before being plated for clonogenic assays and incubated at 37°C and 5% CO₂ for a further 20 days. The effect of UV-C irradiation was determined by calculating the percentage of growth inhibition in cell colony. (A) Shows the number of colonies, where UV irradiation causes a significant (*P<0.05 and **P<0.01) decrease in number of colonies as compared to the negative control. (B) Revealed the ratio of growth inhibition because of irradiation, in which UV irradiation causes a significant (*P<0.05 and **P<0.01) elevation in growth inhibition ratio as compared to the negative control. (Unpaired t-test)

Further experiments were carried out to examine the effects of MIAT silencing on chemotherapeutic drug action in the TNBC cells MDA-MB-231. Transfected cells were treated with Docetaxel, 5-Floururacil (5-FL), Nutlin-3a or Mitoxantrone and cell survival was assessed using the MTS assay. MIAT silencing enhanced the sensitivity to these drugs, especially for those cells transfected with M2 siRNA as revealed in (Figure 6.11). These results suggest that MIAT plays a crucial role in the impaired responses to chemotherapeutic drugs in breast cancer.

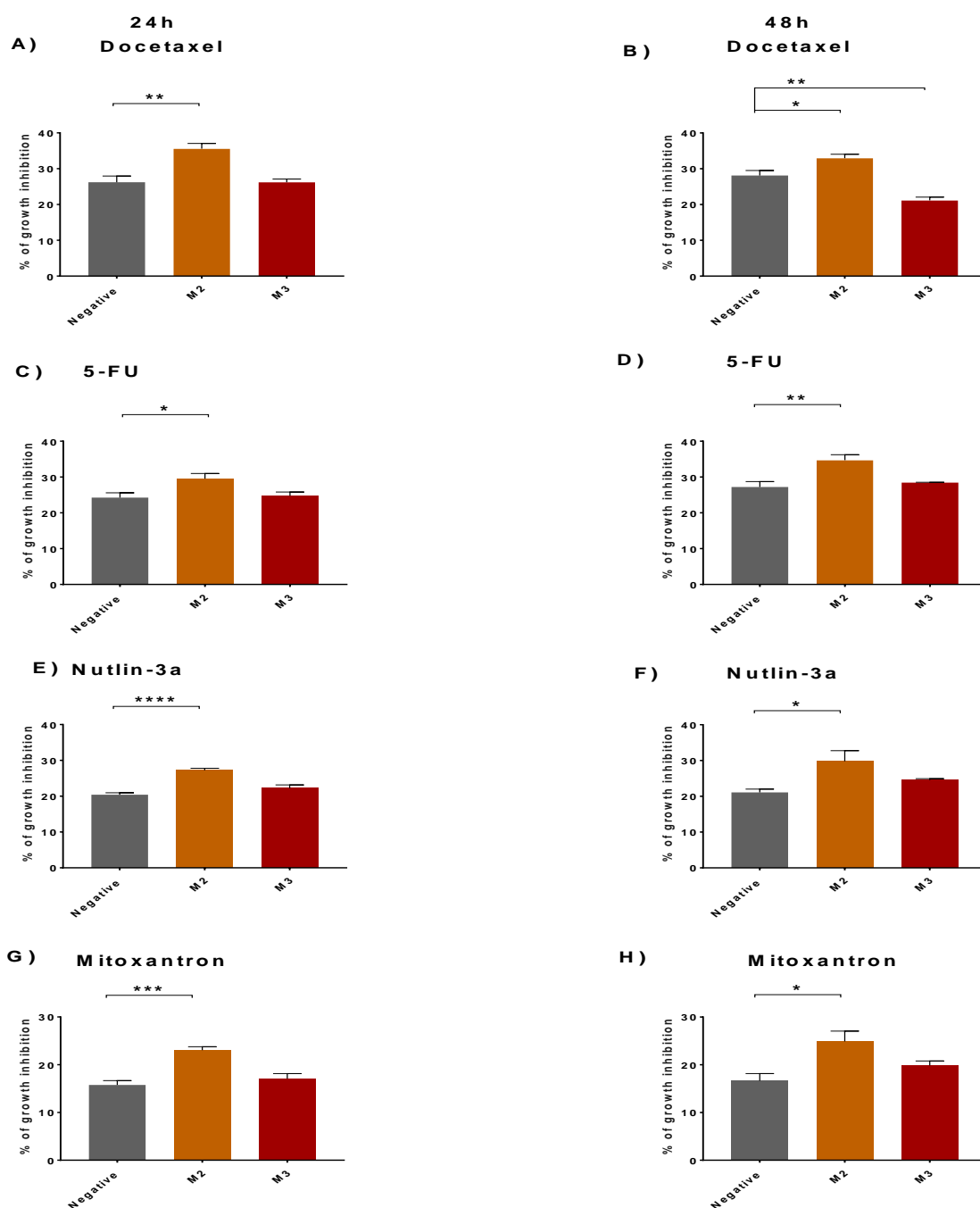


Figure 6.11 The effects of MIAT reduced expression levels on chemotherapeutic drug-induced death of TNBC cells. MDA-MB231 cells were transfected with negative siRNA or one of the MIAT siRNAs (M2 and M3). Transfected cells were harvested and a density of 0.8×10^5 cell/ml and cultured for a minimum 20h in 96 well plates. After that, cells were treated with Docetaxel (5 μ M), 5-Fluorouracil (100 μ M), Nutlin-3a (5 μ M) and Mitoxantrone (50 μ M) or vehicle (25% dimethyl sulphoxide). Cells were incubated in 37° C and 5% of CO₂ for a further 24 and 48h. Cell viability was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS assay) and growth inhibition was calculated relative to the control in the absence of drug. There was a significant and highly significant (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001) elevation in growth inhibition after 24h and 48h of cell treatment. (Unpaired t-test).

6.4 Discussion

The present study demonstrates, for the first time, that MIAT regulates the survival and proliferation of oestrogen receptor-positive breast cancer and TNBC cells. The study further demonstrates that reduction in MIAT expression is associated with an enhanced cell death in response to a range of apoptosis-inducing agents (UV-C irradiation, Docetaxel, 5-FU, Nutlin-3a and Mitoxantrone). The results described here confirm the positive correlation between the expression MIAT and Oct4 and suggest that MIAT regulates the expression of Oct4 pointing to the existence of MIAT and Oct4 axis that controls breast cancer cell fate

.The results revealed that endogenous expression level of MIAT is of critical importance for the survival and growth of breast cancer cells. Modulation of the expression of MIAT was shown to cause significant and specific effects on the survival and proliferation of breast cancer cells. MIAT knockdown in both oestrogen receptor positive and TNBC cells was associated with the consistent and significant decrease in both short and long term viability and the increase in apoptosis levels in the absence of extracellular stimuli. Together, the data supports an important role for MIAT in maintaining the delicate balance between cell survival and cell death in breast cancer cells and points to an oncogene function for this lncRNA in breast cancer.

Oct4 is a transcription factor that contributes with MIAT in forming a regulatory feedback loop (Sattari et al, 2016; Mohamed et al, 2010; Nobili et al, 2017; Ghosal et al, 2013). The relationship between Oct4 and MIAT was first reported in mouse ES cells (Mohamed et al., 2010). Gomafu, the mouse homologue of MIAT, has been shown to bind to Oct4 gene leading to an increase in its expression and Oct4 was

