AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATING THE ROLE OF MIR-187-3P IN MOLECULAR APOCRINE BREAST CANCER

by
ADNAN MOHAMAD JOUMAA

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, Cell Biology, and Physiological Sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
June 2024

AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATING THE ROLE OF MIR-187-3P IN MOLECULAR APOCRINE BREAST CANCER

by

ADNAN MOHAMAD JOUMAA

Approved by:

Dr. Rihab Nasr, Professor Department of Anatomy, Cell Biology, and Physiological Sciences	Advisor
Dr. Nadine Darwiche, Professor Department of Biochemistry and Molecular Genetics	Member of Committee
Dr. Wassim Abou-Kheir, Professor Department of Anatomy, Cell Biology, and Physiological Sciences	Member of Committee
Dr. Georges Daoud, Associate Professor Department of Anatomy, Cell Biology, and Physiological Sciences	Member of Committee

Date of thesis defense: June 21, 2024

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to all those who have contributed to the realization of this project.

First, I want to thank God for leading me in this road and providing me with resilience, strength, patience and wisdom.

Professor Rihab Nasr, I shall write a whole book thanking you for being my advisor. Your support, patience, and expert guidance are the bases for this project's accomplishment and hopefully success. Your belief in me and your dedication to my growth as a researcher have been truly inspiring. Not only, you helped in accomplishing my thesis project, but also in pursing my future career as a medical student. Thank you for always pushing me to achieve my goals and for being a source of encouragement and motivation.

I sincerely appreciate that **Dr. Nadine Darwich, Dr. George Daoud, and Dr. Wassim Abou Kheir**, who are my thesis committee, gave up their valuable time to read through my manuscript and offer valuable feedback.

To my family, who have been my support system throughout this entire journey, I owe the deepest gratitude. Your love, encouragement, and belief in me have been the driving force behind every step I am taking. **Mom and Dad**, thank you for helping me grow as a respectful and successful man alongside my siblings.

I want to extend my appreciation to my lab mentor, **Dr. Ghada Chamandi**, whose guidance and support have been the basis for accomplishing my project. I am grateful for the hours you have dedicated to providing guidance, feedback, and encouragement, whether it was reviewing experimental designs, troubleshooting challenges, or celebrating successes. Your kindness and funny yet firm personality helped me enjoy my time at the lab and overcome tough challenges.

My lab partner **Ms. Mira Mardirossian**, I cannot imagine this journey without you or with someone other than you. Working alongside you in the lab has been an absolute pleasure. We shared successful, funny, and even tough times together. Thank you for your troubleshooting ideas and for responsibility in doing my tasks during my vacations.

I would like thank all DTS Basic Research Core Facilities members. Big thanks to **Dr. Layal El Hajjar** for helping me understand and perform several experiments. I appreciate your kindness and support and dedicating your time for helping me. **Mr. Abdallah Kurdi,** thank you for helping me in the bioinformatics part of the project.

Finally, gratitude to all my professors and friends that believed in my success and supported me through this whole journey.

ABSTRACT
OF THE THESIS OF

Adnan Mohamad Joumaa for Master of Science
Major: Physiology

Title: Investigating the Role of miR-187-3p in Molecular Apocrine Breast Cancer

Background: Breast cancer (BC) is a health problem that is reported to have the highest incidence and mortality rates of cancer among females worldwide and in Lebanon. Moreover, BC heterogeneity renders it from being diagnosed or treated as a single entity. As such, there has been several attempts to classify BC. The expression of hormonal receptors is one of the methods being used for subtyping BC. These receptors are estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Thus, based on the expression of these receptors, BC is classified into 4 molecular subtypes. The most aggressive subtype is negative for the three receptors and is called triple negative BC (TNBC). TNBC itself is also heterogenous and it overlaps with distinct subtypes, namely Molecular Apocrine (MA) and Luminal Androgen (LAR) BCs. These subtypes which overexpress Androgen receptor (AR) are, even today, still misdiagnosed and poorly treated. The discovery of new biomarkers that can help in differentiating subtypes might serve as a helpful tool. A significant amount of research has proved these micro-RNAs (miRNAs) are dysregulated in different types of cancers including BC, and consequently could be involved in cancer initiation and/or progression. Moreover, some miRNAs exhibit distinct dysregulation patterns across BC subtypes, offering potential diagnostic value. Using publicly available data and in silico analysis done on a cohort of ER-negative tissues, our lab found that miR-187-3p is significantly upregulated in samples classified as MABC compared to non-MABC samples. In addition, miR-187-3p is reported as a key player in the tumorigenesis of several types of cancer by controlling proliferation, invasiveness, migration, and aggressiveness of cancerous cells. Building upon these findings, we investigated the role of miR-187-3p in the tumorigenesis of MABC using in vitro BC cellular models.

