AMERICAN UNIVERSITY OF BEIRUT

UNVEILING THE ROLE OF miR-183-5P AND miR-187-3P IN BREAST CANCER

by
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I dedicate this work to all the warriors who are fighting cancer fiercely especially women with breast cancer in my country- Lebanon.

AN ABSTRACT OF THE THESIS OF

Samira Mohammad Abdallah for Master of Science
Major: Human Morphology

Title: Unveiling the Roles of miR-183-5p and miR-187-3p in Breast Cancer

Background: Breast cancer (BC) is the most common type of cancer in incidence and mortality among women both worldwide and in Lebanon, which renders it a major health problem. Despite extensive research in this field, incidence and mortality rates are significantly increasing. BC is a heterogeneous disease composed of several subtypes characterized by distinct clinical outcomes. Thus, unveiling new players in BC development might lead to better understanding of its tumorigenesis. Studies have reported important roles of microRNAs (miRNAs) in the tumorigenesis and progression of cancer. It has been shown that the dysregulation of these small, endogenously expressed noncoding RNA molecules in BC participates in its tumorigenesis. Recent data in our lab has shown that miR-183-5p is one of the most upregulated miRNAs in estrogen-receptor positive (ER+) Lebanese BC tissues compared to normal adjacent tissues, by a microarray profiling analysis. In addition, an ongoing project in the lab has shown a significant upregulation of miR-187-3p in BC. Thus, our aim was to assess the roles of miR-183-5p and miR-187-3p in ER+ BC tumorigenesis.

Methods and Results: Dysregulation of miR-183-5p and miR-187-3p was validated in ER+ BC tissues downloaded from TCGA database compared to normal adjacent tissues (NAT) using DEseq2 tool. It was shown that both miRNAs are significantly upregulated in ER+ BC. Then, potential shared targets and target pathways between miR-183-5p and miR-187-3p were uncovered using miRTargetLink 2.0 tool. Then, some targets shown to be implicated in BC or in cancer in general were chosen for further investigation. These include MBNL1, BCL6, SMAD4, FGF9, and DAB1. After that, the correlations between miR-183-5p, miR-187-3p and the chosen shared targets expression with overall survival (OS) in ER+ BC were assessed using Kaplan-Meier (KM) plot analysis. An inverse correlation was observed between the effects of miR-183-5p and miR-187-3p expression with that of MBNL1, SMAD4, FGF9, and BCL6 on OS in ER+ BC where a low expression of the miRNAs of interest while a high expression of the targets was correlated with a better OS. In contrast, a direct correlation was observed between the effects of miRNAs and DAB1 expression on OS. This indicated that DAB1 may not be a direct target for these miRNAs in ER+ BC. Accordingly, the study was focused on the other targets (MBNL1, SMAD4, BCL6, and FGF9). After that, the dysregulation of miR-183-5p and miR-187-3p in BC cell lines was assessed by RT-qPCR where MCF-7 was further chosen as an in vitro model. miR-183-5p was shown to be upregulated in MCF-7 cell line while miR-187-3p was shown to be downregulated. So, the expression of the miRNAs of interest was modulated in MCF-7 cells by transfection with miR-183-5p inhibitor and miR-187-3p mimic. MTT assay and PI analysis were done to determine the effects of miR-183-5p downregulation and miR-187-3p overexpression on proliferation and cell cycle progression of MCF-7 cells. Both the downregulation of miR-183-5p and the overexpression of miR-187-3p had no effects on the proliferation of the cells. No effect was detected on cell cycle progression with downregulation of miR-183-5p whereas a slight increase in G2-M phase was

observed with overexpression of miR-187-3p. Finally, RT-qPCR data showed that miR-183-5p downregulation upregulated the expression of SMAD4 and MBNL1 genes, while the overexpression of miR-187-3p unexpectedly upregulated MBNL1 mRNA levels in MCF-7 cells.

Conclusion: Overall, our study suggests the potential of miR-183-5p and miR-187-3p to be active players in estrogen-receptor (ER+) BC through the modulation of different mRNA targets. However, further studies are required to validate their role.

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ABBREVIATIONS

- 3’ UTR 3' Untranslated Region
- AGO Argonaute Protein Family
- AJCC American Joint Committee on Cancer
- AML Acute Myeloid Leukemia
- APAF1 Pro-Apoptotic Apoptosis Protease–Activating Factor 1
- ASR Age-Standardized Rate
- ATG5 Autophagy related 5
- AUBMC Amercian University of Beirut
- BC Breast Cancer
- Bcl2 B-Cell Lymphoma 2
- BCL6 B-cell lymphoma 6
- BRCA 1/2 Breast Cancer type 1/2
- ccRCC Clear Cell Renal Cell Carcinoma
- CDC42 Cell division control protein 42 homolog
- CDK1 cyclin-dependent kinase-1
- CDK5 cyclin-dependent kinase-5
- CircRNAs Circular RNAs
- CLL Chronic Lymphocytic Leukemia
- CRC Colorectal Cancer
- CXCR4 C-X-C Motif Chemokine Receptor 4
- DCIS Ductal Carcinoma In Situ
- DGCR8 Digeorge Syndrome Critical Region 8
- DMEM Dulbecco’s Modified Eagle’s Medium
- DMSO dimethyl sulfoxide
- dNTPs Deoxynucleotide triphosphates
- EMT Epithelial Mesenchymal Transition
- EMT Epithelial to Mesenchymal Transition
- ER Estrogen receptor
- EXP5 Exportin 5
- FBS Fetal Bovine Serum
- FGF9 Fibroblast Growth Factor 9
- FOXK1 Forkhead box protein K1
- FOXO1 Forkhead Box Protein O1
- GAPDH Glyceraldehyde 3-phosphate dehydrogenase
- HCC Hepatocellular Carcinoma
- HER2 Human Epidermal growth factor Receptor 2
- IDC Invasive Ductal Carcinoma
- ILC Invasive Lobular Carcinoma
- ITGB1 Integrin-Beta 1
- KEGG Kyoto Encyclopedia of Genes and Genomes
- KM Kaplan Meier

- LCIS Lobular Carcinoma In Situ
- LncRNAs Long non-coding RNAs
- LRFN1 Leucine Rich Repeat And Fibronectin Type III Domain Containing 1)
- MBNL1 muscleblind-like protein 1
- miRNA, miR microRNA
- MRI Magnetic Resonance Imaging
- MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
- MTX Methotrexate
- NC Negative Control
- NCBI National Center for Biotechnology Information
- NRT No Reverse Transcriptase
- NSCLC Non-Small Cell Lung Carcinoma
- NTC No Template Control
- OncomiRs Oncogenic microRNAs
- OS Overall Survival
- P2RX7 purinoceptor 7
- PACT Protein kinase R-Activating protein
- PAK7 P-21 Activated Kinase
- PARP Poly-ADP Ribose Polymerase
- PBS Phosphate-buffered saline
- PDCD4 Programmed Cell Death 4
- PET Positron Emission Tomography
- PI Propidium Iodide
- PIK3CA Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
- PR Progesterone receptor
- Pre-miRNA Precursor microRNA
- PTPRJ Protein Tyrosine Phosphate Receptor type J
- RETREG1 RETREG1 - Reticulophagy regulator 1
- RGS2 regulator of G-protein signaling 2
- RISC RNA-Induced Silencing Complex
- Rnase Ribonuclease
- RT Room Temperature
- SENP1 Sentrin-specific protease 1
- SHP2 Src homology 2 (SH2) domain-containing tyrosine phosphatase-2
- SIRT6 Sirtuin 6
- SMAD4 Mothers against decapentaplegic homolog 4
- TCGA The Cancer Genome Atlas
- TDLU Terminal Ductal Lobular Unit
- TGF-B Transforming growth factor beta
- Tm Melting Temperature
- TNBC Triple Negative Breast Cancer
- TP Triple Positive
- TRBP Transactivation-Responsive RNA Binding Protein
- TRI Trisol

- UNG Uracil N-glycosylase
- XIAP X-linked inhibitor of apoptosis protein
- YAP1 Yes-associated protein 1
- YY1 Yin Yang-1
- ZEB1 zinc finger E-box binding

Research, as life, is full of ups and downs. We have to focus
on the ups, learn from the downs and keep moving!

This work was done with love, passion, commitment, and a lot of enjoyment!

Samira Abdallah

CHAPTER I

INTRODUCTION

"From small beginnings come great things"

Peter Senge

A. Breast Cancer

1. Epidemiology and Risk Factors

a. Worldwide

According to Globocan 2020, breast cancer (BC) is the most prevalent type of cancer in the world and the most common cause of cancer death among women occupying 24.5 % of all new cancer incidence cases and 15.5% of all cancer mortality cases (1) (Figure 1A). This increase is due to the hybridity of BC where its occurrence is attributed to diverse risk factors. These factors include demographic factors like gender (it affects females more), and age (affecting elderly people more often), reproductive factors like early age of menarche, and age of menopause, hereditary factors as genetic factors and family history, and lifestyle factors which include obesity, overweight, and smoking. Albeit BC occurs everywhere in the world, its incidence shows some variation where it is higher in developed countries. It varies from 27 per 100,000 in Middle Africa and East Asia to 92 per 100,000 in Northern America (2).

b. Lebanon

Akin the worldwide status, BC is the most widespread cancer type and the top cause of cancer mortality amidst Lebanese women. According to GLOBOCAN 2020, BC in Lebanon has increased incidence and mortality rates accounting for 33.7 % and 24.3 % respectively (Figure 1B). Two recent studies have shown that BC incidence amid Lebanese women is the highest in the Middle East and among the highest in the world (3, 4). It has boosted from 20 per 100,000 women in 1990 to 78.7 per 100,000 women in 2012 (3). The last estimation of average Age-Standardized Rate (ASR) of BC incidence in Lebanon is 96.5 per 100,000 which is higher than that of Jordan (51 per 100,000), Kuwait (56.1 per 100,000), Qatar (53.8 per 100,000) and Saudi Arabia (24.5 per 100,000) on the regional level. It is from the highest on the international level as well hanging only behind Denmark (97.3 per 100,000) and transcending Italy (90.1 per 100,000) and Germany (90.9 per 100,000) (4). This increase in BC incidence in Lebanon over the years has developed from the wide execution of entrenched mammography screening programs, and awareness campaigns on a national level. Uptake of Mammography screening in Lebanon has increased from 7% in 2001 to 18% in 2005 and is becoming more culturally acceptable among Lebanese women (3). Other factors like reproductive factors (such as the increase in women's mean marital age and age of menopause, the decrease in fertility rate and age at menarche, and the use of hormonal replacement therapy) are also behind this increase in BC ASR in Lebanon. In addition, adoption of westernized lifestyle instead of the healthy Mediterranean diet, decreased breastfeeding, and active and passive smoking could be accused for the high BC incidence in Lebanon (3, 4). Obesity, as well, has been manifested as a risk factor linked to a higher incidence of BC in post-menopausal women (5).

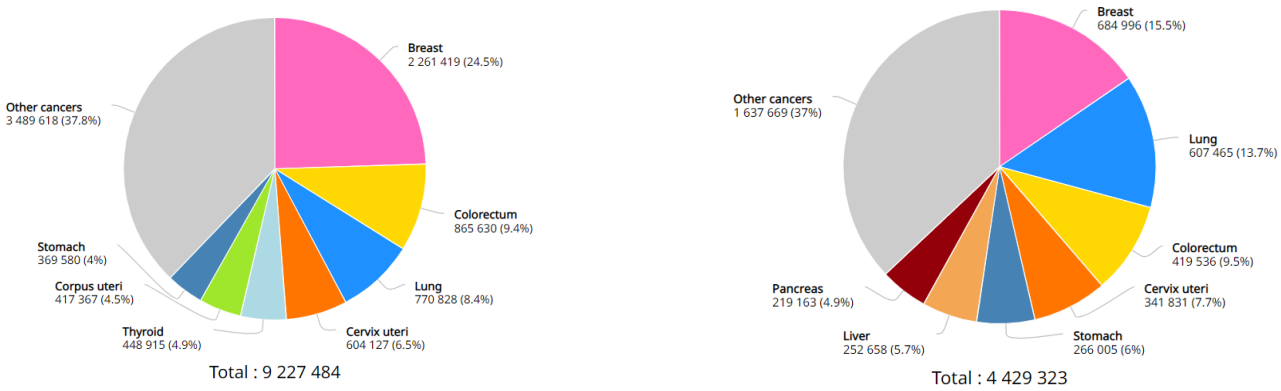
The median age at diagnosis of BC in Lebanon is 50 years which, among other Arab countries, is shown to be a decade earlier than that of western countries according to WHO statistics (6). Of all women with BC in Lebanon, a high percentage present at an age younger than 40 years old. This significantly high prevalence is in stark contrast with the West. The biological behavior of BC is disparate in young women than in their older counterparts. It is shown to be more hostile and accompanied by a worse prognosis (7). El Saghir et al have reported this by a study done on 1,320 BC patients at the American University of Beirut Medical Center- Lebanon between the years 1990 and 2001 where it has been shown that a high percentile of young patients had higher tumor grade, more positive axillary lymph node status, more development of metastasis and worse survival in comparison to their older counterparts despite adjuvant hormonal therapy. In consequence, young age at presentation was shown to be a bad prognostic factor for BC for positive-lymph node and positive-hormone receptor patients (8). This peculiarity of BC epidemiology in the Lebanese population was not linked to BRCA gene mutations. Several studies have proposed that, in spite of the fact that the median age at diagnosis in Lebanon is lower than Westernized populations, BRCA mutation cases are observed to be low (9). El Saghir et al have shown, in a study conducted on 250 Lebanese BC patients, that only 5.6% had deleterious mutations in BRCA1 and BRCA2 genes (10). Farra et al has displayed, in a recent study done on a cohort of 281 patients containing young patients with breast and ovarian cancer at the American University of Beirut Medical Center, that only 7.8% carried BRCA deleterious mutations with a prevalence of c.131G>T mutation in BRCA1 (9). Thus, the major cause behind the high incidence of BC in young females in Lebanon is not BRCA gene mutations. In this regard, it is crucial to look for alternative gene mutations or epigenetic factors that might contribute to BC tumorigenesis.

Worldwide

Incidence

Mortality

A



Lebanon

Incidence

Mortality

B

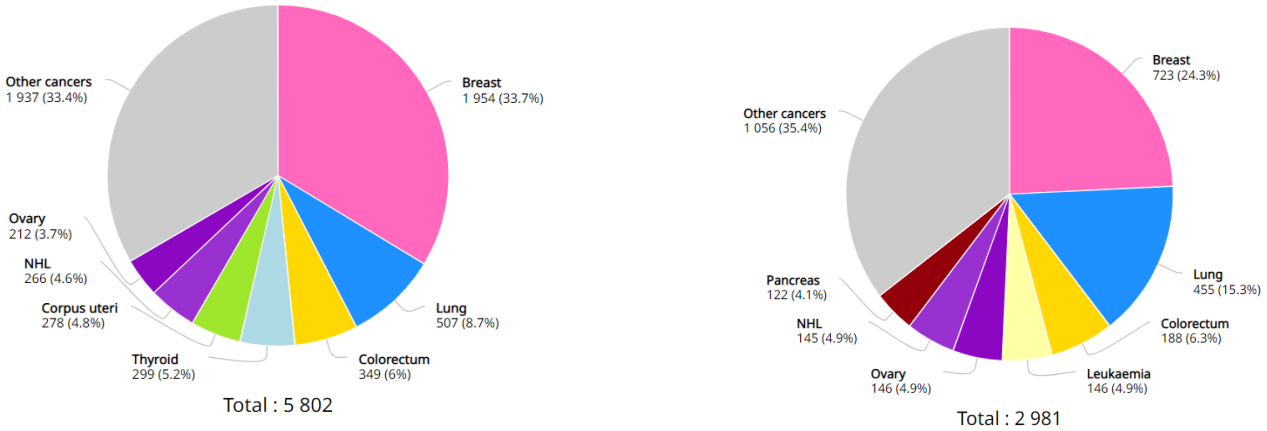


Figure 1 . Breast Cancer Incidence and Mortality among Women in the World (A) and in Lebanon (B). Breast cancer has the highest incidence and mortality rates among females in the World and in Lebanon (GLOBOCAN, 2020).

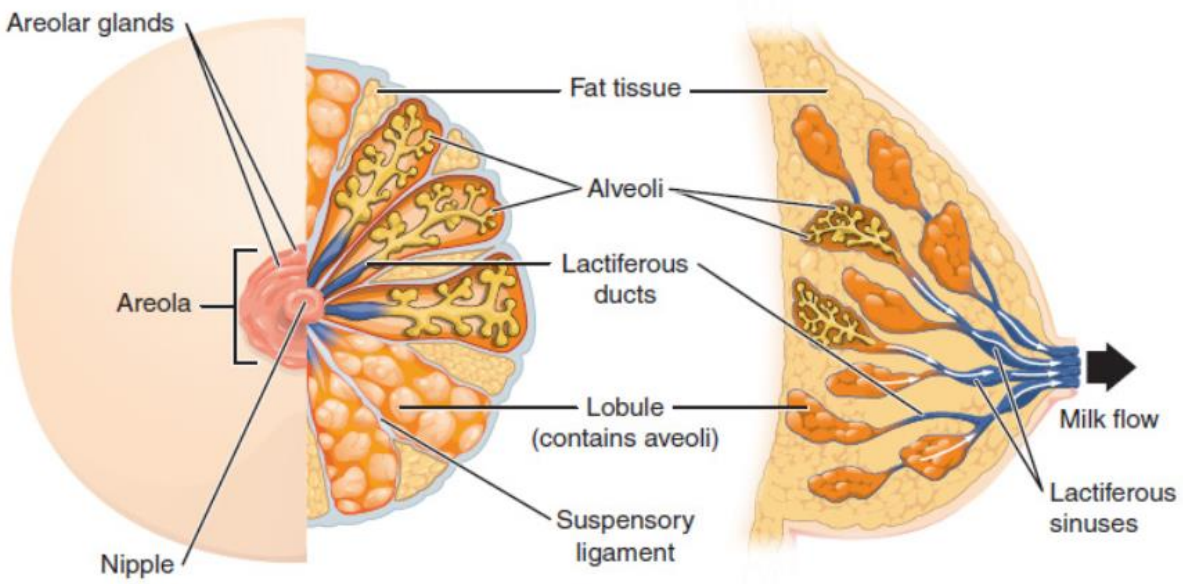
2. ***Mammary Gland Development and Anatomy***

A deeper understanding of normal mammary gland location, anatomy and development is essential to understand BC occurrence and progression. The female breast lies above the pectoralis muscle on the anterior thoracic wall extending on the bottom from the second to the sixth rib (11). The breast is a complex organ composed of parenchyma consisting of ducts and lobes along with supporting stroma that consists of fat, nerves, lymphatics, arteries and veins. The ductal system of the mammary gland encompasses branching epithelial structure where the primary ducts form terminal ductules via an ample branching process. At the end of the terminal ductules exist small secretory sac like structures called alveoli. Regarding the glandular tissue, it is made up of a network of 15-20 lobes where each lobe is composed of several lobules. Two cell layers, a myoepithelial layer facing the basement membrane and a luminal one facing the lumen, cover the ducts of the mammary gland. These glandular and ductal systems are embedded in a highly fibrous stroma (Figure 2A) (12, 13).

The development of the breast follows a stepwise progression and consists of three main stages: fetal, pubertal, and reproductive (14). Starting from the fifth week of fetal life, breast buds, where a meshwork of tubules are formed, generate as an ingrowth of the ectoderm (11). Then, mature lactiferous ducts evolve from the network of rudimentary tubules opening into a superficial mammary aperture that transforms into a nipple during infancy (Figure 2B) (11). Until puberty, the mammary glands remain rudimentary, consisting of only the lactiferous ducts (15). After that, it undergoes substantial remodeling processes during puberty, pregnancy, lactation, and involution following lactation. At the onset of puberty, branching morphogenesis begins where a huge proliferation takes place leading to the formation of a branched ductal network that fills the fat pad. This process is under the influence of estrogen and progesterone and pituitary growth factors.