also find to bind and positively regulates Goma fu transcription in mouse ES cells, and thus, they both constitute a regulatory feedback loop (Mohamed et al, 2010). Sattari et al (2016) confirmed the existence of a regulatory feedback loop between MIAT and Oct4 in aggressive chronic lymphocytic leukaemia and that both molecules act on suppressing apoptotic cell death in malignant mature B cells. Oct4 over-expression increases the cell proliferation and inhibits the apoptosis in myeloid cell with mature B cell phenotype and in cancer stem cell-like cells. Oct4 down-regulation increases cell death and inhibits proliferation (Sattari et al, 2016; Hu et al, 2008; Wang et al, 2013). The present work also supports the existence of a MIAT and Oct4 axis involved in the regulation of breast cancer cell survival. It also confirms that MIAT regulates the expression of Oct4 and that both molecules are required for breast cancer cell survival. These observations are in agreement with a number of studies, which report the important role of MIAT in regulating the cell growth and proliferation (Sattari et al, 2016; Yan et al, 2015; Shen et al, 2016). MIAT lncRNA may act as a regulator of gene expression at transcriptional and post-transcriptional level (Yan et al, 2015). Besides its role as co-activator for Oct4 mRNA, MIAT lncRNA is considered as one of a ceRNA, where it forms a regulatory feedback loop with miRNA-150-5p and VEGF (vascular endothelial factor) responsible for regulating the biological function of endothelial cells (Yan et al, 2015; Mohamed et al, 2010; Yi et al, 2017). Yan et al. (2015) also found that MIAT up-regulation leads to a suppression in miRNA-150-5p (post transcriptionally regulatory factor) resulting in the over-expression of VEGF mRNA and hence promoting the angiogenesis (Yan et al, 2015). The increase in the basal apoptosis levels caused by the reduced levels of MIAT transcripts could be related to the inhibitory role of miRNA 150-5p which prevents the expression of AKT mRNA and hence decrease its anti-apoptotic effects (Shen et al, 2016). Shen et al (2016) have reported a

relationship between AKT and MIAT lncRNA and a role for both molecules in regulating the cell function in human lens epithelial cells (HLEC). The study confirmed that knockdown of MIAT in HLEC leads to growth inhibitory effects, which can be reversed by an injection of AKT activator (Shen et al, 2016). Recent study has also implicated MIAT in the regulation of apoptosis in diabetic retinopathy (DR), a complication of diabetes mellitus (Zhang et al. 2017). Recent studies using high glucose stimulated rat retinal Müller cells have shown that activation of nuclear factor κ B (NF- κ B) promotes the expression of MIAT (Zhang et al., 2017). The increase in MIAT expression was associated with an increase in apoptosis. MIAT suppression reversed the high apoptosis induced by high glucose, indicating that MIAT suppression might serve as protectant in diabetic retinopathy (Zhang et al, 2017). Interestingly, the increase of MIAT expression in high glucose stimulated rat retinal Müller cells led to a decrease in the expression levels of miR-29b, a biomarker for Diabetic retinopathy. miR-29b regulates the expression of the transcription factor SP1 which is involved in PI3K/Akt/Sp1 pathway. The study showed that MIAT controlled the cell apoptosis in DR might be partly through absorbing miR-29b and inhibiting its function, meanwhile regulating the expression of SP1. Therefore, MIAT regulates apoptosis in diabetic retinopathy via a regulatory loop of NF- κ B / MIAT/ miR-29b / Sp1 (Zhang et al. 2017; Yu et al. 2015)

MIAT silencing affected the cell cycle profile of breast cancer cells. In MCF7 cells, there was an increase in the percentage of cells in G1 suggesting that more cells are arrested in G1 and prevented from entering the S and G2/M phases. In MDA-MB-231, reduction in MIAT expression levels caused a decrease in the number of cells in S and G2/M phases but the elevation in G1 phase was not significant. The effects of MIAT down-regulation on the cell cycle might be explained by the effects of MIAT silencing on the levels of Oct4. Down-regulation of Oct4 leads to the

activation of p21 protein, which regulates cell cycle progression at G1 and S phase (Lee et al, 2010). P21 protein is responsible for preventing the continuation of the cell cycle, and hence arresting the cells in G1 phase in embryonic stem cells (Lee et al, 2010). In addition, miRNA 150-5p overexpression is reported to lead cell cycle arrest and an increase in the rate of apoptosis in pancreatic cell cancer (Sun et al, 2013). MIAT lncRNA is a ceRNA that represses the miRNA 150-5p and therefore its down-regulation might lead to cell cycle arrest by the subsequent activation of miRNA150-5p. miRNA150-5p enhances apoptosis and regulates the expression of genes involved in the regulation of cell cycle (Sun et al, 2013).

The consequences of reduced MIAT expression for breast cancer cell survival have also been addressed here. The results demonstrate that reductions in MIAT expression are consistently associated with an enhanced cell death in response to a range of apoptosis-inducing agents (UV-C irradiation, Docetaxel, 5-Fluorouracil, Nutlin-3a and Mitoxantrone). Reduced MIAT expression enhanced growth inhibition induced by UV irradiation in oestrogen receptor-positive breast cancer and TNBC cells. TNBC cells transfected with one of the MIAT specific siRNAs (M2 siRNA) enhanced the growth inhibition induced with the chemotherapy drugs. Thus, reduction in endogenous MIAT levels may enhance the responses of breast cancer cells to certain death-inducing stimuli, including conventional chemotherapeutic agents. The effects of MIAT down-regulation on increasing the sensitivity of TNBC cells to chemotherapy drugs might be caused by the decreased levels of Oct4. Over-expression of Oct4 promotes prostate cancer cells resistance against chemotherapy (Linn et al, 2010). At the same time, chemotherapy drugs play an important role as a repressor for Oct4 expression and hence increase the sensitivity of the cells by inhibition the emergence of drug resistance (Yang et al, 2012b). The fact that only one MIAT siRNA caused an enhanced response to chemotherapeutic

drugs might be due to the fact that siRNA down-regulation of MIAT with the M3 siRNA lasted for a short time. Previous studies have reported that, for unknown reason, it is very difficult to silence MIAT with high efficiency using siRNAs (Mohamed et al, 2010). This was first showed in ES cell study (Mohamed et al, 2010) and later in malignant mature B cells (Sattari et al. 2016). Sattari et al (2016) reported up to 50% reduction in MIAT expression levels after repetition the transfection for three times. The study also reported an increased in apoptosis levels in the malignant B cells transfected with MIAT siRNAs. Similar approach was used during the course of this study where transfection with MIAT siRNAs was repeated two times within a 24h interval. No difference in the extent of MIAT down-regulation was observed when compared to the cells transfected with MIAT siRNAs once. Although further study by applying different approach for MIAT silencing is needed to further determine the functions of MIAT in breast cancer cell survival, the present study indicates that MIAT may play an oncogenic role in breast cancer.

Chapter highlights

1. MIAT down-regulation suppresses the expression of Oct4 transcripts.
2. MIAT down-regulation leads to the decrease in the short and long-term survival of oestrogen receptor-positive breast cancer and TNBC cells. These effects were associated with an increase in the rate of basal apoptosis levels.
3. MIAT down-regulation enhances the response of oestrogen receptor-positive breast cancer and TNBC cells to UV-induced cell death.
4. MIAT down-regulation increases the sensitivity of TNBC to chemotherapeutic drugs.

Chapter 7

General Discussion and Concluding Remarks

Breast cancer is a heterogeneous disease characterised by abnormal growth in breast tissues. It represents the most common worldwide disease and the leading cause of death among women in less developed countries (Cancer, I.A.F.R.O, 2013). The aetiology of breast cancer is attributed to a certain factors including hereditary, hormonal, reproductive in addition to environmental factors, like diet, life style, smoking and occupational exposure (Debruin and Josephy, 2002). Therefore, understanding these risk factors and their roles in genetic and epigenetic modulation might lead to establishing new methods for determining the prognostic and therapeutic biomarkers that play an important role in breast cancer management. Many studies have investigated the role of long noncoding RNAs (lncRNAs) in different types of cancer, including breast cancer (Su et al, 2014; Zhang et al, 2016 c; Jiang et al, 2016 a). LncRNAs are a major component of the human transcriptome and are increasingly recognised to play essential regulatory roles in essential biological processes and consequently their dysregulation may contribute in many human diseases, including cancer (Liz and Esteller, 2016). Indeed, many lncRNAs have been identified to play important role in cancer and it is now widely acknowledged that many more lncRNAs are likely to be of crucial importance in the pathogenesis of the disease. Therefore, gaining better insights into lncRNA biology may lead to better understanding to pathological characteristics and mechanisms and thereby provide novel opportunities for the diagnosis and treatment of this important disease.

Of particular, interest in this regard, the two lncRNAs NEAT1 and MIAT, which are associated with sub-nuclear structures, with an increasing number of evidence implicating them in the pathogenetic mechanisms of different types of cancer. To illustrate these points, the work reported in this thesis focuses on the role of these two lncRNAs in breast cancer.