Methods and Results: The resemblance of MDA-MB-453 and MDA-MB-231 to MABC and non-MABC tumors respectively, was checked by determining AR expression and basal expression of miR-187-3p in both cell lines. Then miR-187-3p were significantly modulated in the chosen cell lines by transfection either with mimic or inhibitors. Proliferative ability of cells was determined by doing MTT assay. Downregulation of miR-187-3p reduced MDA-MB-453 cell proliferation and its upregulation promoted cell proliferation of MDA-MB-231 cells. miR-187-3p increased the invasive ability of MCF-10A, MDA-MB-453, and MDA-MB-231 cells significantly. Then miR-187-3p predicted targets were selected based on databases and literature review. Of these targets, MBNL1 and AGO1 were determined to be as potential targets of miR-187-3p.
Conclusion: Overall, our results suggest that miR-187-3p could play the role of an oncomiR in MABC and non-MABC, increasing their invasive and proliferative abilities by potentially targeting MBNL1 and AGO1. However, further studies should be done to

validate its role and to validate the miRNA-mRNA interaction with potential targets. This research may yield promising insights into MABC, advancing the development of specific diagnostic and therapeutic strategies.

TABLE OF CONTENTS

ILLUSTRATIONS

Figure

TABLES

Table

ABBREVIATIONS

- 3’UTR 3’ Untranslated Region
- AGO Argonaute Protein
- AJCC American Joint Committee on Cancer
- AR Androgen Receptor
- ARID3B AT-rich interactive domain-containing protein 3B
- ATCC American Type Culture Collection
- ATP Adenosine Triphosphate
- BAK1 BCL2 antagonist/killer 1
- BBC3 BCL2 Binding Component 3
- BC Breast Cancer
- BCL2 B-Cell Lymphoma Gene 2
- BCL6 B-cell lymphoma 6
- BL1/2 Basal-like 1/2
- BMF BCL2 Modifying Factor
- BRCA 1/2 Breast Cancer Gene 1/2
- C1S Complement C1S
- ccRCC Clear Cell Renal Cell Carcinoma
- CDK Cyclin-dependent kinase
- cDNA Complementary DNA
- CENPA Centromere Protein A
- CLDN1 Claudin-1
- CLL Chronic Lymphocytic Leukemia
- C-Myc Cellular myelocytomatosis oncogene
- CRC Colorectal Cancer
- DAPI 4',6-diamidino-2-phenylindole
- DBNL1 Drebrin-like 1
- DCIS Ductal Carcinoma in Situ
- DEGES Differentially expressed gene elimination strategy
- DEPC Diethyl Pyrocarbonate
- DGCR8 DiGeorge Syndrome Critical Region 8
- Dicer RNase III Endonuclease
- DMSO Dimethyl Sulfoxide
- DNA Deoxyribonucleic Acid
- Drosha Ribonuclease III Enzyme
- E2F3 E2F Transcription Factor 3
- EGF Epidermal Growth Factor
- EMT Epithelial to Mesenchymal Transition
- ER Estrogen Receptor
- ERBB2/3 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2/3