The lateral branches give rise to the terminal ductal-lobular unit (TDLU) which is the basic functional unit of the breast constituting of terminal ducts and lobules with numerous blind-ended secretory ducts, called acini. Each acinus is lined with an inner layer of luminal epithelial cells and an outer layer of contractile myoepithelial cells. Most of the breast space is occupied by this tree-like pattern of ducts. The rest is occupied by the adipose tissue, blood vessels, immune cells, and fibroblasts (14, 16). Upon pregnancy, prolactin and progesterone work together to promote growth of milk-producing alveolar cells, a process known as alveologenesis, to have a functional developed glandular tissue (14, 16). During lactation, milk is produced by the alveolar luminal epithelial cells upon the contraction of the myoepithelial cells surrounding them. Upon weaning, demand for milk deteriorates which initiates involution where the mammary gland returns to its pre-pregnancy state (11, 14).

A



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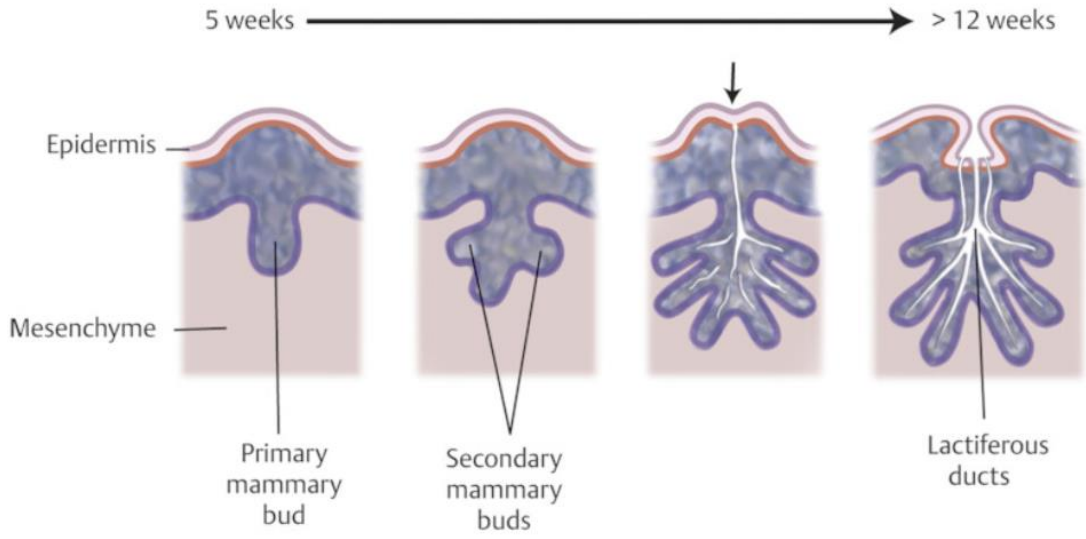


Figure 2. Mammary Gland Anatomy (A) and Development (B). The breast consists of ducts and lobes along with a supporting stroma. Each lactiferous duct of the breast branches into ductules. At the end of the terminal ductules exist one or more alveoli, (B) From the fifth week of fetal life, breast buds emerge as an ingrowth of the ectoderm. Then, secondary breast buds continue development to form lactiferous ducts that open into a nipple. Figure adopted from: RadiologyKey.com

3. Breast Cancer Origin and Evolution

The breast is characterized by cellular plasticity which increases its susceptibility to carcinogenesis (16). Genetic and epigenetic alterations are at the basis of BC. BC initiates when the cells in the breast break free of the check points that control their proliferation, differentiation, migration and death due to mutations activating specific proto-oncogenes and/or inhibiting specific tumor suppressor genes (17). The first step in BC progression is the hyper-proliferation of luminal cells forming in situ carcinoma in the ducts or lobules. Next, if left untreated, the in situ carcinoma spreads in these interconnected structures and progresses to invasive carcinoma where the arrangement of the myoepithelial cells become altered and the basement membrane become degraded. The last stage is metastasis where the BC cells acquire the ability to transmigrate through blood vessels or lymphatic vessels and metastasize to other organs (Figure 3) (18-22).

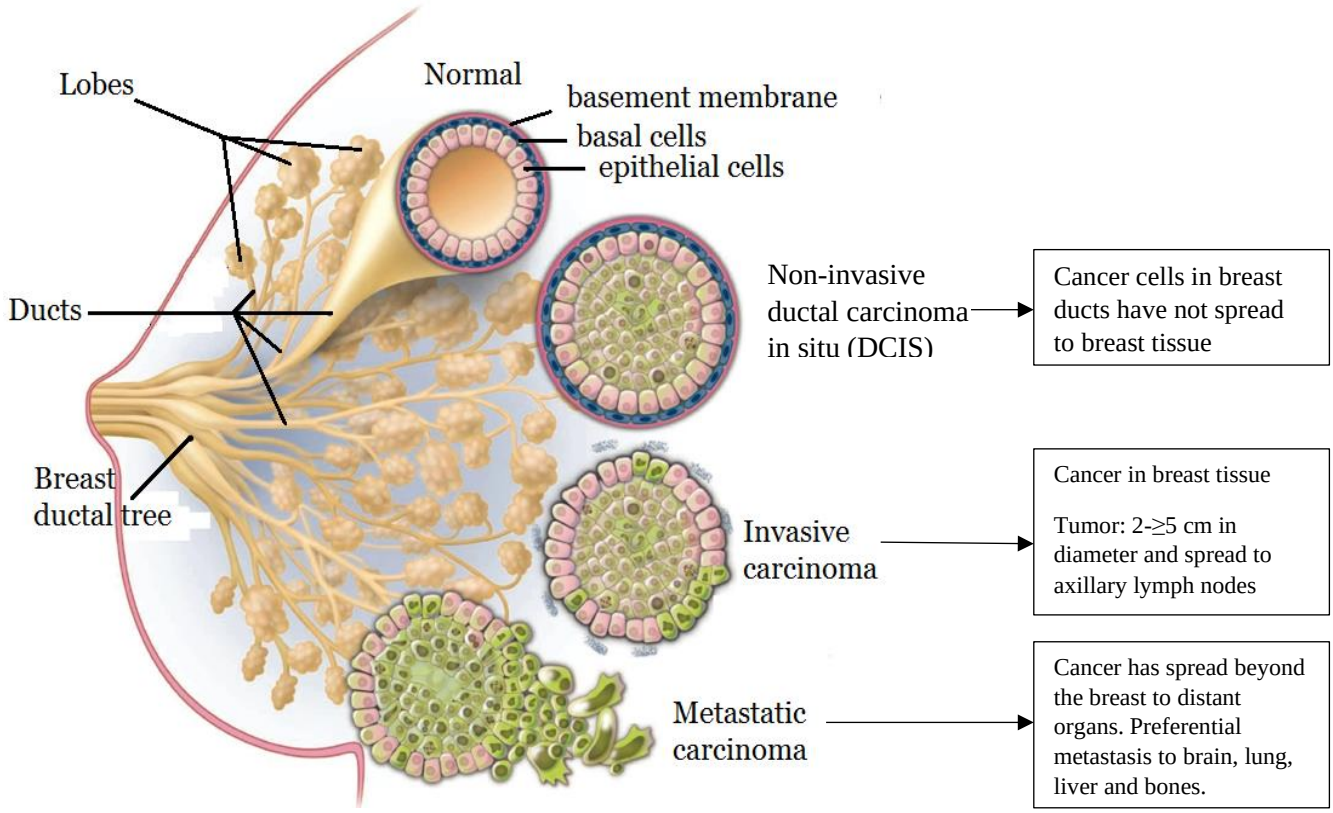


Figure 3. Breast Cancer Development and Tumorigenesis. Figure modified from CapeRay.com

4. Breast Cancer Classification and Staging System

- Breast Cancer Classification

BC is not a single entity but rather a heterogeneous disease made of different subtypes. It is classified according to its histopathology, immunopathology, mRNA expression profile, and miRNA expression (Figure 4) (23).

Histopathologically, BC is divided based on the pathologic growth pattern and sub-divided according to tumor cell type, immunohistochemical profile, architectural features and extracellular secretion (24). Ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC), are the most common histotypes of non-invasive and invasive carcinomas respectively, followed by Lobular carcinoma in situ (LCIS) and Invasive Lobular carcinoma (ILC) (Figure 4) (24, 25). The residuum are less frequent histotypes and include apocrine, micropapillary, papillary, medullary, tubular, mucinous, and cribriform (24, 25).

Immunopathologically, BC is classified based on special receptor markers. These receptors include estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). The immunopathological subgroups arise from different combinations of these three receptors as follows: ER+ (ER+, HER2-), HER2+ (ER-, HER2+), triple negative- (TN) (ER-, PR-, HER2-), and triple positive- (TP) (ER+, PR+, HER2+). These receptor-based subtypes could be expressive of the overall prognosis and therapeutic response (23). BC cases with ER- and/or PR- status have shown a worse prognosis than BC cases that are ER+/PR+ (26, 27). BC subtypes that are HER2+ have shown more aggressive clinical behavior that HER2- subtypes (27), and TNBC have shown poorer prognosis than triple positive (Figure 4) (28).

Molecularly, BC is classified into five intrinsic subtypes based on the gene expression profile, each with a specific clinical outcome (Figure 5) (29-32). Luminal A (ER+, PR+, HER2-) is marked by a low histological grade, a good prognosis, low expression of proliferation related genes and has the best response to endocrine therapy. However, Luminal B tumors (ER+ and/or PR+, HER2+ or HER2-) are marked by a high histologic grade, a poor prognosis, and a high expression of proliferation related genes. In addition, this subtype is sensitive to endocrine therapy but to a lesser extent than Luminal A subtype (33). The third subtype is the HER2-enriched BC (ER-, PR-, HER2+). This subtype is characterized by a high histologic grade, poor prognosis, and a good response to HER2-monoclonal targeted therapy (33). Basal-like subtype (ER-, PR-, HER2-) is similar to TNBC and is characterized by a high tumor grade, a very bad prognosis, no response to endocrine therapy and sensitivity to poly-ADP ribose polymerase (PARP) inhibitors (34). The fifth subtype is the normal-like tumor. This subtype is characterized by a similar gene expression as normal epithelial cells and has a good prognosis (Figure 5) (35).

Furthermore, further BC stratification has emerged with the discovery of miRNAs (Figure 4) (36, 37).

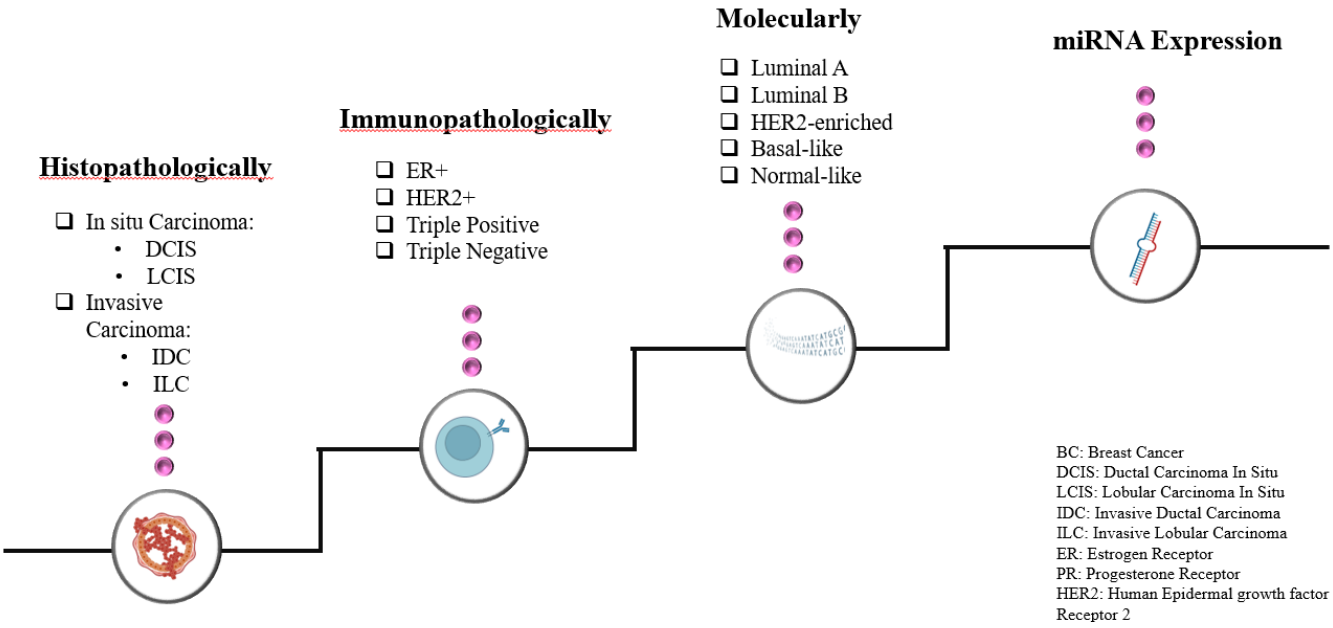


Figure 4. Summary of BC Classification. Images Source: BioRender.com

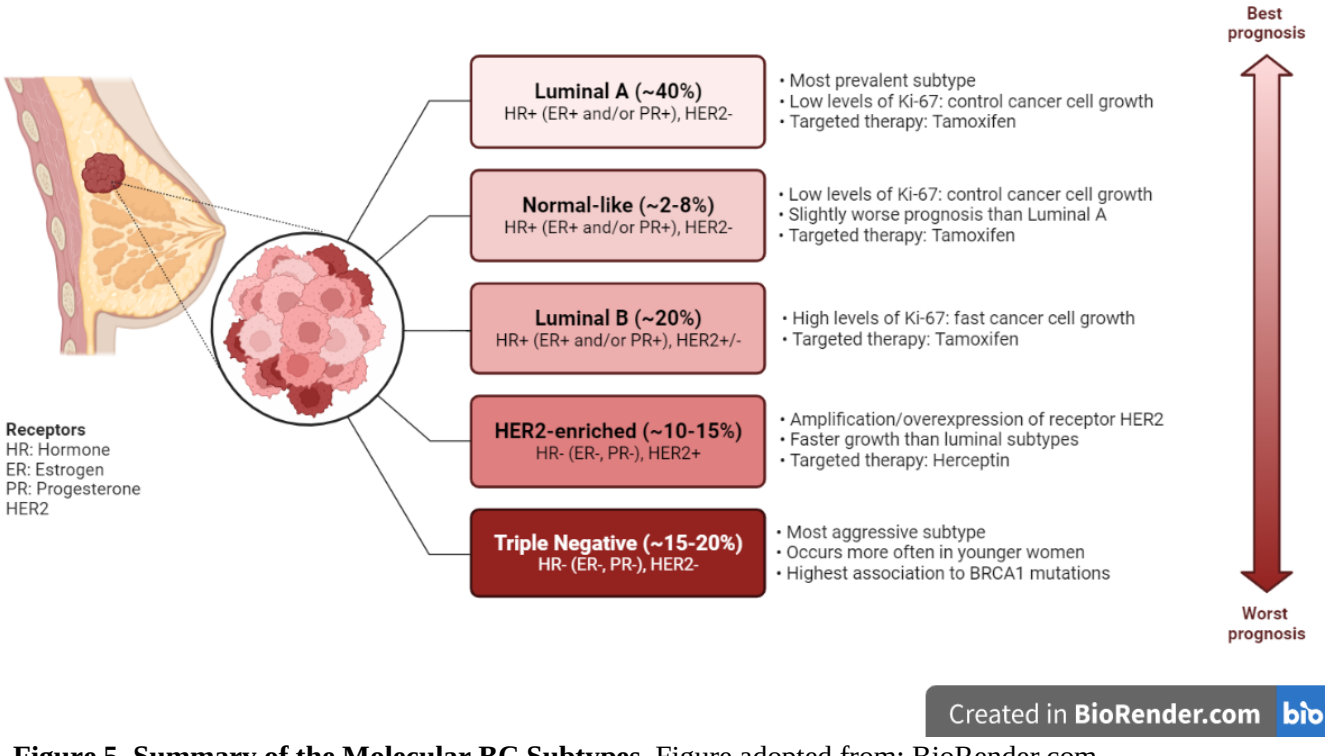


Figure 5. Summary of the Molecular BC Subtypes. Figure adopted from: BioRender.com

- Breast Cancer Staging System

According to the American Joint Committee on Cancer (AJCC), BC is staged into nine stages (0, IA, IB, IIA, IIB, IIIA, IIIB, IIIC, IV) based on the different combinations of the major morphological features of the tumor affecting prognosis. These features include the primary tumor size (T), regional lymph node involvement (N), and distant metastasis status (M). According to the primary tumor size, BC is classified from T0 to T4; T0 designated for no measurable tumor size, T1 to T3 for a primary tumor size varying from ≤20 mm to >50 mm, and T4 for a tumor with a direct extension to chest wall and/or skin. The regional lymph node includes spreading of BC to the ipsilateral axillary, supraclavicular, and internal mammary lymph nodes. Accordingly, BC is stratified into N0 for no zonal lymph node metastasis to N3 for immense lymph node involvement. Distant metastasis encompasses the involvement of contralateral lymph nodes, brain, liver, lungs, and bones. The staging is designated as M0 for no existence of metastasis and M1 for distant metastasis (38). In addition, biomarkers (tumor grade, hormone receptor, and HER2 status), multigene panels (Oncotype DX), and prognostic staging (clinical and pathological) were incorporated in AJCC eighth edition that further stratify BC (Table 1) (39).

Table 1. Breast Cancer Staging System, Eighth Edition of AJCC. Table adopted from AJCC

TNM Staging System, 8th edition	
Tumor Size (T)	T0: No proof for primary tumor T1: tumor ≤20 mm T2: tumor >20 mm but ≤50 mm T3: tumor >50 mm T4: tumor of any dimension with direct extension to chest wall and/or to skin
Nodal Staging (N)	N0: No zonal lymph node metastasis N1: metastasis to movable ipsilateral axillary lymph nodes N2: metastasis to ipsilateral lymph nodes or to internal mammary nodes N3: immense lymph node involvement
Metastasis (M)	M0: No distant metastasis M1: distant metastasis
Biomarkers	Tumor grade, Hormone receptor, HER2 status
Multigene panels	Oncotype DX
Prognostic Staging	○ Clinical ○ Pathological

5. ***Breast Cancer Diagnosis and Treatment***

It was demonstrated by several studies that BC mortality rates could be significantly reduced by early diagnosis accompanied with the appropriate treatment. Early detection techniques include Mammography (the contemporary standard BC screening test), Ultrasound (A supplementary medical imaging technique for mammography), Magnetic Resonance Imaging (MRI) (recommended for young women with dense breasts), Positron Emission Tomography (PET) (the most accurate visualizing metastasis) and Breast Biopsies (distinguishing between cancerous and benign tissues). However, these techniques have some limitations that restrict their efficiency as BC diagnosis methods (Table 2). Mammography, for example, is not recommended for women under the age of 40 and those with dense breasts, is less sensitive to tumors less than 1 mm (about 100,000 cells) and does not give any clue regarding the eventual aftermath or consequence of the disease. As for MRI, it is very expensive to be used regularly and it is correlated with over-diagnosis because of its low precision. Although PET is the most precise method for imaging disease metastasis or its response to therapy, it involves exposure to ionizing radiation which has harmful effects on humans specifically at high levels of exposure (40). A definitive method for BC diagnosis is a breast biopsy increasing the diagnostic accuracy. However, a major limitation other than being expensive is that it is invasive (41). Biomarkers for BC detection are useful in mirroring tumor growth process but they are associated with poor early detection due to low sensitivity and specificity (40, 42). Thus, novel diagnostic and prognostic methods are essentially needed for discovering new remedial methods that enhance the quality of life of BC patients (43). As such, differentially expressed circulating microRNAs in the body fluids of BC patients give hope as a feasible and a useful method for BC diagnosis (44, 45).