7.1The role of Nuclear enriched abundant transcript 1 (NEAT1) in breast cancer

7.1.1 Expression of NEAT1 in breast cancer

NEAT1 is encoded by gene located on chromosome 11q13.1. It is a nuclear-restricted lncRNA where it constitutes the essential structural element of the paraspeckles, sub-nuclear domains implicated in mRNA nuclear retention (Hutchinson et al, 2007; Bond and Fox, 2009). The role of NEAT1 in cancer has been controversial with some studies reporting an oncogenic role and others suggesting that NEAT1 is a tumour suppressor. However, many studies confirmed that NEAT1 is over-expressed in different types of human cancers and its high expression in the cancerous tissues is shown to be associated with prognosis and overall survival in a number of cancers (Adriaens, 2016; Chakravarty et al, 2014; Choudhry et al, 2015).

According to comprehensive gene expression studies, the expression level of lncRNAs is characterised to be tissue- and disease- specific and lower than protein coding genes (Derrien et al, 2012). Such patterns of tissue- and disease- specific expression made many lncRNAs potential candidates to be used as diagnostic and prognostic biomarkers and for monitoring therapeutic responses (Fu et al, 2016; Malih et al, 2016). HOTAIR, H19 and KCNQ1OT1 lncRNAs have been considered as important biomarkers in breast cancer diagnosis. HOTAIR and H19 are reported to show high expression levels in invasive carcinoma (IC) rather than ductal carcinoma in situ (DCIS), while HOTAIR and KCNQ1 Opposite Strand/Antisense Transcript 1 (KCNQ1OT1) lncRNAs show high expression in tumour cells (IC) (Zhang et al, 2015). The current study confirms that NEAT1 is over-expressed in human breast cancer. While previous studies have reported an increased in NEAT1

expression levels in different types of tumours (Qian et al,2016; Choudhry et al,2015; Adriaens et al, 2016), the current study reveals for the first time that the expression patterns of the long and short isoforms of NEAT1 isoforms differ in breast cancer. NEAT1_1 short isoform was found to be significantly up-regulated in breast cancer and its expression was specifically elevated in advance stages of breast cancer and in ER, PR +ve, HER –ve molecular subtype. On the other hand, the expression levels of NEAT1 long isoform were found to be slightly decreased in all the stages, such decrease in the levels of NEAT1_2 was statistically insignificant apart from the levels in TNBC where the decrease was found to be significant.

Variation in the expression of NEAT1 isoforms has been reported previously and studies have shown that the expression of these isoforms is cell specific (Nakagawa et al, 2011). The short isoform NEAT1 _1 is widely and ubiquitously expressed, whereas the expression profile of NEAT1 _2 is found to be restricted to a sub-population of cells originated from certain tissues such intestinal epithelium (Nakagawa et al, 2011). In addition, Chai et al. (2016) reported that HuR, an RNA binding protein and miR-124-3p are responsible for regulation of NEAT1_1 expression and stability in ovarian cancer (Chai et al, 2016). Evidence suggests that HuR promotes ovarian cancer cells growth and invasion by enhancing the over-expression of NEAT1 _1, whilst miR-124-3p suppresses the expression of NEAT1 _1 leading to the inhibition of ovarian cancer cell growth (Chai et al, 2016). Additionally, an oncogenic role of NEAT1 in colorectal cancer has also been reported (Wu et al, 2015). NEAT1_1 was found to be over-expressed in advanced stages and in metastatic tissues (Wu et al, 2015). Overexpression of NEAT1 _1 is also associated with poor prognosis because of its role in enhancing cell growth and invasion (Wu et al, 2015; Li et al, 2017b).

Interestingly, NEAT1_1 overexpression was found to be highly predominant in samples from breast cancer with the molecular subtype ER, PR +ve and HER -ve and down-regulated in triple negative cells. Such observations might be related to the effects of oestrogen receptor overexpression in this molecular subtype of breast cancer, particularly ER α which regulates the expression of different genes in breast cancer including the expression of NEAT1 (Chakravarty et al, 2014). ER α also regulates NEAT1 expression in prostate cancer (Chakravarty et al, 2014; Romano et al, 2010; Lin et al, 2004). ER α -dependent NEAT1 over-expression leads to the chromatin modification which is responsible for guiding NEAT1 transcripts to the promoting sites and activating the expression of target genes (Chakravarty et al, 2014).

MALAT1/NEAT2 is located approximately 55 kb from NEAT1. MALAT1 is implicated in the pathology of many cancers including lung cancer (Ji et al, 2003). MALAT1 is over-expressed in different types of solid tumours such as lung, breast, colon, hepatocarcinoma, pancreatic and prostate cancer and its over-expression has been shown to increase cell proliferation and promotes invasion and metastasis (Tripathi et al, 2013; Meseure et al, 2016; Ma et al, 2015). Abnormal expression of MALAT1 affects the alternative splicing of pre-mRNA leading to an aberrant expression of genes that are responsible for cell cycle regulation, thereby enhancing the proliferation of tumour cells (Tripathi et al, 2013). The present work also showed that MALAT1 expression is elevated in breast cancer samples and such increase was found significant in the ER,PR +ve and HER -ve breast cancer molecular subtype. MALAT1 is also an oestrogen dependent transcript and its expression in breast and prostate cancer is regulated by oestrogen receptors namely ER α / ER β (Aiello et al, 2016). This explains the increased expression of MALAT1 in this molecular subtype of breast cancer. The present study also showed that the expression of MALAT1 in

breast cancer samples was found to correlate positively with NEAT1_1 expression. Evidence of positive correlation between the expression of NEAT1 and MALAT1 has been reported previously (Naganuma and Hirose, 2013; Nakagawa et al, 2012). Such correlation between the expression of the two genes suggests that coordinate dysregulation of these two lncRNAs might play an important role in cancer. Their differential expression according to breast cancer stages and molecular subtypes indicates that these two lncRNAs could potentially be used as markers for prognosis prediction and targeted therapy of breast cancer.

7.1.2 Effects of modulation of NEAT1 expression levels on breast cancer cell survival

One of the aims of the current study was to investigate the effects of NEAT1 silencing on the survival of breast cancer cells. Lipid polymers mediated transfection of NEAT1 specific siRNAs into MCF7 cells led to an unexpected increase in the expression levels of both NEAT1 isoforms. Further experiments revealed that NEAT1 siRNAs delivered using the lipid polymers were effective in silencing the cytoplasmic NEAT1 transcripts and in increasing the expression levels of nuclear NEAT1. These results demonstrated that cellular NEAT1 transcripts reside in the cytoplasm and nucleus and suggested that the cytoplasmic NEAT1 transcript might exert negative feedback on the expression of nuclear NEAT1. Therefore, a decrease in the expression of cytoplasmic NEAT1 was associated with an increase in the expression levels of the nuclear NEAT1. The increase in the expression of nuclear NEAT1 resulted in an increase in short and long-term survival and a decrease in apoptosis. Such observations suggested that NEAT1 might function as an

oncogene. Nucleofection mediated delivery of NEAT1 siRNAs resulted in down-regulation of NEAT1 transcript levels. The reduced level of NEAT1 transcripts was associated with decreased short and long-term viability. Reduction in NEAT1 endogenous levels altered the cell cycle and inhibited cell migration of both triple-negative and oestrogen receptor-positive cells, confirming the oncogenic role of NEAT1 in these cells. ASOs mediated silencing confirmed these observations and provided further evidence that NEAT1 is required for survival in these breast cancer cells.

A number of studies have confirmed the oncogenic role of NEAT1 and reported the effects of NEAT1 over-expression in promoting cell proliferation and suppression of apoptosis (Ma et al, 2016; Xiong et al, 2017; Yu et al, 2017; Ke et al, 2016; Peng et al, 2016). Studies have also reported the effects of NEAT1 down-regulation on inhibition cell growth and survival (Ke et al, 2016; Lo et al, 2016a). The oncogenic property of NEAT1 might be attributed to its role as transcriptional regulator. NEAT1 accumulates at the chromatin site of target genes and causes epigenetic modification, which in turn leads to over-expression of these genes, as reported in prostate cancer (Chakravarty et al, 2014). The other possible explanation of NEAT1 effects might be due to its property as competing endogenous RNA (ceRNA) which acts as a sponge for miR-377-3p leading to up-regulation of the E2F3 gene in non-small cell lung cancer (NSCLC) and hence increases the activity of E2F3 signalling pathway, which is responsible for promoting cell proliferation (Sun et al, 2016). Additionally, Wang et al, (2016) found a regulatory NEAT1/miR-107/CDK6 loop which stimulates cell proliferation in laryngeal squamous cell carcinoma (Wang et al, 2016). NEAT1 in these cells causes suppression to miR-107 and enhances the activity of CDK6 that leads to cell cycle progression (Wang et al., 2016b).