- FAM Fluorescein amidites
- FBS Fetal Bovine Serum
- FOXA1 Forkhead box A1
- GC Gastric Cancer
- HCC Hepatocellular Carcinoma
- HER2 Human Epidermal Growth Factor Receptor 2
- HuR Human Antigen R
- IDC Invasive Ductal Carcinoma
- IL6R Interleukin 6 receptor
- ILC Invasive Lobular Carcinoma
- IM Immunomodulatory
- INPP5B Inositol polyphosphate-5-phosphatase B
- ITPR2 Inositol 1,4,5-trisphosphate receptor, type 2
- JUN Jun-Proto-Oncogene
- KIT Proto-oncogene c-KIT
- LAR Luminal Androgen Receptor
- LCIS Lobular Carcinoma in Situ
- LIN28B Lin-28 Homolog B
- LRFN1 Leucine Rich Repeat And Fibronectin Type III Domain Containing 1
- LSM Laser Scanning Microscope
- MA Molecular Apocrine
- MABC Molecular Apocrine Breast Cancer
- MAPK14 Mitogen-activated Protein Kinase 14
- MBNL1 Muscle blind Like Splicing Regulator 1
- MCTS Multicellular Tumor Spheroids
- MES Mesenchymal Like
- miRNA, miR micro-RNA
- MMP13 Matrix Metallopeptidase 13
- MRI Magnetic Resonance Imaging
- mRNA Messenger RNA
- MSL Mesenchymal Stem Like
- MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
- MYC Myelocytomatosis Oncogene
- Myt Myelin transcription factor 1
- NC Negative Control
- NCBI National Biotechnology Information
- NRT No Reverse Transcriptase
- NSCLC Non-Small Cell Lung Cancer
- OC Ovarian Cancer

- OncomiR — Oncogenic miRNA
- OS — Overall Survival
- PBS — Phosphate Buffer Solution
- PCa — Prostate Cancer
- PCR — Polymerase Chain Reaction
- PDCD4 — Programmed cell death protein 4
- PFA — Paraformaldehyde
- PFA — Progression Free Survival
- Pol II — Polymerase II
- PR — Progesterone Receptor
- Pre-miRNA — Precursor miRNA
- Pri-miRNA — Primary miRNA
- PTEN — Phosphatase and Tensin Homolog
- RAS — Rat Sarcoma
- RB2 — Retinoblastoma 2
- RISC — RNA-Induced Silencing Complex
- RNA — Ribonucleic acid
- RNase — Ribonuclease
- RNU6B — RNA, U6 small nuclear 6, pseudogene
- RT — Room Temperature
- RT-qPCR — Real-Time Quantitative polymerase chain reaction
- S100A4 — S100 Calcium Binding Protein A4
- SEM — Standard Error of the Mean
- SKI — SKI proto-oncogene
- SMAD2 — Mothers against decapentaplegic homolog 2
- SNAI2 — Snail Family Transcriptional Repressor 2
- SOX4 — SRY-box transcription factor 4
- SPRY1 — Sprouty RTK Signaling Antagonist 1
- STAT3 — Signal transducer and activator of transcription 3
- TACC1 — Transforming Acidic Coiled-Coil Containing Protein 1
- TBP — TATA-box binding protein
- TCGA-BRCA Collection — The Cancer Genome Atlas Breast Invasive Carcinoma
- TGF-β — Transforming growth factor-beta
- Tm — Melting Temperature
- TNBC — Triple Negative Breast Cancer
- TP53 — Tumor Protein 53
- TP53INP1 — Tumor protein p53-inducible nuclear protein 1
- TRBP — Transactivation-responsive RNA Binding Protein
- TRI — Trizol
- VEGF-A — Vascular endothelial growth factor A

- XPO5/EXP5 Exportin 5
- ZEB1/2 Zinc finger E-box binding homeobox 1/2

CHAPTER 1

INTRODUCTION

1.1. Overview on Breast Cancer

1.1.1. Epidemiology and Risk Factors of Breast Cancer

1.1.1.1.Worldwide

Breast cancer (BC) is the uncontrolled growth of malignant breast cells forming a tumor. This disease continues to be a critical health concern that can occur in both men and women, but more common in women. According to GLOBOCAN, in 2022, BC was reported as the most common and deadly cancer among females worldwide occupying 23.8% of all incident cancer cases and 15.4% of all cancer mortality rates [1] (Figure 1 A). The incidence rate of BC exceeds other types of cancer not only in developed countries (54.1 per 100,000), but also in developing ones (30.8 per 100,000) [2]. These high rates could be attributed to increased detection or prevalence of BC risk factors that include demographic, reproductive, hormonal, genetic, and lifestyle factors [2]. Demographically, females are affected more than males, and elderly are affected more than young individuals. Moreover, the reproductive risk factors are early age of menarche and late menopause. Also, hormonal factors like taking contraceptive pills and postmenopausal therapy have a role in increasing the risk of developing BC [2]. In addition, BRCA1 and BRCA2 (breast cancer genes) gene mutations increase the risk of BC especially in individuals having a family history of BC. Studies have also shown the positive correlation between developing BC and smoking, consuming alcohol, and being obese. Other risk factors include diabetes, air pollution, and radiation [3].