Radio-, chemo-, hormonal-, targeted-therapies, and surgical excision are used as treatments for BC (46). Targeted therapy is used for BC expressing HER2 receptors however hormonal therapy is used for those expressing estrogen and/or progesterone receptors (25, 47). Surgical excision is performed either by removing the entire breast or a part of it (46).

Table 2. The Uses and Limitations of the Conventional BC Diagnosis Techniques. Adopted from Wang et al, 2017

Type	Usage	Limitations
Mammography	○ The contemporary standard breast screening technique ○ Mass screening ○ Catch all types of tissues (bone, soft tissues and blood vessels) at the same time	○ Not recommended for women<40 y/o ○ Less effective for females with dense breasts ○ Less sensitive with small tumors ○ Ionizing radiation
Ultrasound	○ A supplementary imaging technique for mammography ○ To assess chunks found in mammography	○ Image with low resolution ○ Low specificity and sensitivity
Magnetic Resonance Imaging	○ For young women with dense breasts	○ Expensive ○ Low precision
Positron Emission Tomography	○ The most precise method ○ For imaging disease metastasis or its response to therapy	○ Ionizing radiation
Biopsy	○ To distinguish between cancerous and benign tissues	○ Invasive ○ Expensive

B. microRNAs

microRNAs (miRNAs) are a category of small, evolutionarily conserved, endogenously expressed noncoding RNA molecules that play a fundamental role in the regulation of gene expression in almost all key cellular processes (43, 48-50). The story of

miRNAs has started in 1993 with *lin*-4, the first discovered microRNA in *Caenorhabditis elegans*. The Ambros group has determined that *lin*-4 regulates *lin*-14 mRNA translation where they have shown that the former encompasses small RNAs containing antisense complementary sequences to the 3' untranslated region (3'-UTR) of lin-14 mRNA leading to a decrease in lin-14 protein levels (51). Since then, more microRNAs have been discovered and their role in gene expression of different species, including humans, has been established (52).

1. microRNA Biogenesis

microRNA encoding genes are located all over the human genome (53) and they are either intronic, originated from introns of protein encoding genes, or intergenic, transcribed independently and regulated by their own promoters (53-55). Animal miRNAs are transcribed monocistronically, as individual genes or polycistronically, as gene clusters that are then refined to multiple miRNAs (53, 54, 56).

microRNA biogenesis and maturation is a two-step process taking place first in the nucleus and then is continued in the cytoplasm (57). Within the nucleus, miRNA genes are transcribed by RNA polymerase II into primary microRNA (pri-miRNA), 100-120 nucleotides in length (53). The resulting pri-miRNA transcript is a double-stranded RNA having a stem-loop structure that enciphers miRNA duplexes in the arm of the stem (58).

microRNA processing is categorized into canonical and non-canonical pathways (Figure 6).

- The canonical pathway

In the canonical pathway, pri-miRNA is recognized and cleaved by a microprocessor complex constituted of Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8), an RNase III enzyme and its RNA-binding protein cofactor respectively, into a hairpin intermediate structure called precursor miRNA (pre-miRNA), 60-80 nucleotide in length. Pre-miRNA is then translocated to the cytoplasm by the transporter protein Exportin-5 (EXP5) which is only functional in a complex form with Ran-GTP, a GTP binding nuclear protein (53-55, 57-60). In the cytoplasm, pre-miRNA is further processed by Dicer, an endoribonuclease enzyme (RNase III) that cleaves the terminal loop producing a mature miRNA duplex. Dicer processing is regulated by several RNA-binding proteins like the cofactors TRBP (transactivation-responsive RNA binding protein) and PACT (protein kinase R-activating protein), which form a stable complex with Dicer and stimulate its role. TRBP aids in a proper and specific recognition of the pre-miRNA from a blend of other RNA types in the surroundings and in its time efficient processing. PACT, which forms a more stable complex with Dicer than TRBP, is indispensable in the processing of Dicer (53). The duplex, with its two strands: the “guide” strand, the one that has a relatively unstable 5’ end or that has a 5’ U at nucleotide position 1, and the “passenger” strand, is then loaded onto an Argonaute protein family (AGO) to form the RNA-Induced Silencing Complex (RISC). The guide strand is preserved in the AGO protein and is ready to regulate gene expression by binding to the target mRNA at its 3’ end. Whereas the passenger strand, named miRNA*, is cleaved out or unwinds without cleavage (53-55, 57-59, 61). Certain miRNA* may not be degraded rapidly and may target mRNAs and play important functional roles (58).

- The non-canonical pathway

Non-canonical pathways are categorized into Drosha-independent and Dicer-independent pathways. In the Drosha-independent pathway, Mirtrons generated from the cleavage of short introns, are exported to the cytoplasm by Exportin 5, processed by Dicer and loaded onto AGO protein. In the Dicer-independent pathway, pre-miRNAs are generated from short hairpin RNA (shRNA) molecules and loaded onto AGO2 protein to be processed further (58, 62). In both pathways, the miRISC acts as a gene silencer by interacting with complementary sequences on the corresponding mRNA. Imperfect base pairing between the 5' seed region of the miRNA and the 3' UTR of the target mRNA results in mRNA degradation by endonucleocytic cleavage or translation blockage by deadynelation and decapping (63, 64).

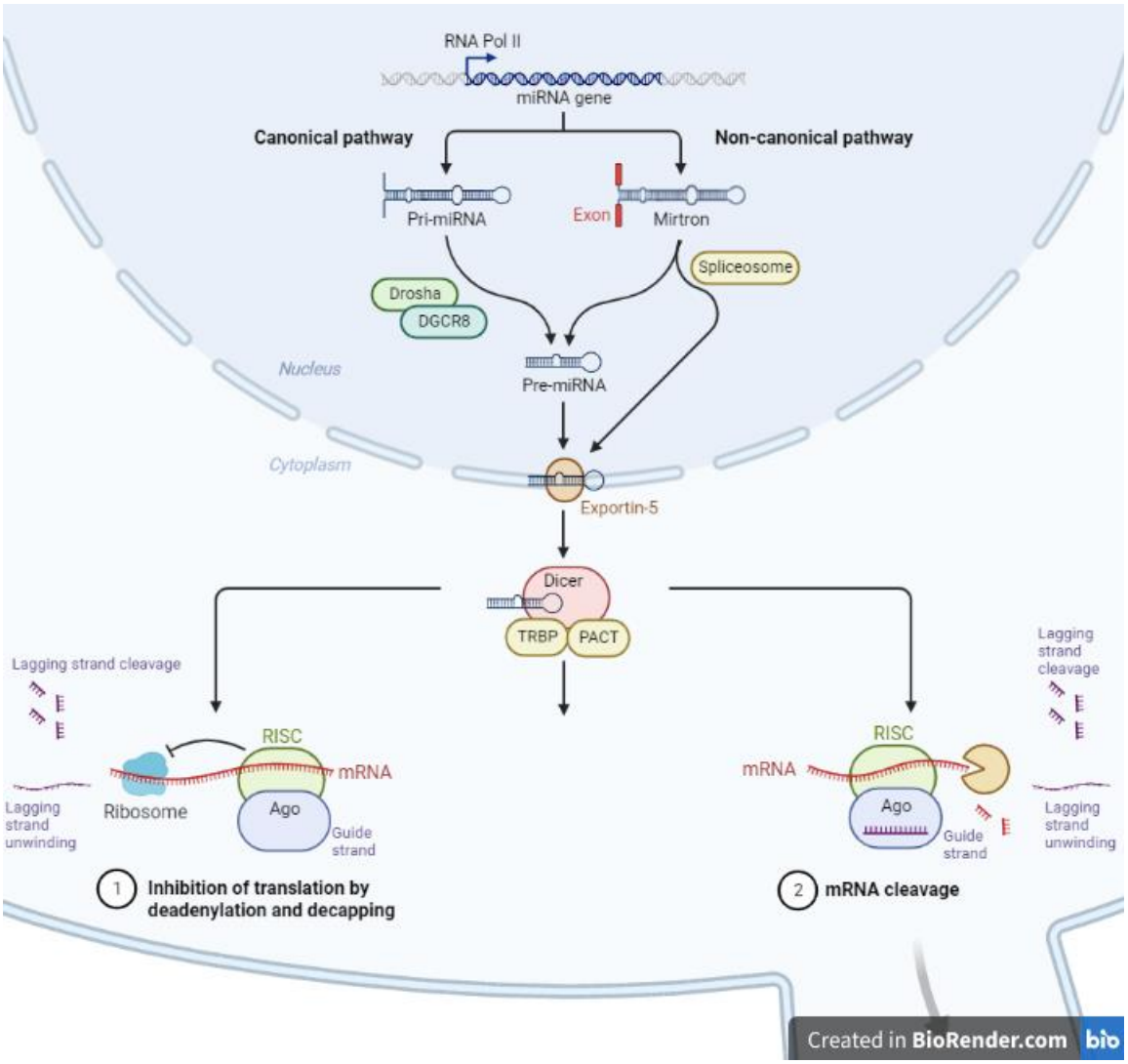


Figure 6. miRNA Biogenesis with its Canonical and Non-Canonical Pathways. The synthesis of miRNA starts in the nucleus. miRNA intronic and intergenic genes are transcribed by RNA polymerase II to give a primary miRNA (pri-miRNA). In the canonical pathway, pri-miRNA is then processed by DROSHA and DGCR8 to form the precursor miRNA (pre-miRNA) Pre-miRNA is then exported to the cytoplasm with the help of exportin (EXP5) in a Ran-GTP dependent manner. In the cytoplasm, pre-miRNA is further processed by DICER with the help of TRBP and PACT to form a miRNA duplex that is loaded into argonaute (AGO) protein forming the RNA-Induced Silencing Complex (RISC). In DROSHA-independent non-canonical pathway, mirtrons are exported to the cytoplasm by EXP5, processed by DICER and loaded into AGO protein. In the DICER-independent non-canonical pathway, pre-miRNAs are transported to the cytoplasm by EXP5 and loaded onto an AGO protein. In all pathways, the guide strand is preserved in the complex and interacts with the target mRNA leading to its degradation, deadenylation or decapping thus inhibiting translation.

2. microRNAs in Biological Processes

microRNAs have regulatory functions in various biological processes such as differentiation, development, apoptosis, angiogenesis and immune responses by modulating specific target genes (65). In addition to the miRNAs role in the mammary gland development and lactation (66), several studies have discussed their role in the development of the muscular and nervous systems as well as in the regulation of the immune system. For example, miR-124 and miR-128 have tissue specific expression in neurons, miR-26 and miR-29 have the strongest expression in astrocytes, and miR-181a has an important role in T-cell selection and sensitivity to antigens (65, 67).

3. microRNAs and Cancer

miRNAs are dysregulated in cancer and involved in the whole process of tumorigenesis. The first correlation between miRNA and cancer was reported in Chronic Lymphocytic Leukemia (CLL) in 2002 where Dr. Croce's group have found that the deletion of miR-15a and miR-16-1 gene clusters resulted in tumor proliferation and progression. Further study divulged the potential of these miRNAs as tumor suppressors by targeting Bcl-2, an anti-apoptotic protein. After that, numerous studies have emerged correlating miRNAs with other types of cancer (68, 69). miRNAs are classified as tumorsuppressors or oncogenes based on their corresponding downstream targets. Onco- and tumor suppressor- miRNAs' aberrant expression in cancer has been described by various mechanisms (70). First, it could be dictated by the fragile location of their corresponding coding genes (70, 71). Such locations increase the tendency of miRNA genes to chromosomal abnormalities (translocations, deletions, amplification). Second, miRNA expression could be

under the epigenetic control and DNA-binding factors affecting the miRNA genes' promoter regions. For example, hyper-methylation is an epigenetic process that leads to the downregulation of miRNA genes. miR-9-1 gene in BC is an example of this process (72). Histone deacetylation and tri-methylation are two other epigenetic processes that downregulate miRNA genes as in the case of miR-29 in B-cell lymphomas (73). On the contrary, there are epigenetic processes that upregulate miRNA genes. Histone acetylation is an epigenetic modification that leads to the overexpression of miRNA genes as in the case of miR-224 in hepatocellular carcinoma (HCC) (74). In addition, P53, a tumor suppressor and a gene expression regulator, is an example of a DNA-binding factor that sticks to a specific conserved consensus in the promoter region enhancing the expression of miR-34 family (75). Third, gaps in miRNA biogenesis that affect the key players in this process (DROSHA, DICER, DGCR8, TRBP, and AGO) could be at the basis of miRNA dysregulation (76). For example, it has been shown that there is an association between repetitive dysregulation of DROSHA and DICER with different types of cancer, miRNA differential expression and poor prognosis (77-79).

OncomiRs are the class of miRNAs that enhance tumor growth and development (Figure 7) (80). miR-17/92 cluster was the first miRNA to be reported as an oncomiR in which it targets the pro-apoptotic protein, Bim, leading to enhanced proliferation of lymphocytes (81). Other oncomiRs are miR-21 and miR-155 where they were shown to be upregulated in CLL patients (82). In addition, they were shown to be oncomiRs in other types of cancer. miR-21 plays an oncomiR role in BC by downregulating programmed cell death 4 (PDCD4) gene, a protein that suppresses metastasis (83). miR-155 has been shown to act as an oncomiR in colorectal and breast cancers by targeting the protein tyrosine phosphate receptor type J (PTPRJ), a tumor suppressor protein and the suppressor of cytokine signaling 1 gene, respectively (84, 85).

Tumorsuppressor-miRNAs are the class of miRNAs that inhibit tumor development by negatively regulating oncogenes (Figure 7) (86). This class of miRNAs is downregulated in cancerous cells and tissues (80, 86). miR let-7 is one example of tumorsupressor miRNAs that is downregulated in cancers. Its decreased expression results in oncogenic loss of differentiation (86). miR-9 and miR-505-5p are additional examples of tumorsuppressors in colorectal and cervical cancers, respectively. miR-9 fulfills its suppression by targeting CXCR4 and miR-505-5p manages the proliferation, migration and invasion of the tumor by targeting cyclin-dependent kinase 5 (CDK5) (87, 88).

In addition, there are miRNAs that have a dual role and can function as both an oncogene and a tumorsuppressor such as miR-186. The targets of miR-186 could be tumor suppressors encoding pro-apoptotic factors (FOXO1, APAF1, P2RX7, RETREG1), metastasis inhibitors (AKAP12, NR5A2), and anti-proliferative factors (PTEN, PPM1B). Or they could be oncogenic encoding for anti-apoptotic factors (PAK7, SIRT6, ATAD2, SHP2), metastasis promoters (SENP1, ZEB1, Cdc42, FOXK1, YAP1), and pro-proliferative factors (XIAP, CDK1, YY1, Jagged1). In addition, miR-186 has been shown to increase cisplatin-, taxol-, and methotrexate (MTX)-sensitivity, which are common chemotherapeutic drugs, in glioblastoma, ovarian cancer, and colorectal cancer (CRC), respectively, by targeting different genes. Thus, the role of miR-186 is dictated by the ratio of the targeted oncogenic/tumor suppressor genes expression, depending on the context (89).

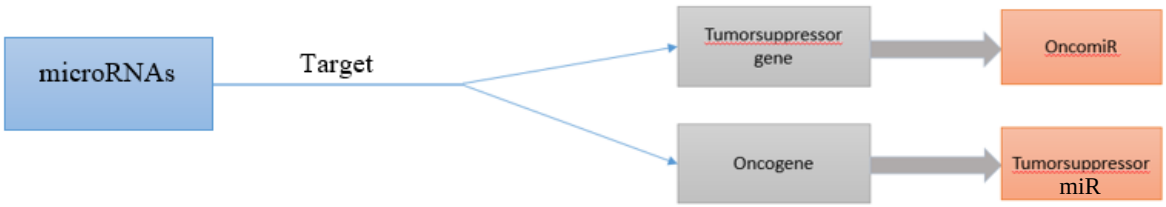


Figure 7. miRNA Potential as an Oncogene or as a Tumor Suppressor.

4. microRNAs and Breast Cancer

miRNAs dysregulation in BC was first reported by Iorio et al in 2005 through miRNA profiling of breast tumors versus normal tissues (90). Later, miRNAs dysregulation has been shown to play an important role in the stratification of BC. A study by Mattie et al. revealed distinct miRNA subsets distinguishing HER2+ from ER+, HER2+ from HER2-, and ER+ from ER- BC. They showed that a subset of miRNAs encompassing miR-107, miR-153, and miR-195 was specific to HER2 status while another subset including miR-142-5p, miR-205, and miR-25 was specific to ER/PR status (91). After that, a substantial number of differentially expressed miRNAs have been shown to distinguish further the molecular subtypes of BC. A subset of miR-20a, miR-106a, and miR-17-5p was differentially expressed in basal-like BC. However, miR-10a and miR-10b were differentially expressed in Luminal A subtype (36). In addition, dysregulated miRNAs have been shown to regulate oncogenic and tumor suppressor pathways consequently being active players during different stages of BC. They have been shown to regulate apoptosis, angiogenesis, cell cycle progression, invasion, metastasis, and epithelial to mesenchymal transition (EMT) (92). miR-16, for example, was identified as one of the downregulated miRNAs in BC and it was shown

to play a tumor suppressor role regulating cell cycle progression by targeting genes implicated in cell cycle and proliferation (93, 94). miR-126 is another miRNA playing the role of tumor suppressor in BC. It was shown to be implicated in vasculogenesis, angiogenesis, proliferation, and prognosis prediction (93-95). On the contrary, miR-9 was shown to be upregulated in invasive lobular carcinomas and implicated in angiogenesis, invasion, and metastasis. In addition, the oncogenic miR-21 was shown to be upregulated in BC and implicated in apoptosis. Suppression of miR-21 reversed EMT via the induction of E-cadherin expression and the down-regulation of mesenchymal markers, thereby suppressing proliferation, survival, and invasion in BC by upregulating PTEN, PDCD4, and RECK genes (93, 94).

Interestingly, it has been shown that miRNAs are present and stable in several biological fluids including blood, plasma, serum, saliva, urine, breast milk, tears, amniotic fluid, seminal plasma, peritoneal and pleural fluid, bronchial lavage and colostrum (96-99). Remarkably, miRNAs can act as diagnostic, prognostic, and therapy predictive biomarkers which might be helpful for early BC diagnosis and improved therapy outcomes, in addition to their role in breast tumorigenesis. For instance, miR-155 was reported to be a diagnostic biomarker for early BC where it was shown to be significantly upregulated in BC patients' serum. miR-148b was found to be a potential biomarker for young BC patients and miR-155 was shown to be a significant biomarker for menopausal state, PR and HER2 status. Additionally, miR-10b was correlated with ER and PR status among Lebanese patients (100, 101).

Moreover, several studies have emphasized the role of miRNAs in therapy prediction in BC where their dysregulation could enhance resistance or sensitivity to therapy. BC treatment, as mentioned before, could include radiotherapy, chemotherapy, targeted therapy, and/or hormonal therapy. Dysregulated miRNAs such as miR-21, miR-144, and miR-27a (upregulation), miR-205,

miR-200c, and miR-302 (downregulation) have been shown to cause radioresistance in BC cell lines (102-107). In addition, increased levels of miR-210 in the plasma of HER2+ BC patients were associated with Trastuzumab resistance, a chemotherapeutic regimen used for the treatment of HER2+ BC (108). Figure 8 summarizes the tumor suppressor and oncogenic miRNAs and their functions in BC.