Down-regulation of NEAT1 led to a significant decrease in the short and long term survival of TNBC cancer cells. Such effects might be related to the positive correlation of NEAT1 lncRNA with the activation of PI(3)K/AKT pathway, which is found to be highly activated in basal-like breast tumours (Chin et al, 2013). Activation of PI3K/AKT pathway promotes survival and increase cell proliferation and migration. Peng et al. (2016) reported a direct relation between AKT kinase pathway and NEAT1 expression in colorectal cancer (Peng et al, 2016). In which, downregulation of NEAT1 leads to inactivation of AKT kinase pathway that has a direct impact on the cell cycle, cell survival and apoptosis (Peng et al, 2016; Chin et al, 2013).

Reduced transcripts levels of NEAT1 led to an alteration in the cell cycle profile of breast cancer cells. The effects of NEAT1 down-regulation on the expression of some genes involved in the regulation of cell cycle as shown in Chapter 5, might be responsible for the alteration of cell cycle profile. Reduced NEAT1 levels suppressed the expression of cell cycle positive regulators and increased the expression of genes involved in the negative regulation of cell cycle. Negative regulator of cell cycle cause arresting of the cells in G1 phase and prevent cell cycle progression to S phase (Yang et al, 2017b; Wang et al, 2016 ; Li et al, 2016; Li et al, 2017a).

Reduced levels of NEAT1 were associated with an increase in migration ability of the cells and a reduction in levels of MALAT1 expression. The reduction in MALAT1 expression supports the finding in chapter 3 where positive correlation was found between NEAT1 and MALAT1 in breast cancer samples. The effects of NEAT1 silencing on migration could be attributed to the low expression of MALAT1. MALAT1 promotes the migration of cancerous cells, a fact confirmed by Gutschner (2013). In addition, Song et al, (2017) confirmed the role of NEAT1 in promoting cell invasion and migration in colorectal cancer. Increased levels of NEAT1 lead to the

suppression of miR-662 and hence over-expression of Zinc finger E-box-binding homeobox 2 (ZEB2), a transcription protein that involved in epithelial-mesenchymal transition process (Song et al, 2017).

The current study investigated the consequences of reduced NEAT1 expression levels on cell death induced by a number of apoptotic stimuli. Reduction in NEAT1 expression levels is consistently associated with an increased cell death in response to a range of apoptosis agents including UV-C irradiation, Docetaxel (anti-mitotic drug), 5-FU (antimetabolite that prevent DNA synthesis), Nutlin-3a (Mdm2 antagonist) and Mitoxantrone (antitumor antibiotic, a cell cycle specific drug) (Longley et al, 2003; Herbst and Khuri, 2003; Tabe et al., 2009; Fox, 2004). Down-regulation of NEAT1 enhanced the breast cancer cells response to UV-induced cell death and such response was associated with a significant increase in the expression levels of pro-apoptotic gene BAD. Reduced expression of NEAT1 had positive impact upon the responses of breast cancer cells to some of these conventional therapeutic agents, which could explain why NEAT1 overexpression is associated with poor prognosis in cancer patients (Yang et al, 2017a). Future experiments are required to study the effects of NEAT1 down-regulation on the breast cancer cell response to PI3K/mTOR inhibitors since NEAT1 is reported to be involved in the activation of PI(3)K/AKT pathway (Zhu et al, 2008).

Overall the present study support an important role for NEAT1 in the control of breast cancer cell survival and suggests that NEAT1 may act as an oncogene in breast cancer cells.

7.1.3 Effects of NEAT1 on gene expression

Two strategies were used to investigate the effects of NEAT1 silencing on breast cancer cell gene expression. Breast Cancer and Cell Cycle RT2 Profiler™ PCR arrays were used to investigate the effects of NEAT1 silencing on the genes involved in cell cycle regulation and breast cancer. RNA sequencing was also used to study the effects of NEAT1 silencing on global gene expression in breast cancer cells.

NEAT1 silencing has resulted in the change of expression of a number of genes involved in the control of cell cycle and breast cancer. Interestingly, the reduction in NEAT1 expression level affected the expression of genes involved in the regulation of cell cycle checkpoints and enhanced the expression of genes involved in the negative regulation of the cell cycle. These genes include BRCA1, BRCA2, CDKN1A, CDKN2A and TP53. BRCA1, BRCA2 and TP53 are among six genes that confer a high risk for hereditary breast cancer. The others are PTEN, CDH1, and STK11. Germline mutations in these genes are the most common known causes of hereditary breast cancer (Walsh et al, 2006). BRCA1 and BRCA2 play important role in the regulation of transcription, DNA damage repair and recombination, control of cell cycle checkpoint apoptosis (Yoshida and Miki, 2004; Powell and Kachnic, 2003). A signalling pathway involving BRCA1/NEAT1/miR-129-5p/WNT4 is important in breast cancer initiation (Lo et al, 2016b). BRCA1 deficient cells induce the expression of NEAT1 and this suppress the activity of miR-129-5p by DNA methylation at the CpG Island in miR-129 genes. Down-regulation of miR-129-5p leads to upregulation of WNT4 which stimulates the oncogenic WNT pathway (Lo et al, 2016b). At the same time, overexpression of BRCA1 leads to co-activation of the Tp53-mediated gene expression like p21 and GADD45 that

contribute in suppress the cell cycle progression (MacLachlan et al, 2002; Yoshida and Miki, 2004).

TP53 is a tumour suppressor that plays an essential role in the cellular response to DNA damage and in the induction of apoptosis, cell cycle arrest and DNA repair (Vogelstein et al, 2000; Isik et al, 2014). The fact that down-regulation of NEAT1 leads to the over-expression of such important tumour suppressor genes provides further evidence about the importance of NEAT1 in the pathogenesis of breast cancer. Up-regulation of CDKN1A and CDKN2A expression in cell with reduced NEAT1 levels provides evidence that NEAT1 is involved in the regulation of cell cycle and cell proliferation. CDKN1A and CDKN2A are cyclin dependent kinase inhibitors. The sequential activation of cyclin and cyclin dependent kinases complexes controls the transition from phase G1 to the phase S of the cell cycle (Ekholm and Reed, 2000). These complexes phosphorylate and inactivate the negative regulators of G1 transition into S phase such as members of the retinoblastoma protein (Rb) family. The inactivation of Rb protein results in the induction of E2F (E2 factor)-regulated gene expression and cell proliferation. CDKN1A and CDKN2A binds and inhibits the function of these complexes leading to the inhibition of progression from G1 to S phase (Ekholm and Reed, 2000).

One the other hand, decrease of the NEAT1 transcript levels led to the down-regulation of the expression of CCND2, CCND1, CCNE1 and MKI67. CCND1 and CCND2 belong to the D-type cyclin family (Patil et al, 2009). Both genes play important role in promoting the cell transition from G1 to S phase (Patil et al, 2009). Cyclin E, the protein encoded by CCNE1, is the main regulator for transition from G1 to S phase. CCNE1 is a known oncogene in many types of cancer and has been associated with gene amplifications in various types of malignancies (Pils et al, 2014). The protein encoded by MKI67, Ki67, is a marker of cell proliferation.

Therefore, the decrease in the expression of these genes further supports a role for NEAT1 in increasing cell proliferation and survival.