1.1.1.2. In Lebanon

In Lebanon, similar to the global status, BC is the most frequently reported malignancy and major cause of cancer deaths among females throughout the years. A study done on BC among Lebanese females between 2005 and 2015 concluded that BC is the most prevalent cancer accounting for 37% of all cancer cases among females [4]. Still in 2022, BC is the most common cancer among Lebanese females with a 33.6% incidence and 23.3% mortality rate [1] (Figure 1B). In addition, the incidence rate in Lebanon is considered the highest among regional countries like Egypt, Tunisia, and Saudi Arabia [4]. The cause of this high percentage can be attributed to the spread of awareness and BC screening among the Lebanese population [5]. Other factors also have a role in increasing the incidence like younger menarche age, older menopause age, obesity, smoking, and excessive use of hormonal replacement therapy [6-8]. Moreover, protective factors like breast feeding and traditional Mediterranean diet are less prevalent in Lebanon compared to regional countries [7]. However, genetic mutations leading to BC like BRCA gene mutations, are not common among Lebanese females having BC. El Saghir et al. conducted a study on 250 young Lebanese females having BC that are considered at a high risk of having BRCA1 or BRCA2 gene mutations. The results showed that only 14 out of 250 (5.6%) of the patients carried a deleterious BRCA gene mutation [9]. Therefore, it is pivotal to find another gene mutation or other factors that contribute to the development and tumorigenesis of BC among Lebanese females.