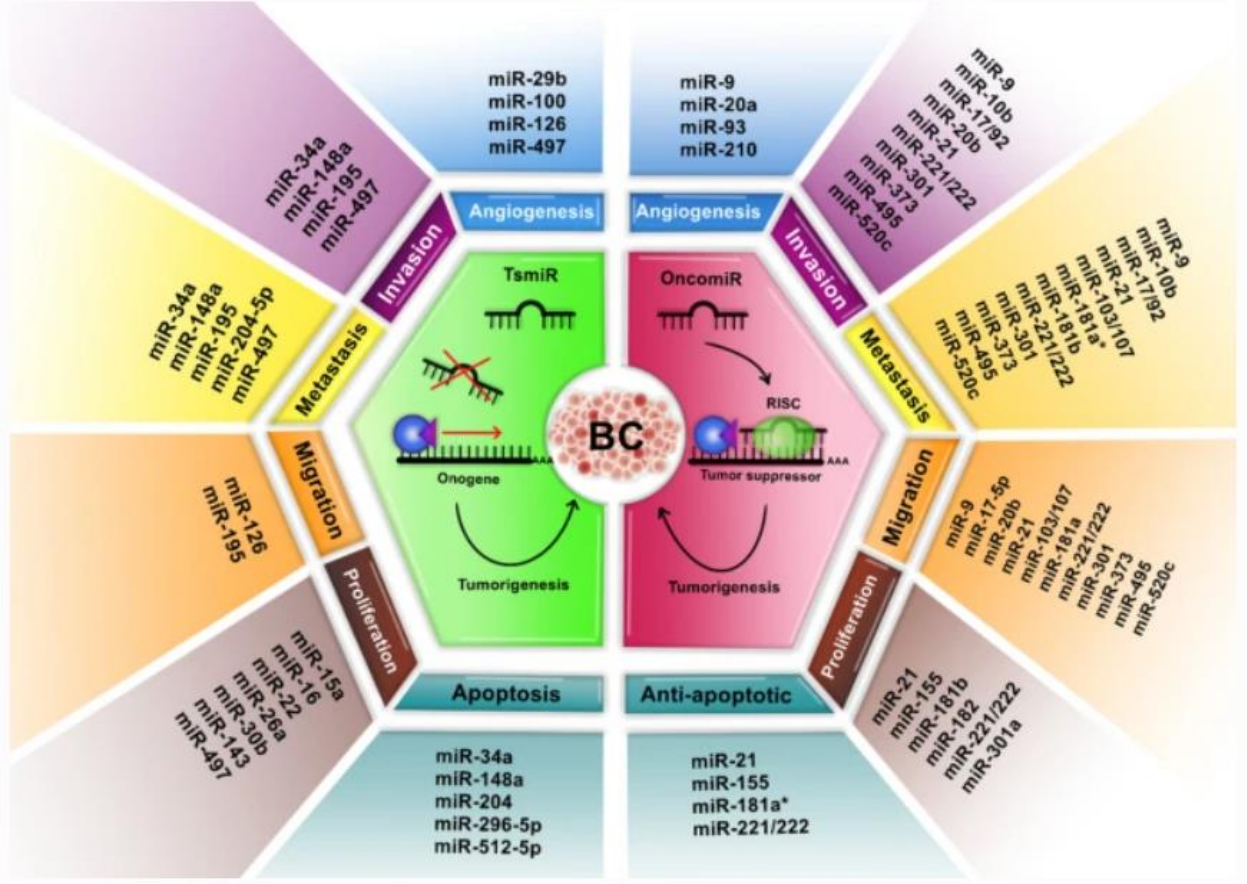


Figure 8. miRNAs Implicated in BC Hallmarks and Tumorigenesis and their Functions. **miRNAs could play a tumor suppressor or an oncogenic role in BC depending on their implicated targets and target pathways. Figure adopted from Nurzadeh et al., A comprehensive review on oncogenic miRNAs in breast cancer, 2021.**

5. Dysregulated miRNAs in Estrogen-Receptor-Positive Lebanese BC Patients

In 2017, Nassar et al. have performed a miRNA microarray and have reported that hsa-miR-183-5p is one of the most significantly dysregulated (up-regulated) miRNAs in Lebanese ER+ BC patients in comparison to normal adjacent breast tissues, with a fold change greater than 2. In addition, the differential expression of miR-183-5p was further validated by an RT-qPCR in 20 tumor and 10 normal adjacent breast tissues where it was shown to be significantly upregulated. This has arisen our interest in investigating this miRNA in BC. Furthermore, based on currently ongoing project in the lab, miR-187-3p has been shown to be dysregulated in BC.

6. miR-183-5p

hsa-miR-183-5p is coded by a member of highly conserved gene cluster located at the 7q31-34 locus of the human chromosome (109). miR-183-5p has been studied in a wide range of diseases as COVID-19, myocardial ischemia/reperfusion injury, diabetic complications, as well as a variety of cancers. miR-183-5p was exhibited to play tumor suppressor and oncogenic roles in many cancers as colorectal (CRC), cervical, lung, gastric, breast, bladder, thyroid, prostate, hepatocellular, and endometrial cancers as well as osteosarcoma and acute myeloid leukemia (AML). Figure 9 summarizes the role of miR-183-5p in these types of cancer (110-126). miR-183-5p role is depicted depending on the type of cancer and implicated pathways. Meng et al. have investigated a tumor suppressor effect of miR-183-5p in Lung cancer. They have shown that the corresponding miRNA mitigates cell proliferation and invasion of lung cancer cells by targeting PIK3CA (118). In prostate cancer, miR-183-5p was shown to have a tumor suppressor role

inhibiting cell adhesion by downregulating ITGB1 (115). On the contrary, miR-183-5p was shown to have an oncogenic effect in CRC where it increases angiogenesis and enhances radio-resistance by targeting FOXO1 and ATG5, respectively (110, 111). Duan et al. have revealed the oncogenic potential of miR-183-5p in HCC where it enhances proliferation, migration, and invasion of HCC cells by targeting PDCD4 (114). miR-183-5p has also an oncogenic role in AML where it was shown to enhance AML cell proliferation via the activation of RAS/RAF/MEK/ERK and PI3K/AKT/FoxO3a pathways by downregulating Erbin gene expression (113). Yan et al. have investigated that miR-183-5p in endometrial cancer downregulates Ezrin expression thus promoting apoptosis and suppressing EMT, proliferation, invasion and migration of human endometrial cancer cells (112). Moreover, miR-183-5p was shown in several studies, among others, to enhance BC development, proliferation, metastasis, and angiogenesis, and inhibits apoptosis by targeting RGS2, four and a half LIM protein1, and PDCD4 (124-126).

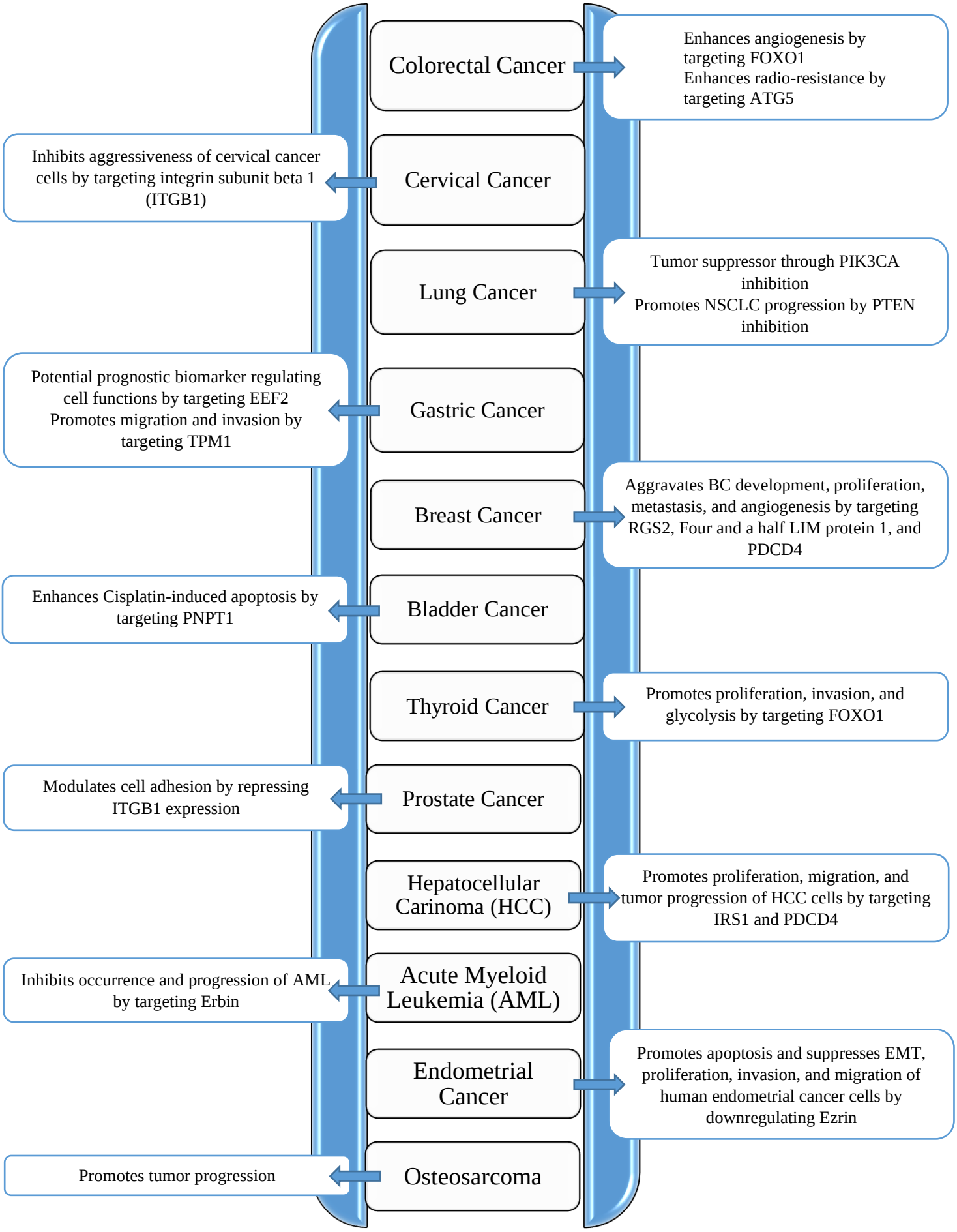


Figure 9. miR-183-5p Role in Different Types of Cancer

In addition, miR-183-5p has been shown to be sponged by several upstream Long non-coding (Lnc) and circular (circ) RNAs in various cancers as cervical, esophageal, gastric, ovarian, and breast cancers. Bai et al. have shown that LncRNA CRNDE acts as an oncogene in cervical cancer through sponging miR-183-5p (127). LncRNA ELFN1-AS1 was shown to promote esophageal cancer progression via miR-183-5p sponging (128). In gastric cancer, miR-183-5p was shown to be sponged by LINC00163 and circ_0000291 to inhibit proliferation, invasion, and metastasis of gastric cells (129, 130). In addition, LEMD1-AS1 was shown to suppress the progression of ovarian cancer cells through miR-183-5p sponging (131). Furthermore, Wang et al. have shown that LncRNA PDIA3P promotes BC development by sponging miR-183-5 (132).

7. miR-187-3p

miR-187-3p is a poorly investigated miRNA, yet, it has been studied in several types of cancer. So far, it has been shown to be a tumor suppressor and down-regulated in various cancer types. Sun et al. have shown that miR-187-3p is significantly down regulated in NSCLC primary tumor tissues and cell lines in comparison to normal ones. Moreover, they have investigated a pivotal role of the corresponding miRNA on NSCLC through inhibiting cell proliferation, migration, invasion, and in inhibiting apoptosis by targeting the oncogenic BCL6 gene (133). Dou et al. have revealed miR-187-3p potential as a prognostic biomarker and a promising therapeutic target in HCC. They have displayed that it is downregulated in HCC tissues and cell lines in addition to its role in inhibiting metastasis and EMT by targeting S100A4 (134). Xu et al. have studied the role of miR-187-3p in ccRCC. They have proved that upregulation of miR-187-3p hinders proliferation, migration, promotes apoptosis ability in human ccRCC cells as well as modulates

immune microenvironment by targeting LRFN1 (135). Furthermore, miR-187-3p was shown to be implicated in response to therapy. Wu et al. have discovered this potential of the corresponding miRNA in TNBC cells where they have shown that miR-187-3p enhances gemcitabine sensitivity by targeting FGF9 gene expression (136).

Moreover, it has been shown that miR-187-3p is implicated in various sponging mechanisms, where it is sponged by LncRNAs and circRNAs in cancers as HCC, NSCLC, thyroid, and pituitary adenoma. The sponging mechanisms and their effects in several types of cancer are summarized in Figure (10) (137-140).

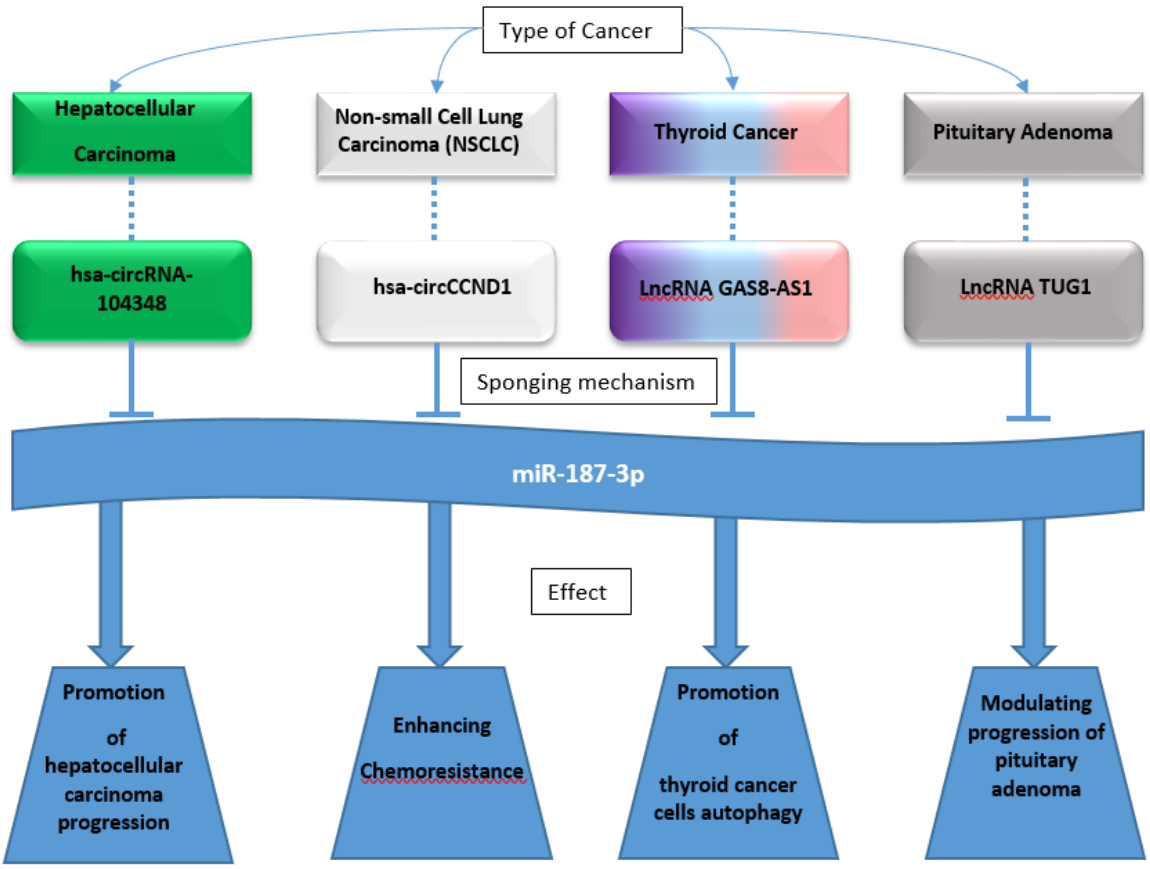


Figure 10. The LncRNA/circRNA-miRNA Sponging Axes for miR-187-3p in Several Types of Cancer and their Effects. hsa-circRNA-104348 functions as a competing endogenous RNA (ceRNA) to promote HCC progression by targeting miR-187-3p/RTKN2 axis and activating Wnt/β-catenin pathway. hsa-circCCND1 regulates oxidative stress to enhance chemoresistance in NSCLC by sponging miR-187-3p. In thyroid cancer, miR-187-3p/ ATG5 axis is one of two targeted axes by the long non-coding RNA GAS8-AS1 to promote thyroid cancer cells autophagy. LncRNA TUG1 in pituitary adenoma enhances progression of the tumor by sponging miR-187-3p.

C. Aim of the Study

Recently, our lab group has shown a panel of dysregulated miRNAs in ER+ Lebanese BC patients by performing a miRNA microarray analysis. Interestingly, hsa-miR-183-5p was one of the most significantly upregulated miRNAs. This has arisen our interest to study the effect of miR-183-5p in BC. Because we believe that the choice of one miRNA is not adequate in dictating the real effect of miRNAs, we decided to do one step further in BC research and study the co-effects of two miRNAs, one of which is hsa-miR-183-5p, in BC tumorigenesis. The choice of the second miRNA was based on the results of an ongoing project in the lab. It has been shown that miR-187-3p is dysregulated in BC. So, for this study, we chose to investigate the miRNAs "hsa-miR-187-3p" with "hsa-miR-183-5p" in ER+ BC for they have shown to be significantly dysregulated. We will conduct this study to better understand the effects of these two miRNAs in ER+ BC tumorigenesis. For this purpose, the dysregulation of miR-183-5p and miR-187-3p in ER+ BC will be validated by an in silico analysis by downloading data from TCGA database and analyzing them using DEseq2 software. Then, potential shared targets and target pathways between miR-183-5p and miR-187-3p will be looked for through another in silico analysis using miRtargetlink2 database. Further selection will be performed on the shared target genes where the ones implicated in BC or cancer will be chosen. After that, in silico Kaplan-Meier analysis will be done on our miRNAs of interest and the chosen potential shared target genes to determine the potential prognostic roles of miR-183-5p, miR-187-3p and their targets in ER+ BC patients. Genes having inverse correlation with the miRNAs will be chosen for further investigation. Furthermore, the aberrant expression of miR-183-5p and miR-187-3p in an in vitro model will be validated. Then, different functional assays will be performed to determine the roles of dysregulated miR-183-5p and miR-187-3p on the cell cycle, proliferation, and progression of ER+ BC MCF-7 cell line. In

addition, the effect of the modulated expression of miR-183-5p and miR-187-3p on the selected potential shared targets will be assessed by RT-qPCR. In essence, this study will unveil the roles of miR-183-5p and miR-187-3p in BC tumorigenesis.

CHAPTER II

MATERIALS AND METHODS

"There is no shortcut to truth, no way to gain knowledge of the universe except through the gateway of the scientific method."

-Karl Pearson

A. Bioinformatics Analysis

1. TCGA Data Analysis

Harmonized miRNA-Seq counts were downloaded from the GDC portal-"TCGA BRCA" project. The downloaded datasets included 1079 BC cases. These were further classified into different BC classes using "citbcmst" R package tool. ER+ BC cases were chosen where 264 Luminal A BC samples, the only ER+ BC available in the database, were obtained versus 27 normal adjacent tissue samples. miRNA differential expression analysis was conducted using DEseq2 R package software between Luminal A BC tissue samples versus normal adjacent tissue samples.