RNA sequencing provided interesting information on the role of NEAT1 in the regulation of gene expression despite the fact that the levels of NEAT1 were reduced by only 35% at the time of RNA collection. Tumour suppressor genes were found up-regulated as a result of reduced NEAT1 levels. These include Interferon-Stimulated Gene 15 (ISG15), Tumour Suppressing Subtransferable Candidate (TSSC4) and Tumor Protein P53 Inducible Protein 13 (TP53I13). BRCA1 Associated ATM Activator 1 (BRAT1), which is involved in DNA damage response was also found to be up-regulated. A number of genes involved in the regulation of immune response, systemic lupus erythematosus and viral carcinogenesis were also up-regulated. A functional role of NEAT1 in controlling the innate immune response to viral and microbial infection have already been reported. NEAT1 expression is induced in HIV-1 infected T cells as well as influenza virus and herpes simplex virus infected epithelial cells (Geng et al, 2016). NEAT1 down-regulation affected genes involved in galactose catabolism, tight junction, fatty acid sphingolipid metabolism. Previous studies have supported a role for NEAT1 in PPAR γ 2 splicing during adipogenesis (Chen, 2016). Most importantly, the RNA sequencing data has confirmed that the short isoform of NEAT1 may act in *cis* to regulate the expression of nearby genes or in *trans* to regulate genes at other genomic locations across the genome.

While ASOs were effective at causing more than 75% silencing of NEAT1 endogenous levels (Southwell et al, 2014), the RNA sequencing results confirmed that unlike the siRNAs, the silencing effects of these ASOs were short lived. Challenges in silencing lncRNAs have been previously reported due to their varying subcellular localisations, with some residing predominantly in the nucleus, the

cytoplasm or in both compartments. siRNAs have been successfully used to silence cytoplasmic lncRNAs. Their ability to silence nuclear lncRNAs is largely dependent on the method of transfection as shown in chapter 3. The work presented in chapter 3 showed that nucleofection of siRNAs resulted in the silencing of NEAT1 levels. NEAT1 specific ASOs also down-regulated NEAT1 endogenous levels but their effects appeared to be short lived with the NEAT1 levels recovered by the time the RNA was collected for sequencing. The use of antisense LNA™ GapmeRs would have been more efficient at silencing NEAT1. GapmeRs are recommended as an excellent alternative to siRNA for silencing nuclear lncRNAs and are taken up by the cells without transfection reagents (Watts and Corey, 2012). Combining Antisense LNA™ GapmeRs and siRNA reagents have also been shown to have additive effects for the lncRNAs present in both nuclear and cytoplasmic compartments (Lennox and Behlke, 2015) .

Overall, the results presented in chapter 3 confirm that the expression of NEAT1 short isoform is elevated in breast cancer. Chapter 4 provides evidence that NEAT1 plays an important role in the regulation of breast cancer cell fate. The results confirmed that reduced level of NEAT1 is associated with loss of short and term survival and an increase in basal apoptosis in TNBC cells. The data presented in Chapter 5 indicate that NEAT1 regulates gene expression in breast cancer cells. Collectively the results suggest that NEAT1 plays a critical role in various cellular functions and the process of breast cancer. A recent study based on 560 whole-human genome sequences has identified NEAT1 as one of the genes that carries driver mutations in breast cancer (Nik-Zainal et al, 2016). These findings, together with the evidence presented in this thesis further highlight the importance of NEAT1 in the pathogenesis of breast cancer.

7.2 The role of Myocardial infarction associated transcript (MIAT) in breast cancer

MIAT gene is mapped to human chromosome 22q12.1. The gene consists of 5 exons and highly expressed in the nervous system and retinal tissues (Sone et al, 2007; Sattari et al, 2016). The clinical importance of MIAT was highlighted by its association with the susceptibility to myocardial infarction (Ishii et al, 2006; Liao et al, 2016). Evidence implicating MIAT in cancer is now emerging. Recent studies reported an increase in MIAT expression levels in neuroendocrine prostate cancer, an androgen receptor (AR)-negative metastatic neoplasm (Crea et al.,2016) and in an aggressive form of chronic lymphocytic leukemia (CLL) (Sattari et al, 2016). The work in Chapter 6 aimed to investigate the role of MIAT in breast cancer. Initial studies reported in Chapter 3 involved the analysis of MIAT expression level in the breast cancer samples suggested that MIAT might be down-regulated in stages III-IV of the disease and its level showed a significant increase in TNBC samples. Similar to neuroendocrine prostate cancer where MIAT is up-regulated, TNBC is characterised by negative endocrine receptors and is highly metastatic with poor prognosis (Zhang et al, 2012). Jin et al. (2017) reported positive correlation between tumour necrosis factor (TNF α), a pro-inflammatory cytokine that promote tumour growth, and MIAT in osteogenic differentiation of human adipose-derived stem cells (Jin et al, 2017). However, further experiments are required to confirm the increase of MIAT levels in TNBC due to the small sample size. Some samples in stage I and II showed high expression of MIAT, therefore a correlation analysis between the expression of MIAT and NEAT1 short isoform was carried out. The results showed a positive correlation between the expression of NEAT1 and MIAT. Such observations might due to the effects of MIAT on promoting the expression of Oct4.

An Oct4/NEAT1/MALAT1 axis has been reported in lung cancer where Oct4 promotes and enhances the expression of NEAT1 and MALAT1 lncRNAs via Oct4/NEAT1/MALAT1 pathway (Nobili et al, 2017; Jen et al, 2017). Oct4 is over-expressed in MDA-MB-231 cells and therefore it might promote the expression of NEAT1/MALAT1 in TNBC (Ling et al, 2012).

Nucleofection of MIAT specific siRNAs into MCF7 and MDA-MB-231 resulted in significant decrease in MIAT expression levels. The decrease in MIAT expression levels was associated with a decrease in Oct4 levels, in agreement with other studies in other cell types. Such positive correlation between the expression of Oct4 and MIAT has been reported previously in aggressive CLL (Sattari et al, 2016; Mohamed et al, 2010). Both genes are up-regulated in CLL and are essential for the survival and apoptosis resistance of these cells.

Down-regulation of MIAT resulted in an increase in basal apoptosis and a decrease in short and long term survival of breast cancer cells. These observations suggest that MIAT plays important role in promoting cell survival and proliferation, as shown in CLL and neuroendocrine prostate cancer (Sattari et al.2016; Yan et al. 2015; Shen et al. 2016). MIAT is reported to regulate gene expression at transcriptional and post-transcriptional levels (Yan et al, 2015). Besides its role as co-activator for Oct4 mRNA, MIAT is considered as one of a competing endogenous RNA (ceRNA), where it form a regulatory feedback loop with miRNA-150-5p and vascular endothelial factor (VEGF) which is responsible for regulating the biological function of endothelial cell (Yan et al. 2015; Mohamed et al. 2010; Yi et al. 2017). Yan et al. (2015) found that up-regulation of MIAT suppresses miRNA-150-5 which results in over-expression of VEGF mRNA and hence promoting angiogenesis.

MIAT silencing altered the cell cycle profile and caused an increase in percentage of cells in G1 phase and a subsequent decrease in the cells in G2/M phase. These

observations might be due to the fact that reduced MIAT expression level is associated with decreased levels of Oct4. Suppression of Oct4 expression leads to the activation of p21 protein, a cell cycle regulatory protein at G1/S phase (Lee et al, 2010). Active p21 inhibits cell cycle progression leading to the arrest of cells in G1 phase (Lee et al, 2010). In addition, the inhibitory effects of MIAT on the cell cycle progress might be mediated by miRNA150-5p. The decrease in MIAT expression leads to the increase in the levels of miRNA150-5p which leads to the inactivation of genes involved in the progression of cell cycle (Bueno and Malumbres, 2011).

Reduced levels of MIAT lncRNA enhanced cell growth inhibition induced by UV-C irradiation in breast cancer cells and chemotherapeutic drugs such as Docetaxel, 5-FU, Nutlin-3a and Mitoxantrone. The diverse nature of the apoptotic stimuli affected by MIAT silencing in TNBC cells which include the DNA damaging agent (UV-C irradiation), an MDM2 inhibitor (Nutlin-3a), a microtubule stabilising agent (Docetaxel) and a topoisomerase II inhibitor/DNA intercalating agent (Mitoxantrone), indicates that MIAT is involved in a late and common step of activation the apoptotic machinery by these cell death stimuli.