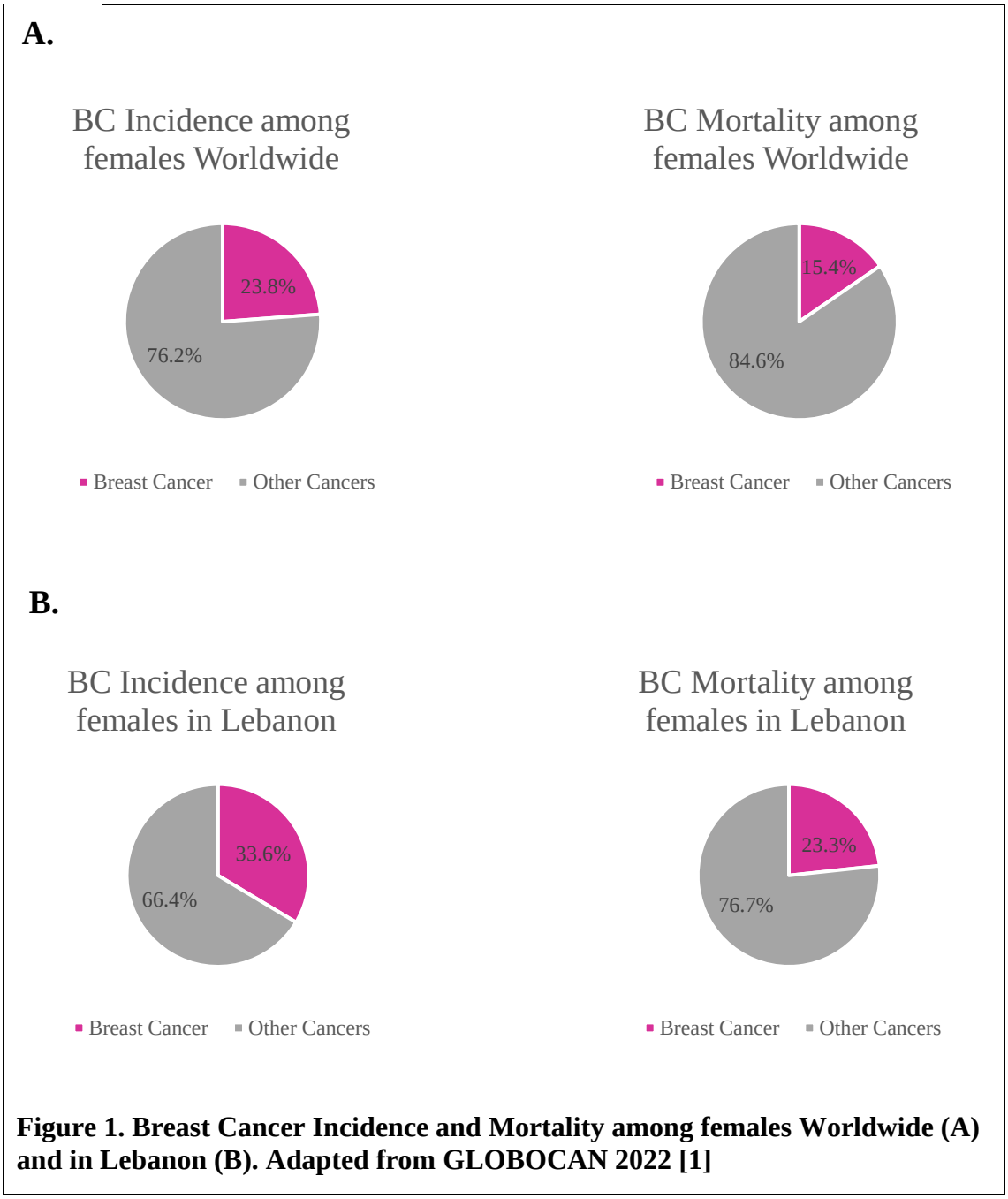


Figure 1. Breast Cancer Incidence and Mortality among females Worldwide (A) and in Lebanon (B). Adapted from GLOBOCAN 2022 [1]

1.1.2. Breast Development and Anatomy

To better understand the occurrence and progression of BC, it is essential to know the anatomy, location, and development of the breast. The female breast lies between the second and sixth rib of the anterior side of the thoracic wall [10]. The breast is a complex organ made up of 15-20 lobes of glandular tissue (mammary glands), ducts,

connective tissue, adipose tissue, lymphatic and blood vessels. Connective tissue separates the lobes and help in supporting the breast [11, 12] (Figure 2 A). At birth, only the ductal system represented by the main lactiferous duct is developed. However, the mammary glands start developing at puberty under the effect of female sex hormones, estrogen and progesterone. Estrogen surge at puberty stimulates the development of the glandular tissue. Moreover, progesterone stimulates the development of the rest of the ductal system. Mammary glands branches into lobules that drain into lactiferous ducts that end at the nipple [13]. These lobules are made up of alveoli that contain secretory epithelial cells. Upon pregnancy, prolactin and progesterone initiate alveologenesis, which is the growth of milk-producing secretory cells. Milk travels through lactiferous duct to reach the lactiferous sinus and exits the breast through the nipples [14, 15] (Figure 2B). By knowing the anatomy of the breast, it is easier to understand the origin and evolution of BC.

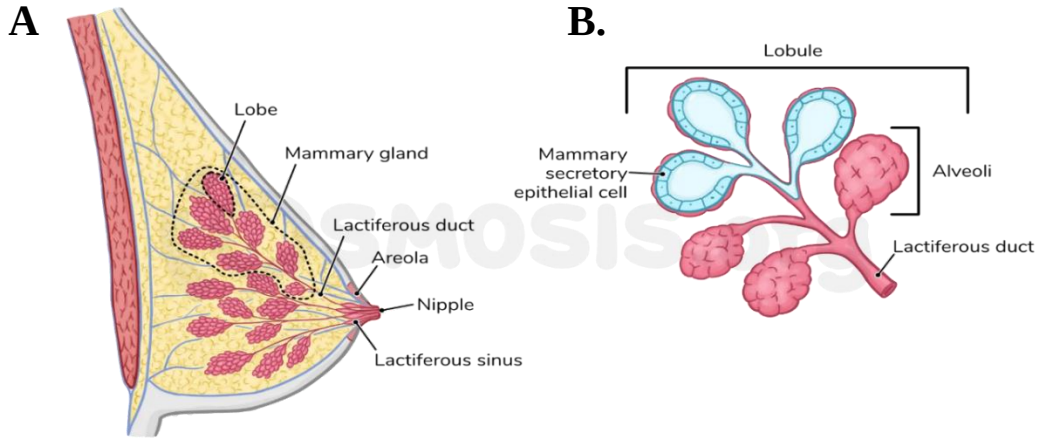


Figure 2. A cross-section of the breast and its contents (A), Lobules' Anatomy (B) adapted from Osmosis [16]

1.1.3. Breast Cancer Origin and Evolution

The breast has the ability to remodel during adulthood due to its cellular plasticity, which increases its susceptibility to carcinogenesis [17]. The initiation of cancer starts by the genetic and epigenetic alterations that affect the signaling pathways of normal breast cells. These pathways control cells' proliferation, migration, and survival among others. Therefore, mutations that activate proto-oncogenes or deactivate tumor suppressor genes, hyperactivate these signaling pathways [18]. Cancerous cells start to proliferate and develop either in the ducts or in the lobules. Thus, BC can be split into ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) (Figure 3). However, both carcinomas start in situ as non-invasive but can become invasive due to several reasons like late detection or inadequate therapy. Invasive breast carcinoma can metastasize at late stages affecting near organs like lymph nodes in the armpit or distant organs like the brain lungs, liver, and bone [19-22].