2. In Silico miRNA-mRNA Targets Prediction

To uncover the potential shared target genes between miR-183-5p and miR-187-3p, miRTargetLink 2.0 tool, which generates convenient network graphs representative of the genes that are targeted by multiple miRNAs, was used. Only predicted target gene networks were obtained where no validated shared genes are present for the corresponding miRNAs indicating that there is no publications yet validating the interaction of miR-183-5p and miR-187-3p with any shared target. The tool has given us around 100 predicted shared target genes based on several

sources like miRcode, CoMeTa, Cupid, BCmicrO, MAMI, and microrna.org, among others. Then, extensive PubMed searches were then performed where the first search was as follows: ((“Neoplasms”[Mesh]) AND (“the name of the miRNA-miR-183-5p/miR-187-3p”[Mesh]) AND (“the name of the predicted shared target gene”[Mesh])), the second one was as follows: ((“Breast Neoplasms” [Mesh]) AND (“the name of the miRNA-miR-183-5p/miR-187-3p” [Mesh]) AND (“the name of the predicted shared target gene”[Mesh])), the third one was as follows: ((“Neoplasms”[Mesh]) AND (“the name of the predicted shared target gene”[Mesh])) and the fourth was as follows: ((“Breast Neoplasms” [Mesh] AND (“the name of the predicted shared target gene”[Mesh])). These searches were conducted to check for validated miRNA-mRNA interaction and if the predicted shared target genes have roles in breast cancer or in cancer in general.

3. Kaplan-Meier Analysis

To determine the effects of miR-183-5p, miR-187-3p and their targets expression on BC survival, kaplan-Meier analysis was done. miRpower web tool was utilized to determine the survival of patients with dysregulated miR-183-5p, miR-187-3p, MBNL1, SMAD4, BCL6, FGF9 or DAB1 expression. Meta data for Kaplan-Meier survival analysis were obtained from https://kmplot.com/analysis/index.php?p=service&cancer=breast_mirna and https://kmplot.com/analysis/index.php?p=service&cancer=breast_rnaseq_gse96058 for miRNA and mRNA expression analysis, respectively. In the Kaplan-Meier Plotter page, filters were adjusted so that patients with ER+ status were selected from the TCGA database. As a result of these filters, 1062 patients were included in miRNA analysis and 2575 patients were included in the mRNA analysis. Kaplan-Meier plots were generated

and a p-value<0.05 was considered a significant correlation between the miRNA/target expression and overall survival in ER+ BC patients.

B. Cell Culture

MCF-7 (ER+, PR+, HER2-) and MDA-MB-453 (AR+, ER-, PR-, HER2-) BC cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Sigma Aldrich) with 10% Fetal Bovine Serum (FBS) (Gibco), 1% sodium pyruvate and 1% penicillin/streptomycin. MDA-MB-231 (ER-, PR-, HER2-) was maintained in DMEM low glucose with 10% FBS (Gibco), 1% sodium pyruvate and 1% penicillin/streptomycin. SUM-185 PE (AR+, ER-, PR-, HER2-) was maintained in Ham's F-12 with 5 µg/ml insulin, 50 µM hydrocortisone (Sigma Aldrich, St. Louis, MO) and 5% FBS. The non-tumorigenic cell line MCF-10A was maintained in DMEM F-12 (Sigma Aldrich) with 5% Horse Serum (STEM CELL Technologies), 20 ng/ml Epidermal Growth Factor (EGF), 0.5 µg/ml hydrocortisone, 100 ng/ml choleratoxin, 10 µg/ml insulin, and 1% penicillin/streptomycin. Cells were incubated at 37°C with 5% CO2.

Table 3. Characteristics of the Used BC Cell Lines (ATCC)

Cell Line	Organism and Age	Origin	Primary Tumor	Receptors
MCF-7	Human, 69 years old	Mammary gland, breast, derived from metastatic site; pleural effusion	Luminal type, invasive ductal carcinoma	ER+ PR+ HER2-
MDA-MB-231	Human, 51 years old	Mammary gland, breast, derived from metastatic site; pleural effusion	Basal type adenocarcinoma	ER- PR- HER2-
MDA-MB-453	Human , 48 years old	Mammary gland, breast, derived from metastatic site; pericardial effusion	Invasive apocrine breast carcinoma	Androgen receptor + ER- PR- HER2 -
SUM-185 PE	Human, age unknown	Mammary gland, breast, derived from metastatic site; pleural effusion	Invasive apocrine breast carcinoma	Androgen receptor + ER- PR- HER2-

C. Total RNA Extraction

Total RNA was extracted from the cells using TRI Reagent (Sigma Aldrich) according to the manufacturer's instructions. 1 ml of TRI Reagent was added per sample, and samples were incubated for 5 minutes at RT to ensure that cells are totally lysed. Then, 200 µl of chloroform was added per 1 ml TRI Reagent, mixed by inverting the tubes for 15 seconds, and centrifuged at 12,000xg for 15 minutes at 4°C. After centrifugation, the mixtures separate into a lower red phenol-chloroform phase, a DNA rich interphase, and a colorless aqueous phase containing the RNA. Then, the aqueous phase from each sample was

transferred to a new tube by angling the tubes by 45°. 500 µl of 100% isopropanol was added per 1 ml TRI Reagent, vortexed for 2 seconds, and incubated at RT for 10 minutes. After that, the samples were centrifuged at maximum speed (21,100xg) for 15 minutes at 4°C to collect the RNA. The supernatants were removed and two steps of washing with 75% ethanol prepared in DEPC (RNase-free water) were done. Each step was followed by centrifugation at maximum speed (21,100xg) for 5 minutes at 4°C. Pellets were air-dried for at least 10 minutes to remove any residuals of ethanol. Pellets were then re-suspended in 40 µl DEPC water and incubated for 15 minutes at 55°C on a heat block. RNA concentration and purity were measured by Denovix Blue Spectrophotometer. Samples with a good quality ratio were stored at -80°C to be used in further experiments.

D. miRNA Expression by Reverse Transcription Quantitative Real Time PCR (RT-qPCR)

Reverse transcription of 10 ng of RNA was completed using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems). Multiplex cDNA master mixes were produced on ice. 2µl of DEPC treated water, 0.1µl of 100 mM dNTPs, 1 µl of 10x Reverse Transcriptase Buffer, 0.13 µl of RNase Inhibitor, 0.67 µl of Multiscribe Reverse Transcriptase enzyme, and 1 µl of each 5x microRNA primers (RNU6B as an endogenous control, miR-183-5p, miR-187-3p), for a total volume of 6.9 µl, were added per reaction sample. 3.1 µl of the 10 ng RNA diluted in DEPC was put to each reaction tube on ice. Reaction samples were well mixed and loaded in the BioRad T100 thermal cycler. The protocol is as follows: 30 minutes incubation at 16°C (priming), 30 minutes incubation at 42°C (reverse transcription), 5 minutes incubation at 85°C

(RT inactivation), and infinite hold at 4°C. cDNA samples were diluted by adding 57 µl DEPC water and stored at -20°C for later use in RT-qPCR.

RT-qPCR for miR-183-5p and miR-187-3p expression was accomplished using TaqMan microRNA Assays and 2x TaqMan Universal Master Mix with no Amperase Uracil N-glycosylase (UNG) (Applied Biosystems). The master mixes were prepared as follows: 5µl of 2x Universal Master Mix, 0.5 µl of the corresponding 20x microRNA probes, and 2µl of DEPC water. 7.5µl of the master mix followed by 2.5µl of each cDNA sample were dispersed in each reaction well of a BioRad 96 well skirted PCR plate. Plate was spun shortly at 2,000xg for 1 minute and then filled into the qPCR 962 machine. The following steps were run: 10 minutes hold at 95°C, 40 cycles of 15 seconds at 95°C (denaturing step), and 60 seconds at 60°C (annealing and extension step). miR-183-5p and miR-187-3p expression was normalized according to the endogenous control RNU6B. Then, the relative expression of the corresponding miRNAs was determined in the transfected cells compared to the negative control (NC inhibitor, NC, and double NC) transfected cells.

E. Transfection of Cells

80,000 cells of MCF-7 were seeded per well in a 12-well plate to reach 60-80% confluency for the different assays. Cells were seeded in duplicates for each condition: control cells (untransfected), cells transfected with miR-183-5p inhibitor to downregulate miR-183-5p, cells transfected with miR-187-3p mimic to upregulate miR-187-3p, cells transfected with both miR-183-5p inhibitor and miR-187-3p mimic, cells transfected with negative control (NC) duplex, cells transfected with NC inhibitor, and cells transfected with both NC duplex and NC inhibior (GenePharma, Shanghai), all of which are FAM-labeled. 3,000 cells were seeded per well in a 96-well plate to measure cell viability by MTT assay. Three plates were seeded for the following time

points: 24, 48, and 72 hours. Cells were incubated overnight to reach 60-80% confluency. Media was replaced with antibiotic-free media two hours prior to transfection. Once the cells were ready, the transfection was done using Lipofectamine RNAiMAX Reagent (Invitrogen) and Opti-MEM Medium (Gibco) according to the manufacturer's instructions. For the 12-well plates, Lipofectamine and 40 pmol of miR-183-5p inhibitor/NC inhibitor and 30 pmol of the miR-187-3p mimic/NC were diluted in 56 µl Opti-MEM. Then, they were incubated for 5 minutes at RT. After that, the diluted samples were added to lipofectamine in a 1:1 ratio and incubated for 10 minutes at RT. 100 µl of each complex was added per well drop by drop in a circular pattern to ensure proper dispersion of the lipid complex. Then, the cells were incubated at 37°C and 5% CO_2 in the incubator. After 24 hours, post transfection the cells were washed with Saline (PBS) (Lonza). Cells for RNA extraction and RT-qPCR analysis were stored at -80°C. Other cells were processed directly for specific assays. For the 96-well plates, lipofectamine and 8 pmol of miR-183-5p inhibitor/ NC inhibitor, and 5 pmol of the mimic/NC were diluted in 25 µl Opti-MEM followed by 5 minutes incubation at RT, the addition of diluted samples to diluted lipofectamine, incubation for 10 minutes and then 10 µl of the final complex was added per well. The plates were incubated for 24, 48, and 72 hours to perform MTT assay.

F. Transfection Efficiency

Cells were transfected and harvested as previously described. 300 µl per condition were transferred to 1.5 ml tubes and resuspended in 400 µl of 1X PBS. Cells were analyzed on Guava EasyCyte8 Flow Cytometer (Millipore) to determine the transfection efficiency by detecting green fluorescence of FAM. Fluorescence intensity was adjusted according to the negative control sample. Green fluorescence (GRN-HLog) versus Side Scatter (FSC-HLin)

was measured and 10,000 events were collected. The percentages of transfected cells were quantified by the GUAVASOFT 2.7 software.

G. MTT Assay

MCF-7 cells were seeded in 96-well plates to measure cell growth and transfected as previously mentioned. 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), a yellow dye which is reduced by cellular enzymes to the blue product formazan, was added per well 24, 48 and 72 hours after transfection. Then, each plate was incubated for 4 hours at 37°C and 5% CO_2 followed by the addition of 100 µl DMSO as a stop solution and incubation for 15 minutes. The absorbance was measured at 595 nm using Multiskan EX from Thermo Fisher Scientific.

H. Cell Cycle Analysis

24 hours post transfection, the cells that were seeded at 80,000 cells per well seeding concentration, were harvested. 80% of cells were transferred to 1.5 ml tube for cell cycle analysis by Propidium Iodide (PI) staining. Cells were centrifuged at 2,000 RPM for 10 minutes. After that, the supernatant was discarded and the cells were fixed by resuspending the pellets with 1 ml of cold 70% ethanol (prepared in 1X PBS). Fixed cells are stored at -20°C until the day of the experiment. On the same day of the experiment, ethanol was removed by spinning down the fixed cells. Cells were then washed with 1ml of 1X PBS, centrifuged, and treated with 100µl per pellet of Ribonuclease (RNase, 0.2mg/ml) to confirm that only the DNA is stained. Samples were then incubated at 37°C for 40-50 minutes then centrifuged to remove

the RNase. Cells were then resuspended in 200µl of 1X PBS + 20µl of PI (1mg/ml). Samples were mixed rigorously and incubated at 4°C for 45 minutes secured from light. Samples were examined on Guava Easy Cyte8 Flow Cytometer (Millipore). Cell cycle of the total cell count was measured by plotting Red Fluorescence Area (RED-HLin) versus Red Fluorescence Width (RED-W), and 10,000 events were collected. Singlets cells (<5,000) were gated. The percentages of cells in each cell cycle phase were quantified and analyzed by the FlowJo software.

I. Primers Optimization of the Selected mRNA Targets

Primers of the selected mRNA shared target genes between miR-183-5p and miR-187-3p were designed using National Center for Biotechnology Information (NCBI). The specificity, amplicon size, and melting temperature (Tm) were checked on PrimerBlast. Primers were optimized on untransfected MCF-7 or MDA-MB-231 cells depending on the expression of each gene in a specific cell line based on the literature.

1. cDNA Synthesis for mRNA Expression

Reverse transcription of 1000 ng of RNA was performed using the iScript cDNA synthesis kit. cDNA master mixes were prepared on ice. 4 µl of 5x iScript Reaction Mix and 1 µl of iScript Reverse Transcriptase were added to a total of 15 µl of 1000 ng RNA diluted in DEPC water. No Reverse Transcriptase (NRT) was included as a negative control. Reaction samples were well mixed and loaded in the BioRad T100 thermal cycler. The following steps were run: 5 minutes incubation at 25°C for the priming step, 20 minutes incubation at 46°C for the reverse transcription step, 1 minute incubation at 95°C for RT inactivation, and an infinite hold at 4°C. cDNA samples were diluted by adding 20 µl DEPC water and stored at -20°C for later use in RT-qPCR.

2. Primers Optimization by RT-qPCR

Different gradients of temperature were used for the optimization of primers. The master mixes were prepared as shown in Table 4. 9 µl of each master mix was added per well of a BioRad 96 well skirted PCR plate followed by the addition of 1 µl of the cDNA sample. NRT and No Template Control (NTC) reaction wells were also included. The plate was briefly centrifuged at 2,000xg for 30 seconds to recover the full volume that needs to be in the bottom of the well. Then, the plate was loaded into the PCR C1000 Thermal Cycler Bio-Rad machine. The following protocol was followed: 10 minutes hold at 94°C, 40 cycles of 15 seconds at 94°C, a gradient of 58-68 for FGF-9 and BCL6 genes and a gradient of 59-65 for SMAD-4, MBNL1, and DAB1 for 30 seconds as well as a melt curve 55°C to 95°C with an increment 0.5°C for 0.05 seconds. The optimal temperature per primer was selected based on the highest and most overlapping melting peaks of the forward and reverse primers. Primers that did not show a clear peak on 2 µM concentration, were tried on a concentration gradient to choose the most suitable concentration.

Table 4. Primer Optimization Performed on Different Temperatures and Concentrations

Primers	BCL6	MBNL1	SMAD4	FGF9	DAB1	GAPDH
SYBR (µl)	5	5	5	5	5	5
FP volume (µL)	1	1	0.45	1	1	1
RP volume (µL)	1	1	0.45	1	1	1
DEPC (µl)	2	2	2	2	2	2
Cdna (µl)	1	1	1	1	1	1
Total (µl)	10	10	10	10	10	10
Temperature Gradient (°C)	57-66	59-65	59-65 57-63	54-56 57-66 58-68	56-65 59-65 57-63	57-66
Concentration Gradient (µM)	No gradient 2 µM	No gradient 2 µM	0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5	0.3, 1, 2, 4	0.3, 1, 2,4	No gradient 2 µM

J. Gene expression of the Selected mRNA Targets by RT-qPCR

RT-qPCR for targets expression was executed using iTaq^{TM} Universal SYBR Green Supermix (BioRad). The master mixes were prepared as shown in Table 4. Using ΔΔCq, the relative expression of the mRNA targets was determined in miR-183-5p inhibitor and miR-187-3p mimic transfected cells compared to NC inhibitor and NC duplex transfected cells, respectively. Protocol was followed according to manufacturer's constructions.

K. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 Software. Student's T-test was used to analyze differences between the two groups. Data presented are the means +/- SEM of two or three different experiments as noted in the figure legends. A p value<0.05 was considered significant.

CHAPTER III

RESULTS

"A Little progress each day adds up to big results"

-Satya Nani

A. In silico Analysis for the Expression of miR-183-5p and miR-187-3p in ER+ BC Patients

In order to check the dysregulation of miR-183-5p and miR-187-3p in ER+ BC patients, 264 Luminal A BC patient samples versus 27 normal samples were taken from TCGA database and analyzed using DEseq2 software. Both hsa-miR-183-5p and hsa-miR-187-3p were shown to be significantly upregulated in Luminal A BC patients versus normal, with an expression fold change more than 4 and a p-value less than 0.01. The results are summarized in Table 5.

Table 5. Significant Dysregulation of miR-183-5p and miR-187-3p in ER+ BC Patients

Dysregulated miRNA	Mode of Dysregulation	Log_2Fold Change	Fold Change	Adjusted P-value
miR-183-5p	Up-regulated	2.19	4.56	<0.01
miR-187-3p	Up-regulated	2.16	4.47	<0.01

B. Selection of Predicted Shared Targets between miR-183-5p and miR-187-3p

miR-183-5p and miR-187-3p shared targets were selected through miRtargetlink 2.0 tool. Extensive PubMed searches were performed to check the occurrence of any validated miRNA-mRNA interaction in BC and other types of cancer, in addition, to check for the role of the resulting mRNA targets in BC in particular and in all types of cancer in general. The targets that have shown to play important roles in BC or in cancer in general and the ones being predicted by a higher number of databases were chosen. Thus, the following predicted shared targets, among other targets, were selected: SMAD4, MBNL1, BCL6, FGF9, and DAB1 (Figure 11). Figure 11. was produced using Word Document 2007 and indicates that each gene is being targeted by both miR-183-5p and miR-187-3p.

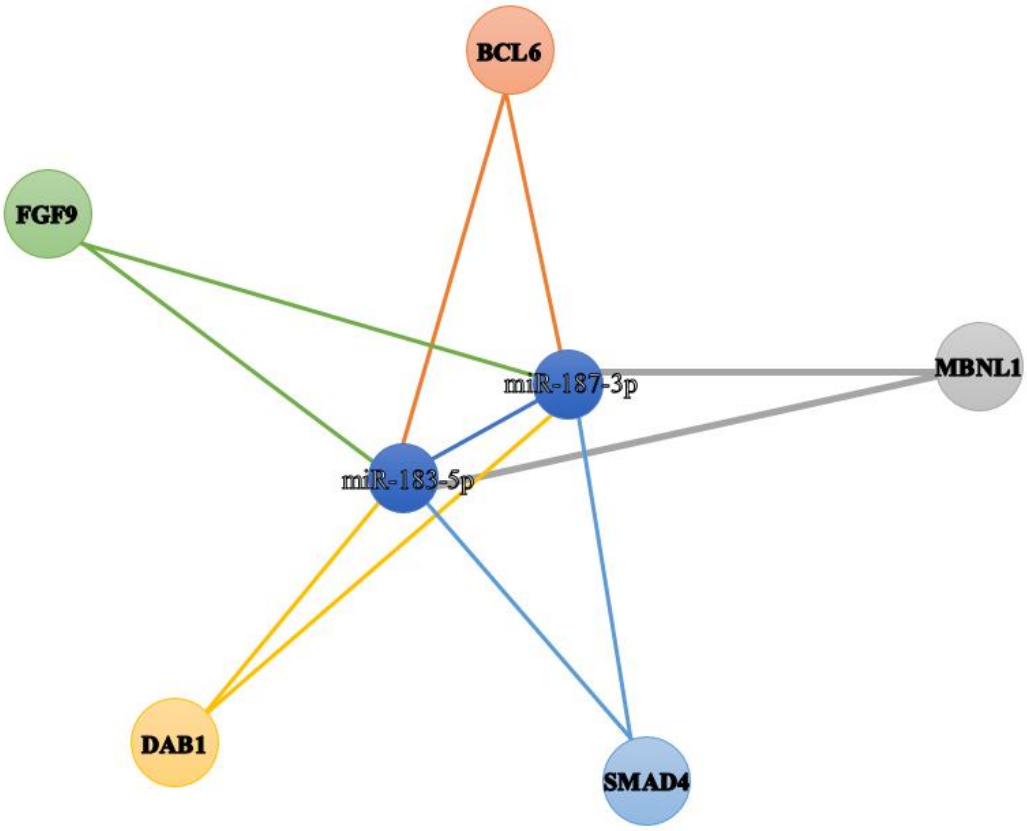


Figure 11. Predicted Shared Target Genes Between miR-183-5p and miR-187-3p.