Overall, the results indicate that the lncRNA MIAT is involved in breast cancer and is required for cell survival. Reduced levels of MIAT result in the loss of survival and the increase in basal apoptosis, suggesting that MIAT may act as an oncogene. In agreement with these findings, Luan et al. (2017) reported that MIAT expression is increased in breast cancer cell lines and advanced breast tumours and its overexpression is associated with TNM stage and lymph node metastasis. Consistent with the data presented in Chapter 6, Luan et al. (2017) showed that reduced levels of MIAT promoted breast cancer cell apoptosis and inhibited cell proliferation, migration, and in vivo tumour growth (Luan et al, 2017). Future studies

will be required to investigate the mechanism (s) by which MIAT regulates cell survival and its potential use as biomarker and specific therapeutic target.

7.3 Concluding remarks

The work presented in this thesis show that NEAT1 and MIAT lncRNAs are key regulators of breast cell survival. Future work should involve the identification of the proteins that directly interact with them. This will provide better understanding of their functions and an insight into their molecular mechanisms. Other recent work has supported these findings and identified several other lncRNAs that act in the same or opposite direction to control cell fate in breast cells. These include the oncogenes HOTAIR, XIST, MALAT and H19 and the tumour suppressor GAS5 (Reviewed by Cerik et al, 2016). Together these findings highlight the importance of lncRNAs in breast cancer and demonstrate that lncRNAs can regulate cell proliferation and cell survival by suppressing or promoting cell death, suggesting a key role for these molecular regulators. Therefore, better understanding of the functions of these lncRNAs and the mechanisms by which such lncRNAs regulate cell death and survival will undoubtedly aid in the development of optimised breast cancer therapies in the future.

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Appendix

Appendix I

Table 5.2 The 67-significantly differentially expressed genes as illustrated by <https://ipathwayguide.advaitabio.com/report/24273/contrast/294>.

symbol	Name	Genomic location	Fold change	p- value
DEGS2	Delta 4-Desaturase, Sphingolipid 2	14q32.2	10	5.000 e-5
RNF223	Ring Finger Protein 223	1p36.33	10	5.000 e-5
HSPB1	Heat Shock Protein Family B (Small) Member 1	7q11.23	4.932	1.000e-4
ISG15	ISG15 Ubiquitin-Like Modifier	1p36.33	5.243	1.000e4
RPLP1	Ribosomal Protein Lateral Stalk Subunit P1	15q23	2.885	2.000e-4
MIF	Macrophage Migration Inhibitory Factor	22q11.23	6.349	4.000e-4
CYBA	Cytochrome B-245 Alpha Chain	16q24.2	3.201	6.000e-4
C14orf80	Chromosome 14 Open Reading Frame 80	14q32.33	4.039	0.001
LINC00116	Long Intergenic Non-Protein Coding RNA 116	2q13	4.106	0.003
CHPF	Chondroitin Polymerizing Factor	2q35	2.801	0.004
CRIP2	Cysteine Rich Protein 2	14q32.33	5.012	0.004
C8orf55	Thioesterase Superfamily Member 6	8q24.3	2.439	0.004
NUBP2	Nucleotide Binding Protein 2	16p13.3	4.271	0.005
H2AFJ	H2A Histone Family Member J	12p12.3	2.768	0.006
RPUSD1	RNA Pseudouridylate Synthase Domain Containing 1	16p13.3	4.303	0.006
DPP7	Dipeptidyl Peptidase 7	9q34.3	3.303	0.006
H1FX	H1 Histone Family Member X	3q21.3	2.76	0.007
PPDPF	Pancreatic Progenitor Cell Differentiation And Proliferation Factor	20q13.33	2.536	0.007
GALK1	Galactokinase 1	17q25.1	4.042	0.007
C9orf16	Chromosome 9 Open Reading Frame 16	9q34.11	3.896	0.007
PPP1R16A	Protein Phosphatase 1 Regulatory Subunit 16A	8q24.3	3.407	0.007
MFSD3	Major Facilitator Superfamily Domain Containing 3	8q24.3	4.349	0.008
PARP10	Poly(ADP-Ribose) Polymerase Family Member 10	8q24.3	3.107	0.009
SCRIB	Scribbled Planar Cell Polarity Protein	8q24.3	2.370	0.009
TFF1	Trefoil Factor 1	21q22.3	2.307	0.014
MRPL41	Mitochondrial Ribosomal Protein L41	9q34.3	2.912	0.014
C9orf142	Chromosome 9 Open Reading Frame 142	9q34.3	2.864	0.014
C9orf24	Chromosome 9 Open Reading Frame 24	9p13.3	3.933	0.015
SPDEF	SAM Pointed Domain Containing ETS Transcription Factor	6p21.31	2.339	0.016
CCDC85B	Coiled-Coil Domain Containing 85B	11q13.1	4.161	0.018
FBXL14	F-Box And Leucine Rich Repeat Protein 14	12p13.33	2.96	0.019
PFKL	Phosphofructokinase, Liver Type	21q22.3	1.703	0.02
TSSC4	Tumor Suppressing Subtransferable Candidate 4	11p15.5	2.457	0.02
E4F1	E4F Transcription Factor 1	16p13.3	2.659	0.021
PKP3	Plakophilin 3	11p15.5	1.935	0.022
FAM195B	MAPK Regulated Corepressor Interacting Protein 1	17q25.3	2.86	0.022
FBXL15	F-Box And Leucine Rich Repeat Protein 15	10q24.32	2.408	0.023
NTHL1	Nth Like DNA Glycosylase 1	16p13.3	2.55	0.025
BTBD2	BTB Domain Containing 2	19p13.3	2.523	0.027
ALKBH7	AlkB Homolog 7	19p13.3	2.523	0.027
FBXL14	F-Box And Leucine Rich Repeat Protein 14	12p13.33	2.96	0.019
PFKL	Phosphofructokinase, Liver Type	21q22.3	1.703	0.02
FAM100A	UBA Like Domain Containing 1	16p13.3	2.886	0.023
ZNF598	Zinc Finger Protein 598	16p13.3	2.582	0.023
FKBP8	FK506 Binding Protein 8	19p13.11	2.285	0.024

SSSCA1	Sjogren Syndrome/Scleroderma Autoantigen 1	11q13.1	2.484	0.027
MED16	Mediator Complex Subunit 16	19p13.3	2.489	0.028
RASSF7	Ras Association Domain Family Member 7	11p15.5	1.999	0.032
FAM195A	MAPK Regulated Corepressor Interacting Protein 2	16p13.3	2.247	0.032
SCAND1	SCAN Domain Containing 1	20q11.23	4.419	0.033
POLRMT	RNA Polymerase Mitochondrial	19p13.3	2.331	0.034
TP53I13	Tumor Protein P53 Inducible Protein 13	17q11.2	2.249	0.034
DGCR6L	DiGeorge Syndrome Critical Region Gene 6 Like	22q11.21	2.245	0.035
FLYWCH2	FLYWCH Family Member 2	16p13.3	2.212	0.036
RPS15	Ribosomal Protein S15	19p13.3	2.241	0.038
MRPL55	Mitochondrial Ribosomal Protein L55	1q42.13	2.346	0.039
BRAT1	BRCA1 Associated ATM Activator 1	7p22.3	1.815	0.039
NPDC1	Neural Proliferation, Differentiation And Control 1	9q34.3	2.258	0.04
MVD	Mevalonate Diphosphate Decarboxylase	16q24.2	2.38	0.04
ISG20	Interferon Stimulated Exonuclease Gene 20	15q26.1	2.188	0.042
BCL7C	BCL Tumor Suppressor 7C	16p11.2	2.227	0.044
COMTD1	Catechol-O-Methyltransferase Domain Containing 1	10q22.2	2.732	0.045
NUDT8	Nudix Hydrolase 8	11q13.2	10	0.047
TIGD5	Tigger Transposable Element Derived 5	8q24.3	2.745	0.048
ANAPC11	Anaphase Promoting Complex Subunit 11	17q25.3	2.130	0.048
RNH1	Ribonuclease/Angiogenin Inhibitor 1	11p15.5	1.834	0.049

Table 5.3 NEAT1 downregulation effect on biological pathway as detected by Reactome database

Pathway name	Submitted entities found	Pathway name	Submitted entities found
Activation of gene expression by SREBF (SREBP)	FASN;MVD	Mitochondrial translation termination	MRPL41;MRPL55
Regulation of cholesterol biosynthesis by SREBP (SREBF)	FASN;MVD	Mitochondrial translation elongation	MRPL41;MRPL55
Defective GALK1 can cause Galactosemia II (GALCT2)	GALK1	Influenza Infection	RPS15;RPLP1;ISG15
Mitochondrial transcription initiation	POLRMT	Metabolism of steroids	FASN;MVD
Transcription from mitochondrial promoters	POLRMT	Mitochondrial translation initiation	MRPL41;MRPL55
Glycogen storage disease type II (GAA)	GAA	Interleukin-12 family signalling	MIF
Lysosomal glycogen catabolism	GAA	Phosphorylation of the APC/C	ANAPC11
Recognition and association of DNA glycosylase with site containing an affected pyrimidine	NTHL1	Peptide chain elongation	RPS15;RPLP1
ChREBP activates metabolic gene expression	FASN	Inactivation of APC/C via direct inhibition of the APC/C complex	ANAPC11
Displacement of DNA glycosylase by APEX1	NTHL1	Inhibition of the proteolytic activity of APC/C required for the onset of anaphase by mitotic spindle checkpoint components	ANAPC11
Attachment of GPI anchor to uPAR	GPAA1	Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	RPS15;RPLP1
Gene and protein expression by JAK-STAT signalling after Interleukin-12 stimulation	MIF	Mitochondrial translation	MRPL41;MRPL55
Cross-presentation of particulate exogenous antigens (phagosomes)	CYBA	Conversion from APC/C:Cdc20 to APC/C:Cdh1 in late anaphase	ANAPC11
Transcriptional activation of mitochondrial biogenesis	POLRMT	Eukaryotic Translation Elongation	RPS15;RPLP1
Galactose catabolism	GALK1	Translation	MRPL41;RPS15;RPLP1;MRPL55
Senescence-Associated Secretory Phenotype (SASP)	H2AFJ;ANAPC11	Glycogen storage diseases	GAA

Depyrimidination	NTHL1	Formation of a pool of free 40S subunits	RPS15;RPLP1
Neutrophil degranulation	PFKL;DPP7;GA A;CYBA;MIF	Negative regulators of DDX58/IFIH1 signalling	ISG15
Cleavage of the damaged pyrimidine	NTHL1	Interferon alpha/beta signalling	ISG20;ISG15
Base-Excision Repair, AP Site Formation	NTHL1	Metabolism of carbohydrates	PFKL;CHPF;G AA;GALK1
Synthesis of Dolichyl-phosphate	MVD	Diseases associated with glycosylation precursor biosynthesis	GALK1
Viral mRNA Translation	RPS15;RPLP1	HSF1 activation	HSPB1
APC-Cdc20 mediated degradation of Nek2A	ANAPC11	Glycogen metabolism	GAA
RHO GTPases Activate NADPH Oxidases	CYBA	PRC2 methylates histones and DNA	H2AFJ
Chondroitin sulfate biosynthesis	CHPF	NS1 Mediated Effects on Host Pathways	ISG15
Selenocysteine synthesis	RPS15;RPLP1	SIRT1 negatively regulates rRNA expression	H2AFJ
L13a-mediated translational silencing of Ceruloplasmin expression	RPS15;RPLP1	Attenuation phase	HSPB1
Glycogen breakdown (glycogenolysis)	GAA	Resolution of Abasic Sites (AP sites)	NTHL1
SRP-dependent cotranslational protein targeting to membrane	RPS15;RPLP1	ERCC6 (CSB) and EHMT2 (G9a) positively regulate rRNA expression	H2AFJ
GTP hydrolysis and joining of the 60S ribosomal subunit	RPS15;RPLP1	Vitamin B5 (pantothenate) metabolism	FASN
Nonsense-Mediated Decay (NMD)	RPS15;RPLP1	DNA methylation	H2AFJ
Nonsense Mediated Decay (NMD) enhanced by the Exon Junction Complex (EJC)	RPS15;RPLP1	Negative regulators of DDX58/IFIH1 signalling	ISG15
VEGFA-VEGFR2 Pathway	HSPB1;CYBA	Interferon alpha/beta signalling	ISG20;ISG15
signalling by BMP	MIF	Metabolism of carbohydrates	PFKL;CHPF;G AA;GALK1
Mitochondrial biogenesis	POLRMT	Diseases associated with glycosylation precursor biosynthesis	GALK1
Eukaryotic Translation Initiation	RPS15;RPLP1	HSF1 activation	HSPB1
Cap-dependent Translation Initiation	RPS15;RPLP1	Glycogen metabolism	GAA
RNA Polymerase I Promoter Opening	H2AFJ	PRC2 methylates histones and DNA	H2AFJ
Host Interactions with Influenza Factors	ISG15	Auto degradation of Cdh1 by Cdh1:APC/C	ANAPC11

Activated PKN1 stimulates transcription of AR (androgen receptor) regulated genes KLK2 and KLK3	H2AFJ	Translation initiation complex formation	RPS15
Influenza Viral RNA Transcription and Replication	RPS15;RPLP1	RNA Polymerase I Chain Elongation	H2AFJ
Class I MHC mediated antigen processing & presentation	FBXL15;CYBA;FBXL14;ANAPC11	Cellular Senescence	H2AFJ;ANAPC11
Antigen processing: Ubiquitination & Proteasome degradation	FBXL15;FBXL14;ANAPC11	Detoxification of Reactive Oxygen Species	CYBA
ROS, RNS production in phagocytes	CYBA	Cellular responses to stress	H2AFJ;HSPB1;CYBA;ANAPC11
RMTs methylate histone arginines	H2AFJ	Ribosomal scanning and start codon recognition	RPS15
Deposition of new CENPA-containing nucleosomes at the centromere	H2AFJ	Asymmetric localization of PCP proteins	SCRIB
Nucleosome assembly	H2AFJ	Regulation of expression of SLITs and ROBOs	RPS15;RPLP1
Formation of the ternary complex, and subsequently, the 43S complex	RPS15	AUF1 (hnRNP D0) binds and destabilizes mRNA	HSPB1
Condensation of Prophase Chromosomes	H2AFJ	HSF1-dependent transactivation	HSPB1
Selenoamino acid metabolism	RPS15;RPLP1	APC/C:Cdc20 mediated degradation of Securing	ANAPC11
DNA Damage Bypass	ISG15	Activation of the mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S	RPS15
Influenza Life Cycle	RPS15;RPLP1	Formation of the beta-catenin:TCF trans activating complex	H2AFJ
Meiotic recombination	H2AFJ	Regulation of APC/C activators between G1/S and early anaphase	ANAPC11
Major pathway of rRNA processing in the nucleolus and cytosol	RPS15;RPLP1	Amino acid synthesis and interconversion (transamination)	PPP1R16A
rRNA processing in the nucleus and cytosol	RPS15;RPLP1	RNA Polymerase I Promoter Clearance	H2AFJ
DNA Damage/Telomere Stress Induced Senescence	H2AFJ	Antiviral mechanism by IFN-stimulated genes	ISG15
APC/C:Cdh1 mediated degradation of Cdc20 and other APC/C:Cdh1 targeted proteins in late mitosis/early G1	ANAPC11	ISG15 antiviral mechanism	ISG15