C. Low Expression of miR-183-5p and miR-187-3p correlates with better survival in ER+ BC Patients

To determine if miR-183-5p and miR-187-3p expression could predict prognosis in ER+ BC patients, Kaplan-Meier analysis was done. ER+ patients from TCGA database were selected. It was found that the overall survival increased with miR-183-5p and miR-187-3p low expression (Figure 12A, 12B).

A. B.

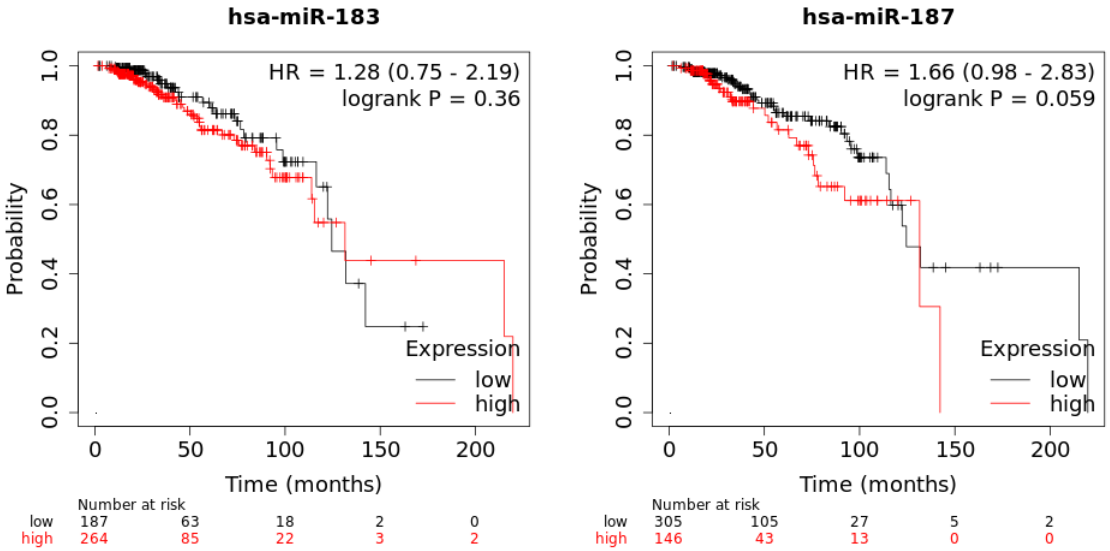


Figure 12. In Silico KM Plot showing the correlation between the Expression of miR-183-5p and miR-187-3p and Overall Survival (OS) in ER+ BC Patients. (A): Correlation of miR-183 expression with OS, (B): Correlation of miR-187 expression with OS. HR: Hazard Ratio

D. High Expression of MBNL1, SMAD4, BCL6, and FGF-9 Correlates with Better Survival in ER+ BC Patients

Using Kaplan-Meier Analysis, the correlation of each of the predicted shared target genes with the overall survival (OS) of ER+ BC patients was tested. It was found that the OS increased significantly with high expression of MBNL1, BCL6, and FGF9 and slightly with SMAD4. On the contrary, OS increased significantly with the low expression of DAB1. This indicates that MBNL1, BCL6, FGF9 and DAB1 could have important roles in ER+ BC patients' prognosis.

In addition, an inverse relationship was observed between the expression of miR-183-5p and miR-187-3p with MBNL1, SMAD4, BCL6, and FGF9 showing that these targets may be downregulated by the corresponding miRNAs and may be direct targets in ER+ BC. However, the correlation of DAB1 with survival in ER+ BC patients was directly proportional to that of miR-183-5p and miR-187-3p ruling out the option of being a direct target for these miRNAs in ER+ BC (Figure 13). Accordingly, in this study, we focused on MBNL1, BCL6, SMAD4, and FGF9.

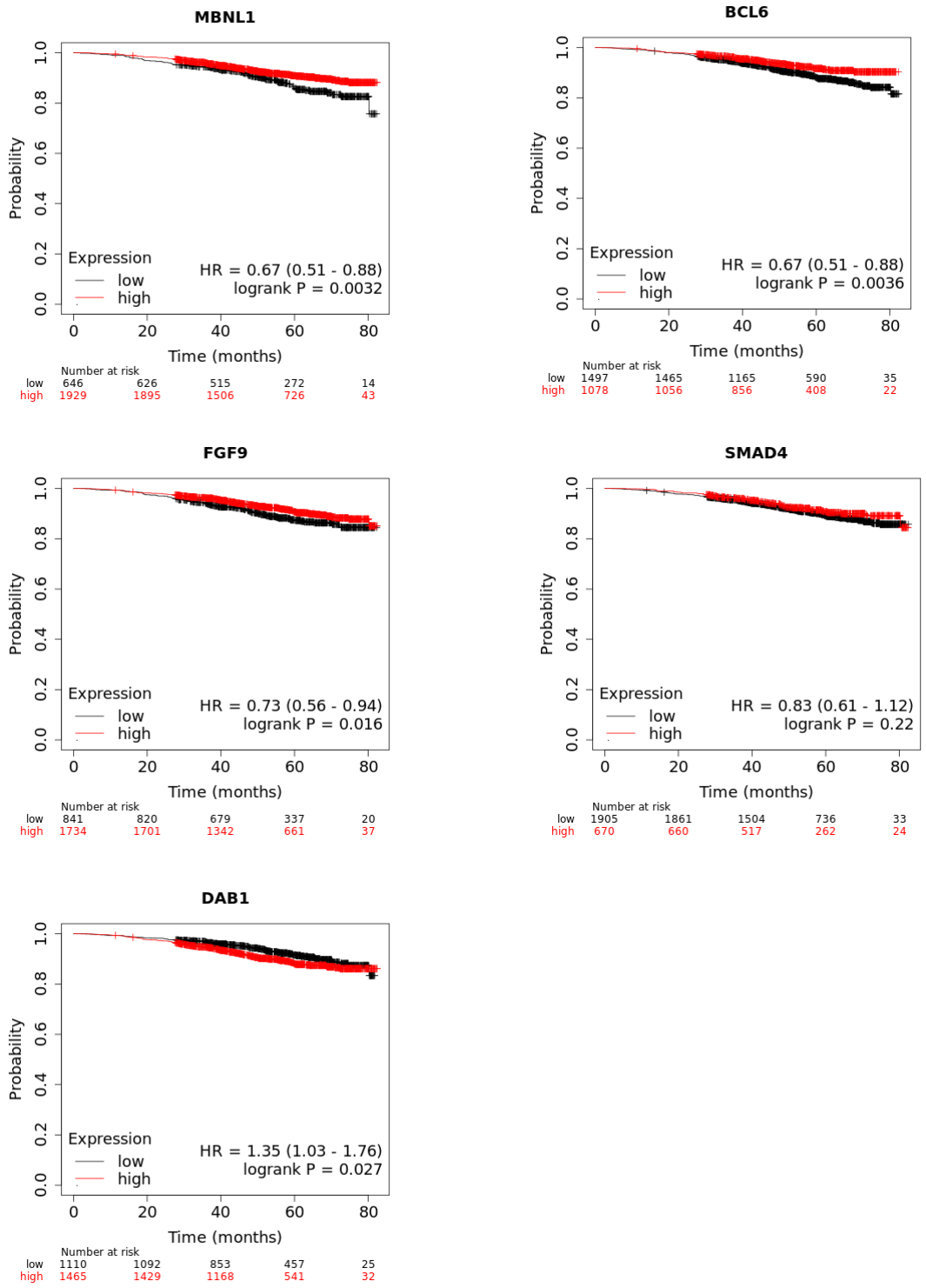


Figure 13. In Silico KM Plot Showing the Correlation Between the Expression of MBNL1, BCL6, FGF9, SMAD4, or DAB1 with Overall Survival (OS) in ER+ BC Patients.

E. miR-183-5p and miR-187-3p Expression in BC Cell Lines

The expression of miR-183-5p and miR-187-3p in different BC cell lines was assessed by performing RT-qPCR compared to the normal non-tumorigenic epithelial cell line MCF-10A and using RNU6B as an endogenous control. Results have shown that miR-183-5p was significantly upregulated in MCF-7 cells while miR-187-3p was significantly downregulated in MCF-7 and MDA-231 cells (Figure 14). Since both miRNAs have shown a strong significant dysregulation in MCF-7 cell line which is ER+, in comparison to other cell lines, it was chosen as an in vitro model for the investigation of miR-183-5p and miR-187-3p roles in ER+ BC.

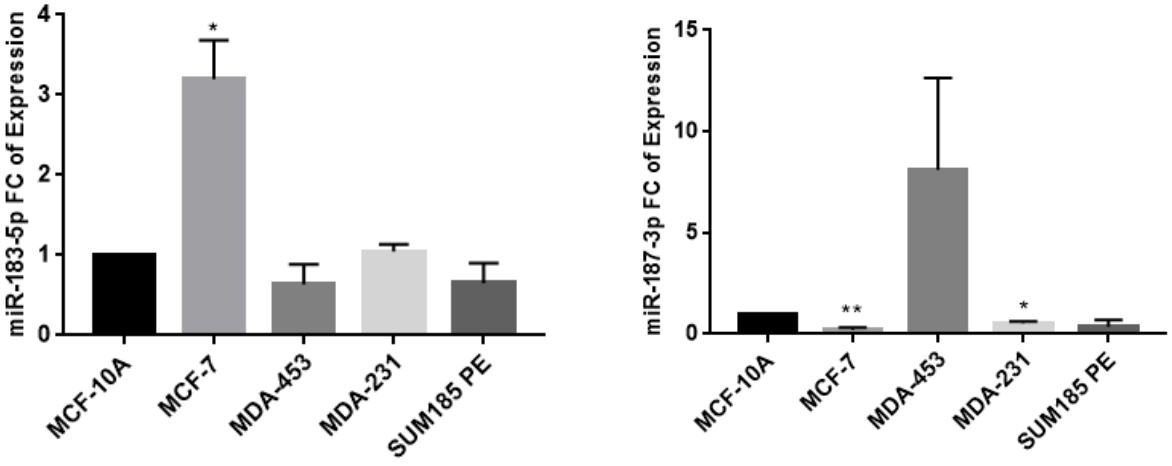


Figure 14. Relative Expression of miR-183-5p and miR-187-3p in Different BC Cell Lines Compared to MCF-10A. RNU6B was used as an endogenous control. Error bars represent SEM (n=2). * denotes $p<0.05$, ** denotes $p<0.01$

F. miR-183-5p Downregulation and miR-187-3p Upregulation in BC Cell line upon Transfection with miR-183-5p and miR-187-3p FAM-labeled Inhibitor and mimic, respectively

To discover the roles of miR-183-5p and miR-187-3p in BC, transfection of MCF-7 cells was performed with miR-183-5p inhibitor and miR-187-3p mimic as previously mentioned. Transfection efficiency for all conditions was validated by flow cytometry, each condition compared to its corresponding negative control, 24 hours post transfection (Figure 15). MCF-7 cell line was significantly transfected with miR-183-5p inhibitor and miR-187-3p mimic (Figure 16A, 16B) compared to the control. In addition, the levels of the corresponding miRNAs were detected as compared to the corresponding NC in each condition by RT-qPCR and using RNU6B as an endogenous control. The downregulation of miR-183-5p was significant in miR-183-5p inhibitor-transfected cells as compared to NC inhibitor (Figure 17A). miR-187-3p was significantly overexpressed in MCF-7 cells transfected with miR-187-3p mimic as compared to NC (Figure 17B).

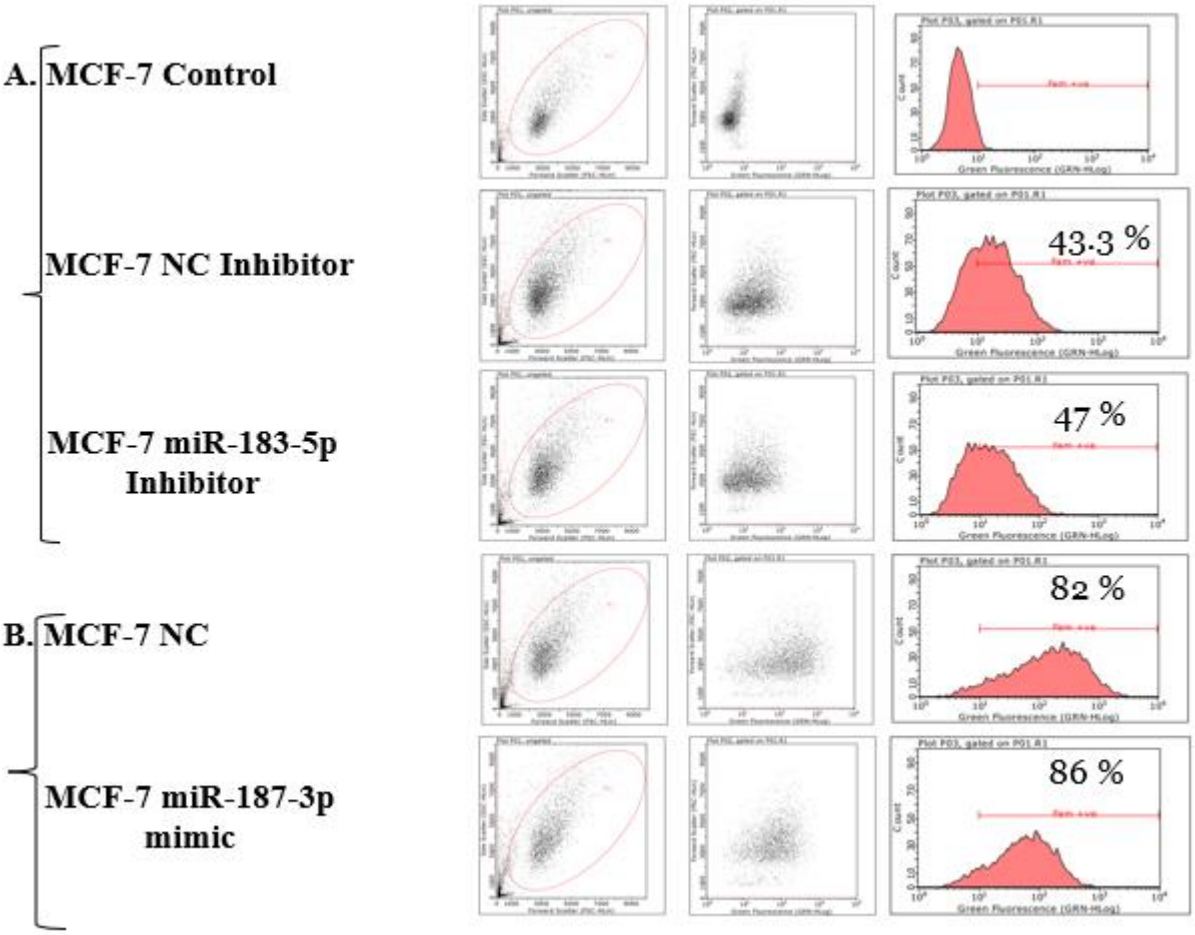
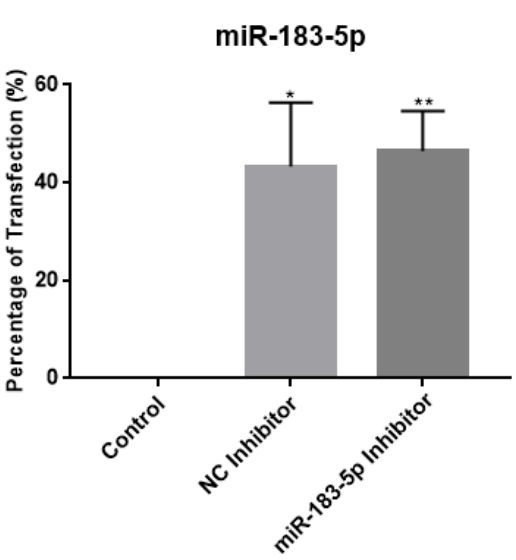


Figure 15. Transfection Efficiency of FAM-labeled miR-183-5p Inhibitor and miR-187-3p mimic. Transfection efficiency was measured by flow cytometry in MCF-7 cell line post transfection. (A): Representative figures of flow cytometric analysis of miR-183-5p inhibitor compared to NC inhibitor, (B): Representative figures of flow cytometric analysis of miR-187-3p mimic compared to NC

A



B

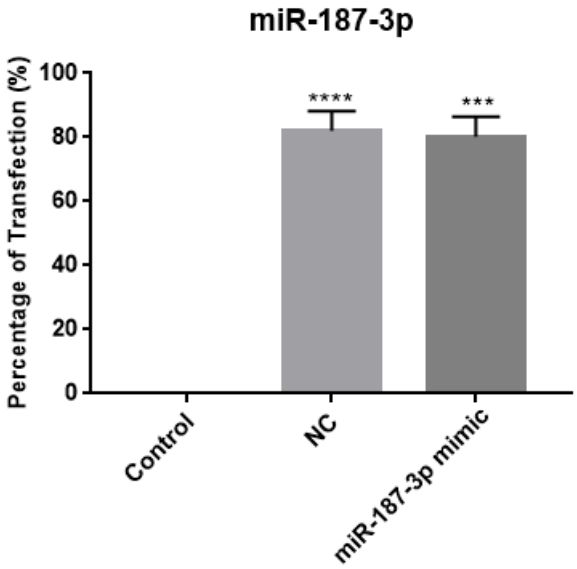


Figure 16. Flow Cytometric Transfection Efficiency of each condition Compared to the Corresponding NC. (A): Percentage of FAM-labeled miR-183-5p inhibitor and NC inhibitor transfected cells as compared to control, (B): Percentage of FAM-labeled miR-187-3p mimic and NC transfected cells as compared to control. Error bars represent SEM (n=3). * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$, **** denotes $p<0.0001$

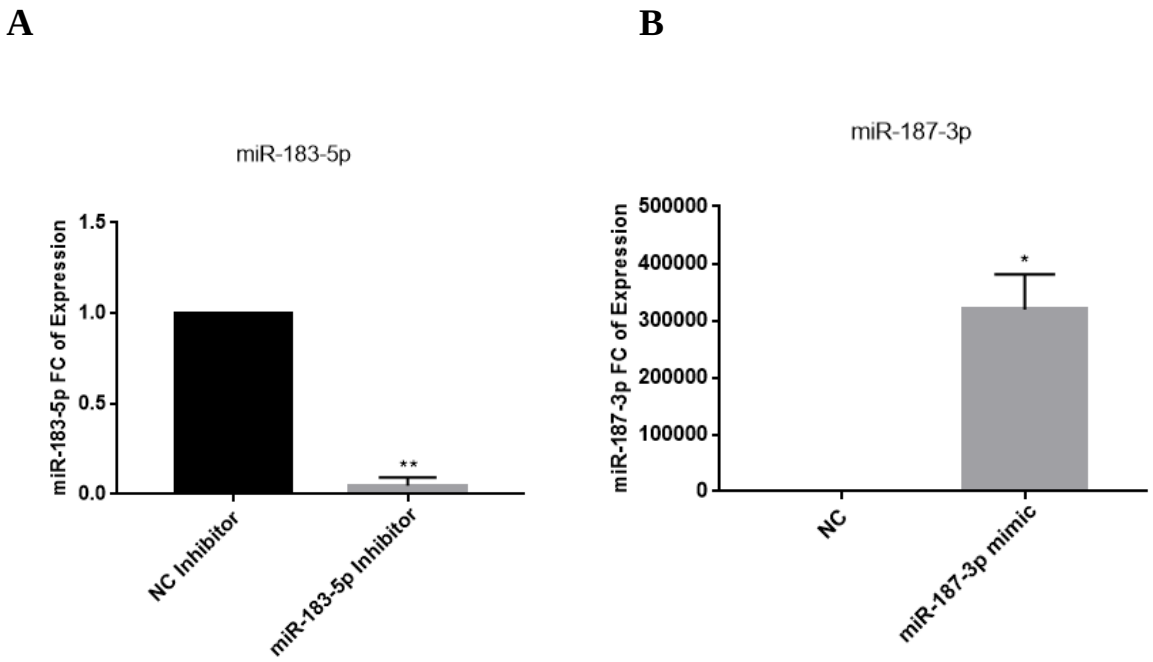


Figure 17. Transfection Efficiency of FAM-labeled miR-183-5p Inhibitor and miR-187-3p mimic by RT-qPCR in MCF-7 cell line. Transfection efficiency was measured by RT-qPCR. (A): RT-qPCR analysis of miR-183-5p levels as compared to NC inhibitor, (B): RT-qPCR analysis of miR-187-3p levels as compared to NC, RNU6B was used as an endogenous control. Error bars represent SEM (n=2). * denotes $p<0.05$, ** denotes $p<0.01$

G. miR-183-5p and miR-187-3p have no effect on MCF-7 Cells Proliferation

To determine the effect of miR-183-5p and miR-187-3p on MCF-7 proliferation, MTT assay was done. Results show that there is no significant difference in the proliferation of transfected MCF-7 cells with the different conditions when compared to the cells transfected with the corresponding negative control at 24, 48, and 72 hours. This indicates that miR-183-5p and miR-187-3p have no significant effect on MCF-7 cells proliferation in the tested conditions (Figure 18).

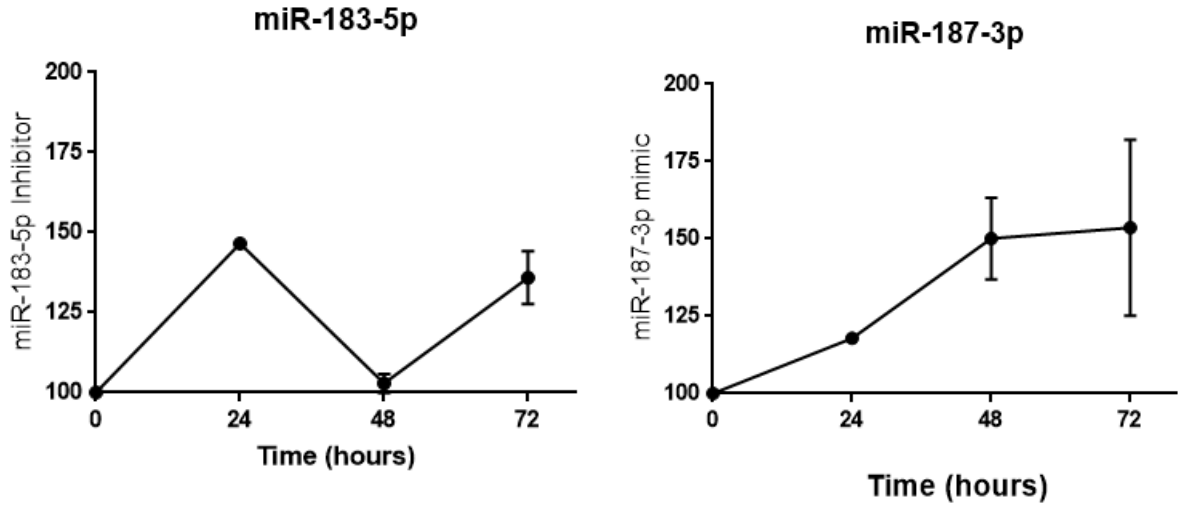


Figure 18. Proliferation of MCF-7 Cells Transfected with miR-183-5p Inhibitor or miR-187-3p Mimic Compared to NC Inhibitor and NC respectively 24, 48, and 72 hours post transfection.

H. Downregulation of miR-183-5p has no Effect on Cell Cycle of MCF-7 while overexpression of miR-187-3p slightly increases G2-M phase

To determine the effect of miR-183-5p and miR-187-3p on cell cycle of MCF-7, PI assay was done. There was no significant difference in the cell cycle phases of miR-183-5p inhibitor-transfected cells compared to NC inhibitor 24 hours post transfection (Figures 19, 20). While there was a slight increase in G2-M phase in MCF-7 cells transfected with miR-187-3p mimic (Figures 19,20). This indicates that miR-183-5p has no effect on the cell cycle progression of the selected cell line at the tested time points while miR-187-3p slightly increases G2-M phase.

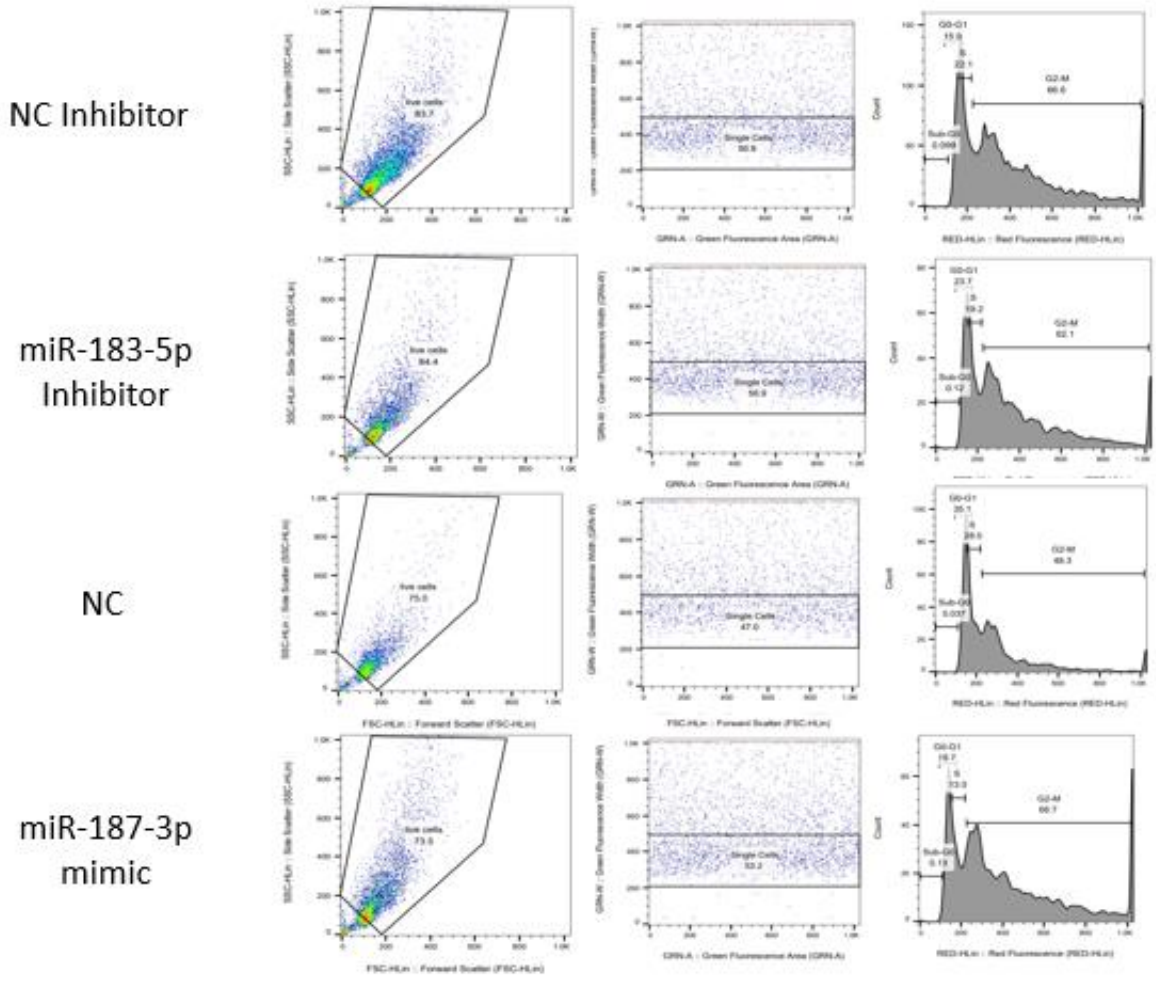


Figure 19. Representative Flow Cytometric Results for the Effects of miR-183-5p and miR-187-3p in Transfected MCF-7 Cell Cycle Progression (n=2)

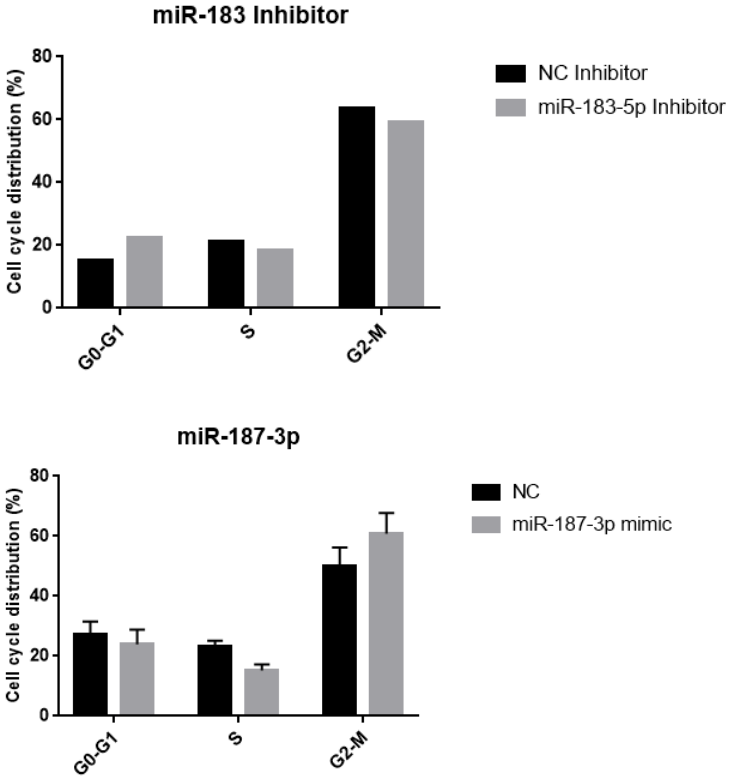


Figure 20. Cell Cycle Analysis of MCF-7 Cells Transfected with miR-183-5p Inhibitor and miR-187-3p mimic in comparison to NC Inhibitor and NC, respectively, by PI Staining Showing the Percentage of Cells in Each Cell Cycle Phase Error bars represent SEM (n=2).

I. Optimization of Annealing Temperature and Concentration of miR-183-5p and miR-187-3p shared targets primers

Primers of the selected target genes were designed on Primer Bank and checked on Primer Blast. The respective melting temperature of the primers are showed in Table 6.

The annealing temperatures of the selected target genes were optimized in MCF-7 by RT-qPCR. Analysis showed that all target genes functioned at 2µM except for SMAD4 where the optimal concentration was shown to be 450 nM after being tried on a concentration gradient. The optimal temperature for all the target genes is 60°C except for MBNL1 which functioned at 61°C (Figure 21). Primer optimization for DAB1 and FGF9 was performed however they did not show proper melting curves.

Table 6. The Sequence and Melting Temperature (Tm) of Primers of GAPDH and Predicted Shared Targets of miR-183-5p and miR-187-3p Designed on PrimerBank.

Gene	Primer	Sequence	Tm (°C)
GAPDH	GAPDH-F	5'-TGGTGCTCAGTGTAGCCCAG-3'	64.6
	GAPDH-R	5'-GGACCTGACCTGCCGTCTAG-3'	66
SMAD4	SMAD4-F	5'-CCACCAAGTAATCGTGCATCG-3'	60
	SMAD4-R	5'-TGGTAGCATTAGACTCAGATGGG-3'	60
BCL6	BCL6-F	5'-GTTTCTAGGAAAGGCCGGACA-3'	62.6
	BCL6-R	5'-TAAACTGCTCACGGCTCACAA-3'	60.5
MBLN1	MBLN1-F	5'-GATCTTGCCGACTGCACCAATG-3'	63.3
	MBLN1-R	5'-TTGCCACGTTGGTACTCTCGAC-3'	63.9
FGF9	FGF9-F	5'-CAACCTGTAAGTGCTGTGTGACC-3'	59.5
	FGF9-R	5'-CATTGCAACGTAACCTGAGGG-3'	60.2
DAB1	DAB1-F	5'-CAACCTGTAAGTGCTGTGACC-3'	60.5
	DAB1-R	5'-CATTGCAACGTAACCTGAGGG-3'	61.1

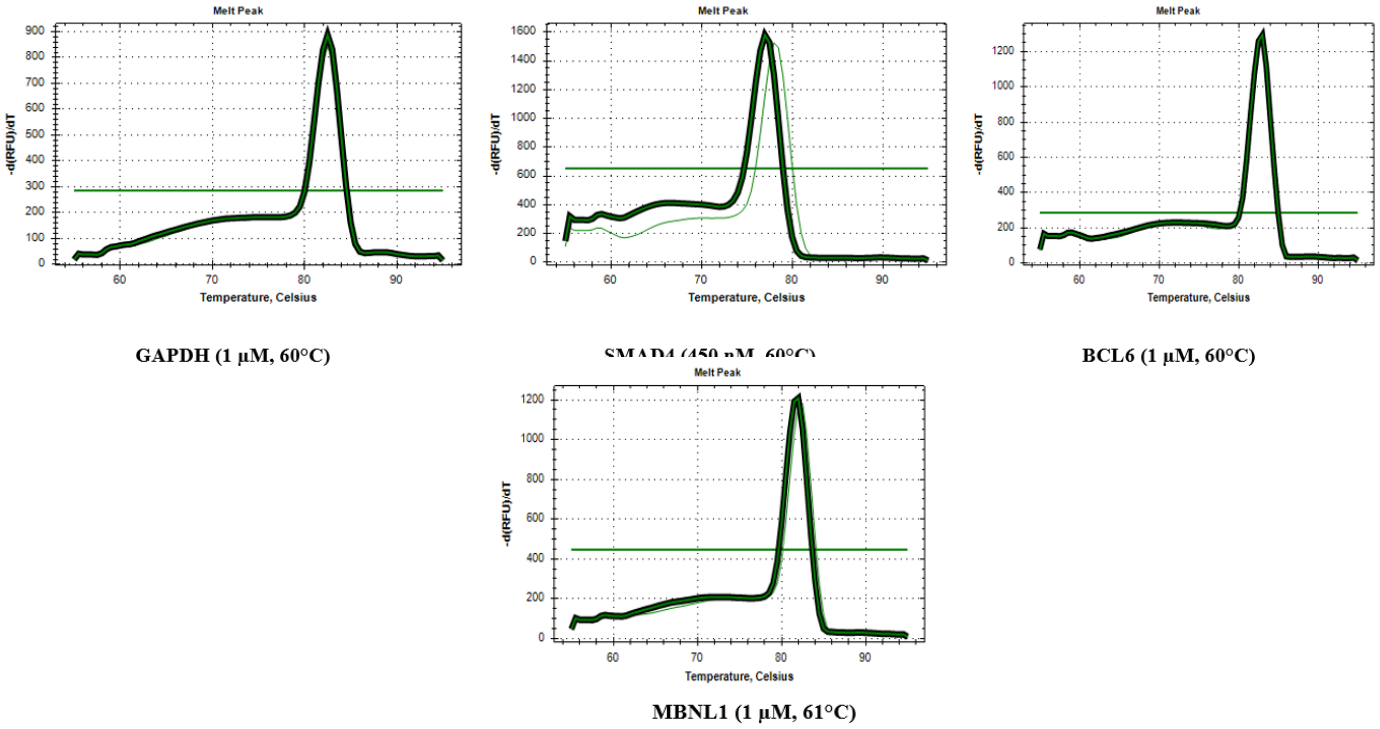


Figure 21. Melting Peaks of the Optimized Target Genes Primers. GAPDH, BCL6 and MBNL1 were optimized at 2 µM with 60°C being the optimal temperature for GAPDH and BCL6 while 61°C being the optimal concentration for MBNL1. SMAD4 was optimized on a range of temperatures and concentrations, the optimal concentration was 450 nM and the optimal temperature was 60°C.

J. SMAD4 and MBNL1 are Potential Targets for miR-183-5p

To discover if miR-183-5p and miR-187-3p target MBNL1, SMAD4, and BCL6, RT-qPCR was done on transfected cells harvested 24 hours post transfection using GAPDH as an endogenous control. Upregulation of SMAD4 and MBNL1 expression was observed in MCF-7 cells transfected with miR-183-5p inhibitor. This indicates that these targets may be potential targets for miR-183-5p. On the contrary, downregulation of BCL6 was observed in MCF-7 cells transfected with miR-183-5p inhibitor. Unexpectedly, a significant upregulation of MBNL1 was observed in MCF-7 cells transfected with miR-187-3p mimic while the other genes showed a non-significant upregulation in these cells (Figure 22).

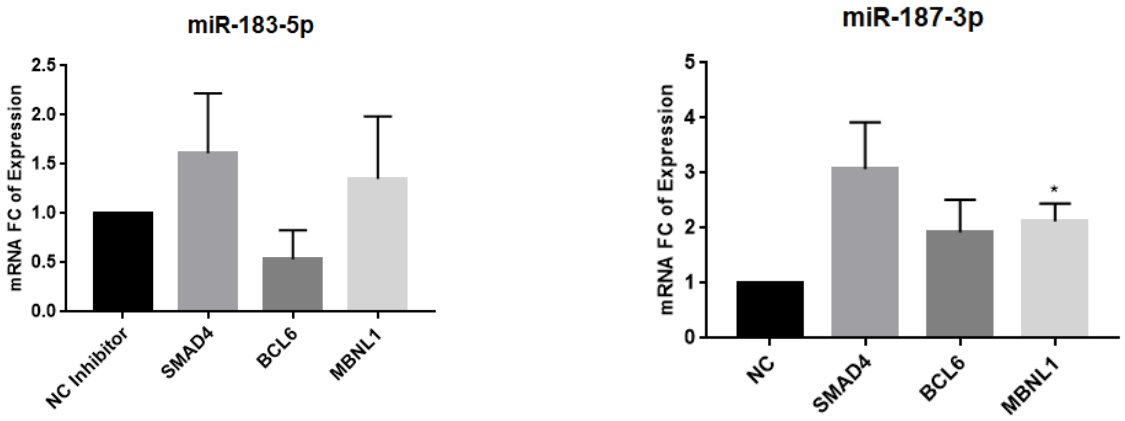


Figure 22. Expression levels of Potential miR-183-5p and miR-187-3p Shared Targets in MCF-7 Transfected Cells compared to NC Inhibitor and NC, respectively 24 hours post transfection by RT-qPCR. GAPDH was used as an endogenous control. Error bars represent SEM (n=2) * denotes $p<0.05$

CHAPTER IV

DISCUSSION

"There is a crack in everything and that's how light gets in"

-Saint Augustin

BC was found to be the cancer with the highest number of cases worldwide and in the Lebanese population (141). In spite of the important role of genetic factors (BRCA1 and BRCA2) in increasing the risk of the disease, it has been shown that epigenetic modifications could play a role in the development of BC (142). In recent years, a huge interest has been devoted to explore the role of miRNAs, which were found to control the expression of numerous oncogenes or tumor suppressor genes in numerous types of cancer including BC. In addition, miRNA expression profile has been investigated as diagnostic and prognostic biomarkers to evaluate tumor initiation, development, and response to therapy in cancer patients (143). Recently, Nassar et al. have performed a miRNA microarray analysis in ER+ Lebanese BC tissues versus normal adjacent breast tissues and have discovered a total of 173 significantly dysregulated mature miRNAs ($p < 0.05$), where miR-183-5p was one of the most dysregulated miRNAs in ER+ tumor tissues with a fold change greater than 5 times in comparison to normal adjacent tissues (144). In addition, miR-183-5p has been reported to play diverse roles in different types of cancer including BC (Figure 9). This has arisen our interest to investigate miR-183-5p in BC tumorigenesis. In addition, currently running projects in the lab have shown the dysregulation of miR-187-3p in BC. Hence, this invigorated our interest to further understand the role of miR-183-5p and miR-187-3p in BC development.