Cdc20:Phospho-APC/C mediated degradation of Cyclin A	ANAPC11	NoRC negatively regulates rRNA expression	H2AFJ
Cholesterol biosynthesis	MVD	Signaling by ROBO receptors	RPS15;RPLP1
APC:Cdc20 mediated degradation of cell cycle proteins prior to satisfaction of the cell cycle checkpoint	ANAPC11	Nicotinate metabolism	PARP10
Chondroitin sulfate/dermatan sulfate metabolism	CHPF	Neddylaton	FBXL15;FBXL14
APC/C:Cdc20 mediated degradation of mitotic proteins	ANAPC11	RNA Polymerase I Transcription	H2AFJ
Activation of APC/C and APC/C:Cdc20 mediated degradation of mitotic proteins	ANAPC11	DDX58/IFIH1-mediated induction of interferon-alpha/beta	ISG15
RUNX1 regulates genes involved in megakaryocyte differentiation and platelet function	H2AFJ	rRNA processing	RPS15;RPLP1
Telomere Maintenance	H2AFJ	APC/C-mediated degradation of cell cycle proteins	ANAPC11
Transcriptional regulation by small RNAs	H2AFJ	Regulation of mitotic cell cycle	ANAPC11
Fatty acyl-CoA biosynthesis	FASN	Negative epigenetic regulation of rRNA expression	H2AFJ
Sphingolipid de novo biosynthesis	DEGS2	Meiosis	H2AFJ
Positive epigenetic regulation of rRNA expression	H2AFJ	Metabolism of water-soluble vitamins and cofactors	PARP10;FASN
RHO GTPases activate PKNs	H2AFJ	Regulation of mRNA stability by proteins that bind AU-rich elements	HSPB1
Diseases of carbohydrate metabolism	GAA	Cellular responses to external stimuli	H2AFJ;HSPB1;CYBA;ANAPC11
Post-translational modification: synthesis of GPI-anchored proteins	GPAA1	PCP/CE pathway	SCRIB
Activation of anterior HOX genes in hindbrain development during early embryogenesis	H2AFJ	Regulation of lipid metabolism by Peroxisome proliferator-activated receptor alpha (PPARalpha)	MED16
Activation of HOX genes during differentiation	H2AFJ	M Phase	H2AFJ;ANAPC11
Interferon Signaling	ISG20;ISG15	Glycosaminoglycan metabolism	CHPF
Reproduction	H2AFJ	Antigen processing-Cross presentation	CYBA

RHO GTPase Effectors	H2AFJ;CYBA	Adaptive Immune System	NPDC1;FBXL15;CYBA;FBXL14;ANAPC11
Synthesis of substrates in N-glycan biosynthesis	MVD	Diseases of glycosylation	GALK1
Cellular response to heat stress	HSPB1	Separation of Sister Chromatids	ANAPC11
Mitotic Prophase	H2AFJ	Ub-specific processing proteases	FKBP8
Formation of the cornified envelope	PKP3	Mitotic Anaphase	ANAPC11
Glucose metabolism	PFKL	Mitotic Metaphase and Anaphase	ANAPC11
Signalling by WNT	H2AFJ;SCRIB	Metabolism of amino acids and derivatives	RPS15;RPLP1;PPP1R16A
Epigenetic regulation of gene expression	H2AFJ	TCF dependent signalling in response to WNT	H2AFJ
Integration of energy metabolism	FASN	Diseases of metabolism	GAA
Organelle biogenesis and maintenance	POLRMT	Signalling by Rho GTPases	H2AFJ;CYBA
Infectious disease	RPS15;RPLP1;ISG15	Keratinization	PKP3
DNA Repair	NTHL1;ISG15	Innate Immune System	PFKL;DPP7;GAA;CYBA;ISG15;MIF
Sphingolipid metabolism	DEGS2	Metabolism of lipids	FASN;MVD;DEGS2;MED16
		Cell surface interactions at the vascular wall	MIF
Metabolism of vitamins and cofactors	PARP10;FASN	Chromatin organization	H2AFJ
Biosynthesis of the N-glycan precursor (dolichol lipid-linked oligosaccharide, LLO) and transfer to a nascent protein	MVD	Chromosome Maintenance	H2AFJ
Beta-catenin independent WNT signalling	SCRIB	RUNX1 regulates transcription of genes involved in differentiation of HSCs	H2AFJ
PPARA activates gene expression	MED16	MAPK6/MAPK4 signalling	HSPB1
Gene Silencing by RNA	H2AFJ	Transcriptional regulation of white adipocyte differentiation	MED16
Signaling by TGF-beta family members	MIF	Glycolysis	PFKL
Oxidative Stress Induced Senescence	H2AFJ	Mitotic Spindle Checkpoint	ANAPC11

Immune System	NPDC1;ISG20;P FKL;DPP7;GAA; FBXL15;CYBA;I SG15;MIF;FBXL 14;ANAPC11	Regulation of HSF1-mediated heat shock response	HSPB1
Chromatin modifying enzymes	H2AFJ	Deubiquitination	FKBP8
Developmental Biology	RPS15;H2AFJ;R PLP1;PKP3; imm16	Metabolism of RNA	RPS15;RPLP1 ;HSPB1
Transcriptional regulation by RUNX1	H2AFJ	Cell Cycle, Mitotic	H2AFJ;ANAP C11
Cell Cycle Checkpoints	ANAPC11	Immunoregulatory interactions between a Lymphoid and a non- Lymphoid cell	NPDC1
Axon guidance	RPS15;RPLP1	Cell Cycle	H2AFJ;ANAP C11
Signaling by Receptor Tyrosine Kinases	HSPB1;CYBA	Asparagine N-linked glycosylation	MVD
MAPK family signaling cascades	HSPB1	Metabolism of proteins	MRPL41;RPS 15;GPAA1;R PLP1;FKBP8; MVD;FBXL15 ;FBXL14;MR PL55
Cytokine signalling in Immune system	ISG20;ISG15; MIF	Fatty acid metabolism	FASN
Metabolism	CHPF;GAA;RPL P1;PPP1R16A; MED16;NUBP2 ;RPS15;PARP10 ;PFKL;FASN;MV D;DEGS2;GALK 1	Disease	RPS15;RPLP1 ;GAA;ISG15; GALK1
Signaling by Interleukins	MIF	Post-translational protein modification	GPAA1;FKBP 8;MVD;FBXL 15;FBXL14
Hemostasis	MIF	Nicotinamide salvaging	PARP10
Gene expression (Transcription)	H2AFJ;POLRMT ;MED16	Base Excision Repair	NTHL1
Generic Transcription Pathway	H2AFJ;MED16	B-WICH complex positively regulates rRNA expression	H2AFJ
RNA Polymerase II Transcription	H2AFJ;MED16	Meiotic synapsis	H2AFJ
Signal Transduction	H2AFJ;HSPB1;S CRIB;CYBA;MIF		

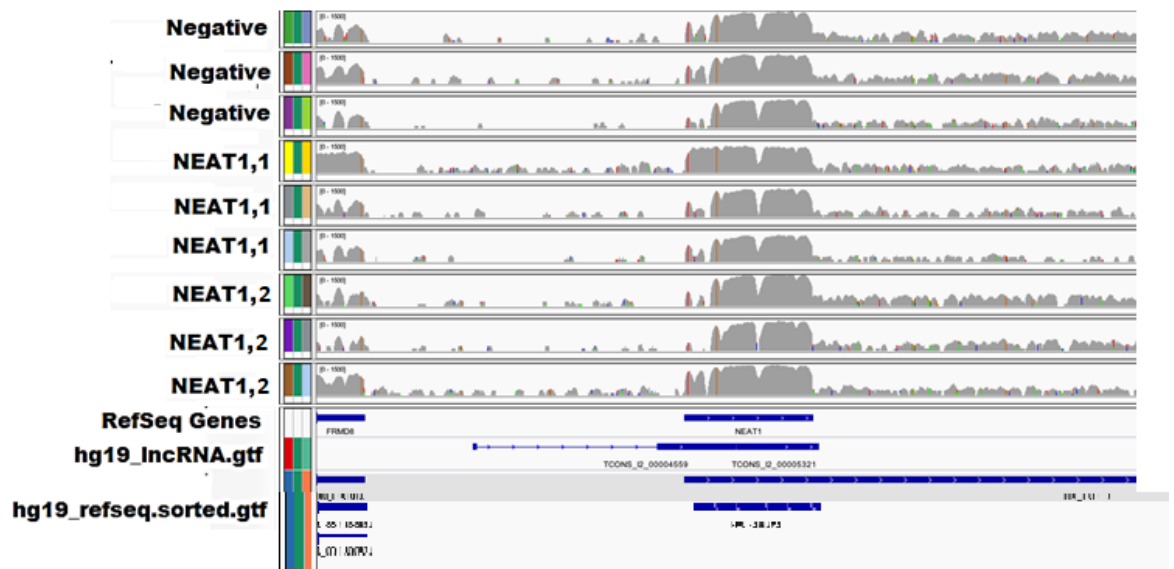


Figure 5.7 Log scale to show the coverage (grey bars) over the NEAT1 short and long isoform. The diagram was obtained from Integrative Genomics Viewer (IGV).