In this study, bioinformatics analysis revealed that miR-183-5p and miR-187-3p share common targets and target pathways that have important prognostic effects on the overall survival of ER+ BC patients. In addition, the analysis showed miR-183-5p and miR-187-3p upregulation in 264 Luminal A (ER+) BC tumor tissues downloaded from TCGA data portal versus 27 normal adjacent breast tissues; subsequently, we measured the expression of these miRNAs in ER+ BC cell line and found that miR-183-5p was significantly upregulated however miR-187-3p was significantly downregulated in MCF-7 cell line in comparison to normal mammary epithelial cell line MCF-10A. Then, the functional roles of miR-183-5p and miR-187-3p were assessed in ER+ BC by performing MTT and PI assays. Our results showed that miR-183-5p and miR-187-3p modulated expressions have no significant effects on the proliferation of MCF-7 cells. miR-183-5p modulated expression has no significant expression on cell cycle progression of MCF-7 cells while a slight increase was observed in G2-M phase in miR-187-3p mimic-transfected MCF-7 cells. Finally, RT-qPCR results have shown that SMAD4 and MBNL1 were upregulated with miR-183-5p inhibitor and all the targets were, unexpectedly, upregulated with miR-187-3p mimic.

In literature, the expression of miR-183-5p and miR-187-3p varied according to the type of cancer. It has been shown that miR-183-5p is upregulated in CRC, ovarian cancer, TNBC, and esophageal cancer (111, 124, 125, 145, 146), among others, which is consistent with our in silico clinical and RT-qPCR experimental results and it was shown to be downregulated in lung cancer and gastric cancer (118, 121). As for miR-187-3p, it was shown to be downregulated in most types of cancer as in TNBC, NSCLC, HCC, prostate cancer, cervical cancer, osteosarcoma, CRC, lung tumors, and glioma (136, 147-155) which is consistent with our RT-qPCR experimental results while both are non-consistent with clinical results obtained by the in silico analysis. These results

reflect the expression of the miRNA only in the patients downloaded from TCGA data portal (264) and not all ER+ BC patients. It was shown that miR-187-3p is upregulated in Oral squamous cell carcinoma (156). The expression of miR-187-3p was controversial in gastric cancer where some studies have reported its upregulation (157, 158) while others have reported its downregulation (152). So, the expression of miRNAs depends on cell type, experimental conditions, and sample size. While our study was the first to report the upregulation of miR-187-3p in ER+ BC and it is one of the first studies to assess miR-187-3p in BC.

KM plots in our study, as previously mentioned, showed a correlation between low expression of miR-183-5p and miR-187-3p with better OS in ER+ BC patients. However, this correlation was not statistically significant. This does not reflect clinical significance where this correlation does not mimic the real effect of these miRNAs in the body of BC patients as it is known that several non-coding RNAs interact with each other to affect several genes. It is worth mentioning that these KM plot analyses are not for our data but for patients all over the world downloaded from TCGA data portal. So, if other miRNAs would be included in KM analysis with a Cox regression analysis, then the correlation of miR-183-5p and miR-187-3p would become significant. In addition, these results reflect the effect of these miRNAs in around 200 ER+ BC patients out of 1069 where the rest were censored from the study for reasons that we do not know. It is clear also that the correlation of miRNAs expression on OS survival is dependent on cancer type where low expression of miR-183-5p was correlated with better OS in CRC, renal cell carcinoma, glioma, and HCC (110, 114, 159-161) which are consistent with our results in ER+ BC. For miR-187-3p, in cervical cancer, CRC and HCC, it was shown that higher rates of OS and relapse-free survival of high miR-187-3p expression group than in the low miR-187-3p expression group were observed (148, 150, 162) which is reverse to what we got in ER+ BC. There are no

studies yet investigating the prognostic value of miR-183-5p or miR-187-3p in BC using KM analysis. However, a study by Mulrane et al. revealed that miR-187-5p is an independent prognostic factor in two cohorts of BC using multivariate Cox regression analysis (163).

In addition, we were interested in checking the correlation between the expression of the chosen predicted shared target genes of both microRNAs, found in the previous in-silico analysis (BCL6, MBNL1, SMAD4, DAB1 and FGF9), and the OS in ER+ BC. If the low expression of miR-183-5p and miR-187-3p has been shown to correlate with better survival, then we expect a correlation between the high expression of the target genes, which supposed to be downregulated by the miRNAs, and better OS in ER+ BC. KM plots showed that the high expression levels of all the target genes, except for DAB1, was associated with better OS in ER+ BC. This inverse effect, which we were expecting, between miR-183-5p, miR-187-3p and the target genes increases their potential as downstream targets of these miRNAs. On the contrary, the low expression level of DAB1 was shown to be associated with better OS in ER+ BC showing a similar effect with the expression of miR-183-5p and miR-187-3p. This rules out the option of DAB1 being downregulated by these two miRNAs and shows that it may not be a direct target in ER+ BC. Moreover, DAB1 may be a direct target in other subtypes of BC where it has been shown in literature that DAB1 is downregulated in ER- BC and negatively correlates with poor prognosis (164). Accordingly, we focused our study on the other targets.

MCF-7 cell line, which is an ER+ BC cell line, was chosen as an in vitro model for our study. So, miR-183-5p and miR-187-3p expression were modulated in MCF-7 BC cell line as follows: miR-183-5p was downregulated by transfection with miR-183-5p inhibitor and miR-187-3p was overexpressed by transfection with miR-187-3p mimic. The inhibitor concentration was optimized by trying different concentrations of the inhibitor to transfect MCF-7 cell line. The one

which gave the highest transfection efficiency without causing cytotoxicity was used. It is important to mention that we did not assure that this is the best concentration of transfection with an inhibitor and this is open for further optimizations. Flow cytometric analysis showed that transfection with miR-183-5p inhibitor, NC inhibitor, miR-187-3p mimic and NC was successfully done in MCF-7. Analysis of miR-183-5p and miR-187-3p levels by RT-qPCR showed that the former was significantly downregulated upon transfection with the inhibitor and the latter was significantly overexpressed upon transfection with the mimic. The usage of miRNA inhibitor is poorly investigated in literature, yet, some studies have used it. Yuan et al. have transfected miR-NC mimics, miR-183 mimics, miR-NC inhibitors or miR-183 inhibitors into MKN28 gastric cancer cells, without specifying the concentration used, to assess the effect of miR-183-5p on proliferation and apoptosis of gastric cells (165). Yang et al. have used miR-183 inhibitors to knockdown miR-183 in PANC-1 cells where they have shown that downregulation of miR-183 inhibited the growth of PANC-1 pancreatic cancer cells *in vitro* and *in vivo*, and increases chemosensitivity to 5-fluorouracil and gemcitabine (166).

Functional assays were performed to assess the functional roles of miR-183-5p and miR-187-3p in BC as previously mentioned. Our results did not reveal any significant effect of modulated miR-183-5p and miR-187-3p expression on the proliferation of MCF-7 cells. This may be due to the fact that MTT is not the best technique to assess proliferation where the absorbance of all the cells (transfected and non-transfected) is being measured. Further sorting of transfected cells may increase the precision of MTT results. There are no studies on the effect of miR-183-5p inhibitor or miR-187-3p mimic modulated expression on proliferation of MCF-7. However, a study has shown the effect of miR-183-5p mimic on MCF-7 cells (126) which significantly promoted proliferation of cells transfected with miR-183-5p mimic in comparison to NC.

Moreover, our PI results showed no effect of miR-183-5p inhibitor modulated expression on cell cycle progression of MCF-7 cells and a slight increase in G2-M phase in cells transfected with miR-187-3p mimic, but without significant difference when compared to that of NC transfected cells. It is worth mentioning that PI experiment in our study lacks another time point (after 48 hours), where we could have a better view of cell cycle progression and we could analyze if the cells are being arrested at G2-M or they are being pushed to divide. In literature, studies reported the effect of miR-183-5p and miR-187-3p mimics modulated expression on cell cycle in TNBC as in MDA-MB-231 cells. A study reported that miR-183-5p mimic upregulation decreased the proportion of cells in G0/G1 phase while it increased the proportion of cells in S phase, 48 hours post transfection in MCF-7 cells (126). So, the difference in reported results and their significance might be attributed to the time points at which the assays were performed. The respective time points were selected in our study based on previous data in our lab that showed that the miRNA expression levels are the highest at 24 hours and decrease at later time points. However, we believe that although miRNA levels decrease with time, its downstream effects are better viewed in later time points.

Before investigating the association of miR-183-5p and miR-187-3p with the potential targets, optimization of the annealing temperature of the designed primers was performed. The optimal temperature and concentration were found for all the target genes except for FGF9 despite the fact that it has been tested on a wide range of temperatures and concentrations. Being a designed primer, not taken from literature, the problem could be with the design and sequence of the reverse and forward primers. Thus, FGF9 was excluded from further investigation although it was from the most important targets for both miRNAs

Finally, we investigated the association of miR-183-5p and miR-187-3p with the potential targets that were determined previously by in the silico analysis and were properly optimized. Among the shared targets that we studied is SMAD4, which was upregulated in our study, but not significantly, in MCF-7 cells transfected with miR-183-5p inhibitor. This gene encodes a member of the SMAD family of signal transduction proteins. SMAD proteins are phosphorylated and activated by transmembrane serine-threonine receptor kinases in response to transforming growth factor (TGF)-beta signaling. TGF-β inhibits cell proliferation in breast epithelial cells and many BC cell lines. SMAD4 protein forms homomeric complexes and heteromeric complexes with other activated SMAD proteins, which then are accumulated in the nucleus regulating the transcription of target genes. A previous study showed that SMAD4 expression in BC is lower than in normal adjacent breast epithelial tissues and suggested that the impairment of TGF-β/SMAD-signaling, because of loss of TβRII or SMAD4, might progress 5-year survival by probably reducing metastases (167). Furthermore, studies have demonstrated that SMAD4 has a possible role in the growth of breast carcinoma and has a potential as a prognostic marker of breast carcinoma (168). SMAD4 has been validated as a direct target for miR-183-5p in ovarian and prostate cancer but not in BC (145, 169). BCL6 is also one of the shared target genes that we studied. BCL6 (B-cell chronic lymphocytic leukemia/lymphoma 6) is as an important oncoprotein and therapeutic target (170). BCL6 is considered as a zinc-finger protein and an effective transcriptional repressor. Previous studies have proved that BCL6 serves a significant role in BC invasion and metastasis. While BCL6 protein is hardly expressed in the normal mammary epithelium, it is overexpressed in breast cancers, mostly high-grade ductal BC (171). The proto-oncogene BCL6 is a principal controller of B-lymphocyte growth and it facilitates proliferative development. In addition, it facilitates the proliferation of B-lymphocytes and inhibits their differentiation into plasma and

memory B cells. Moreover, BCL6 has been informed to stop the differentiation of mammary cells and stimulate the oncogenicity of human BC cells (172). BCL6 has been shown to be a putative target for miR-187-3p in NSCLC without the validation of direct interaction (133) and no studies were found in BC.

The last shared target gene chosen in our study is MBLN1. MBNL1 (muscleblind-like protein 1) gene encodes a protein covering two pairs of conserved RNA-binding CCCH zinc finger domains and has a well-characterized part in pre-mRNA splicing regulation. MBNL1 is required for normal growth of photoreceptor cells and normal muscle differentiation and attachment, MBNL1 is also essential for normal mammalian development. In addition to its role in splicing, MBNL1 has been concerned as a controller of other phases of RNA processing and expression. A previous study found that endogenous MBNL1 can suppress metastasis of human BC cells. MBNL1 directly binds to the 3′ untranslated domains (UTRs) of two genes that are responsible of suppressing metastatic invasion. Furthermore, to support these findings, analysis of *MBNL1* expression in breast tumor samples was performed and it confirmed a significant association between the expression of MBNL1 and metastasis-free survival (173). This is in accordance with the Kaplan-Meier plot showing that the high expression of MBNL1 correlates with better OS. There are no association between MBNL1 and miR-183-5p inhibitor or miR-187-3p mimic in literature.

Importantly, our data showed the upregulation of SMAD4 and MBLN1 upon transfection with miR-183-5p inhibitor in MCF-7 cell line. This indicates the inverse correlation between miR-183-5p expression and the two targets in ER+ BC. Hence, SMAD4 and MBNL1 could be potential direct target genes for miR-183-5p. It is worth validating these results by a luciferase assay. However, upon transfection with miR-187-3p mimic in MCF-7 cell line, all the targets were

showed to be upregulated which is opposite to what we were expecting where a miRNA downregulates the expression of target genes. This indicates that MBNL1, SMAD4, and BCL6 may be indirect targets for miR-187-3p.

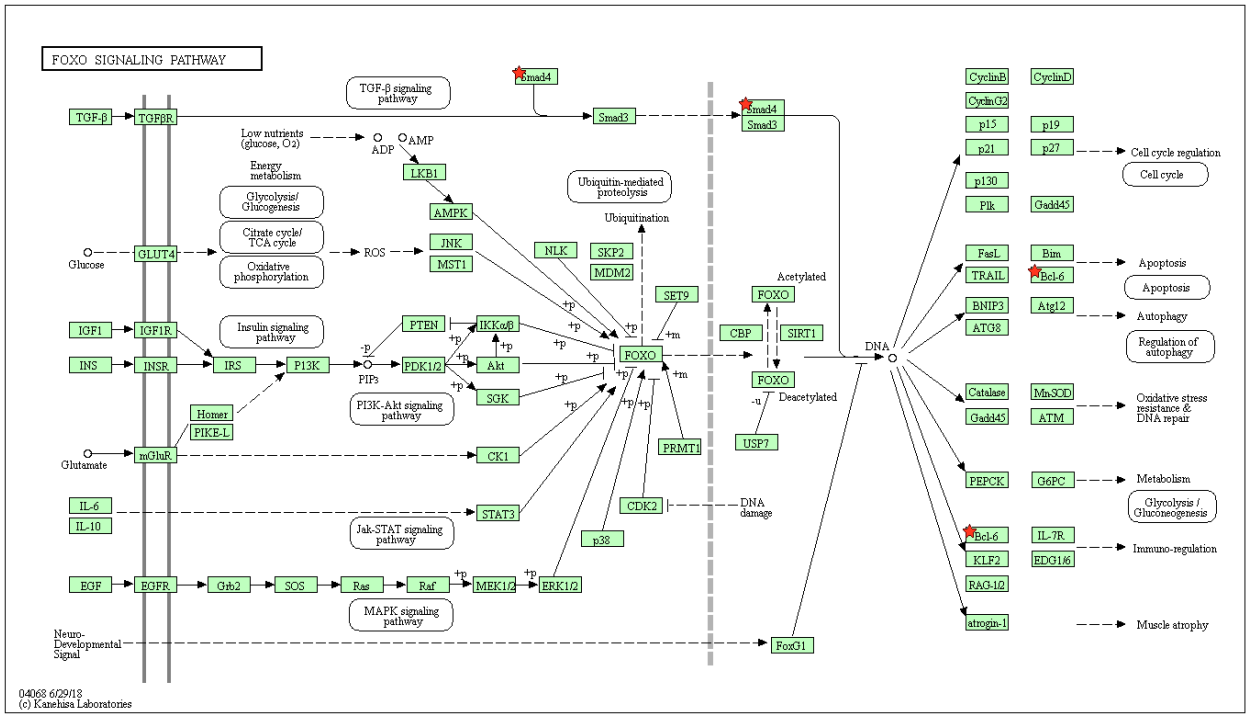


Figure 23. Implication of SMAD4 and BCL6 in FOXO KEGG Signaling Pathway. Red stars indicate the shared target genes.

Interestingly, using the DAVID functional annotation and enrichment analysis tool (2021 update) (174), we were able to conduct Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of our genes of interest where a list of the three genes SMAD4, BCL6, and MBNL1 was submitted in the database. The KEGG pathway analysis of our gene set resulted in one significant KEGG pathway which is the TGF-ß signaling pathway, one of FOXOs signaling

pathways. It has been found that SMAD4 and BCL6 genes interact indirectly in this signaling pathway (Figure 23). In addition, a literature search has showed that TGF-ß signaling pathway plays an important role in enhancing BC metastasis (175-179). This adds the value of SMAD4 and BCL6 implication in BC. So, it is worth investigating the interaction of miR-183-5p and miR-187-3p with these genes in TGF- ß signaling pathway in the future. As for MBNL1, it was not shown to be implicated in any KEGG pathway.

- Limitations and Future Perspectives:

  In the bioinformatics analysis, our results showed a significant upregulation of miR-183-5p and miR-187-3p in 263 ER+ clinical samples downloaded from TCGA database versus normal adjacent breast tissues. However, this was not consistent with RT-qPCR experimental results when the expression of miR-183-5p and miR-187-3p was checked in MCF-7 BC cell line in comparison to MCF-10A. This limitation sheds the light on an important idea that an in vitro model does not always mimic what occurs inside the body. In addition, the clinical samples downloaded from the TCGA database do not reflect the expression of both miRNAs in all ER+ BC patients but only that of the studied cohort and MCF-7 cell line was taken from one patient having ER+ BC.

  Moreover, in this project, transient transfection was used to transfect MCF-7 cells with FAM-labeled miR-183-5p inhibitor and miR-187-3p mimic along with their corresponding negative controls. However, in transient transfection, the transfected material does not get integrated into the cellular genome and is degraded gradually until 96 hours post transfection. In addition, transient transfection depends totally on the cell type and culture medium where it demands optimized conditions of transfection reagents and culture media. This forms a limitation regarding the transfection process as it is worth

mentioning that a stable transfection, where the transfected material integrates into the genome and is passed on to future generations, might show better results than a transient transfection.

Another limitation in this project is the early time points used. When assessing the effect of the modulated expression of miR-183-5p and miR-187-3p on cell cycle progression of MCF-7 cells, PI staining was performed on cells harvested at a single time point only which is 24 hours. It is good to see the effects of these modulated expressions at 48 and 96 hours as well to have a better insight on the modulated cell cycle of MCF-7 and to know if the cells are being arrested at G2-M or they are being pushed to divide. Furthermore, not enough biological replicates (for example n=3) were performed in several experiments. More consistent experiments may give better view of the effects of miR-183-5p and miR-187-3p modulated expression on MCF-7 BC cell line and may increase the significance of the results.

Using these findings as a good template, we are interested to further investigate the co-effects of miR-183-5p and miR-187-3p on ER+ BC. This will be based on specific optimizations regarding the double transfection. As a pursue for this project, we are also interested to look for upstream potential non-coding RNAs (circRNAs/ LncRNAs) that sponge miR-183-5p and miR-187-3p and validate a circRNA/LnRNA-miRNA-mRNA axis for our miRNAs of interest in ER+ BC. In addition, because SMAD4 and MBNL1 have shown to be potential targets for miR-183-5p, it is good to validate the direct interaction between miR-183-5p and these two genes by a luciferase assay. And finally, it is good to look for miRNA-mRNA interactive signaling pathways to know where the effect of the miRNA has started, which genes does it affect directly or indirectly.

In conclusion, our study suggests the potential of miR-183-5p and miR-187-3p as active players in ER+ BC. While further studies are needed to unveil their significant and important roles in this subtype of BC.

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