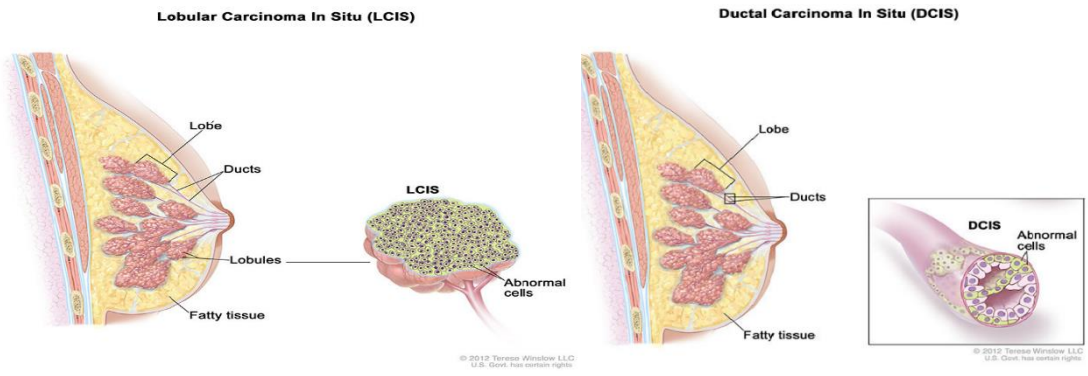


Figure 3. Lobules and Ducts in LCIS and DCIS respectively; adapted from Cancer Care Brevard [23].

1.1.4. Breast Cancer Classification

BC cannot be classified or treated as a single entity due to its heterogeneity that classifies it into several subtypes. Therefore, it is subtyped based on the tumor's histopathology, immunopathology, and mRNA expression [24]. Histopathological classification includes two broad categories; ductal or lobular which can be further classified as in situ or invasive [25, 26]. The most frequent subtypes of in situ and invasive types are ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) respectively. Followed by lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (ILC) [26] [25]. Other less frequent subtypes include apocrine, micropapillary, papillary, medullary, tubular, mucinous, and cribriform [25].

Three hormone receptors are used as markers in immunopathological subtyping of BC; estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Based on these markers, BC cases can be assigned into 4 main categories, ER+ (ER+/HER2-), HER2+ (ER-/HER2+), triple positive (ER+/PR+/HER2+), and triple negative (TNBC) (ER-/PR-/HER2-) [24]. These subtypes have different clinicopathological features and outcomes [27]. HER2+ and TNBC showed more aggressive clinicopathological features and poorer overall survival compared to ER+ and triple positive tumors [27].

Molecularly, BC can be divided into 5 intrinsic subtypes based on mRNA expression (Figure 4). ER+ BC cases are subtyped into luminal A (ER+, PR+, HER2-), luminal B (ER+ and/or PR+, HER2+ or HER2-), and normal-like subtype that has similar gene expression to normal breast epithelial cells [28, 29]. ER- tumors are classified as HER2-enriched BC (ER-/HER2+), or basal-like similar to TNBC. These 5 subtypes differ in survival, demographics, tumor characteristics and prognosis [30-33]

(Figure 4). The majority of tumors are classified as luminal A (40%). These tumors have lower grade, better prognosis, and lower expression of proliferation related genes compared to luminal B tumors (20% of BC tumors) [29]. Normal-like (2-8% of BC tumors) have a better prognosis than luminal B but lower than luminal A [28]. HER2-enriched tumors (10-15% of BC tumors) have a high histologic grade, poor prognosis, and a good response to HER2-monoclonal targeted therapy. Basal-like tumors or TNBC (15-20% of all BC tumors) have the worst prognosis among the other subtypes and are associated with BRCA1 mutations [28, 29]. Having accurate classification criteria of BC tumors can help in the development of accurate diagnostic tools and treatment strategies.

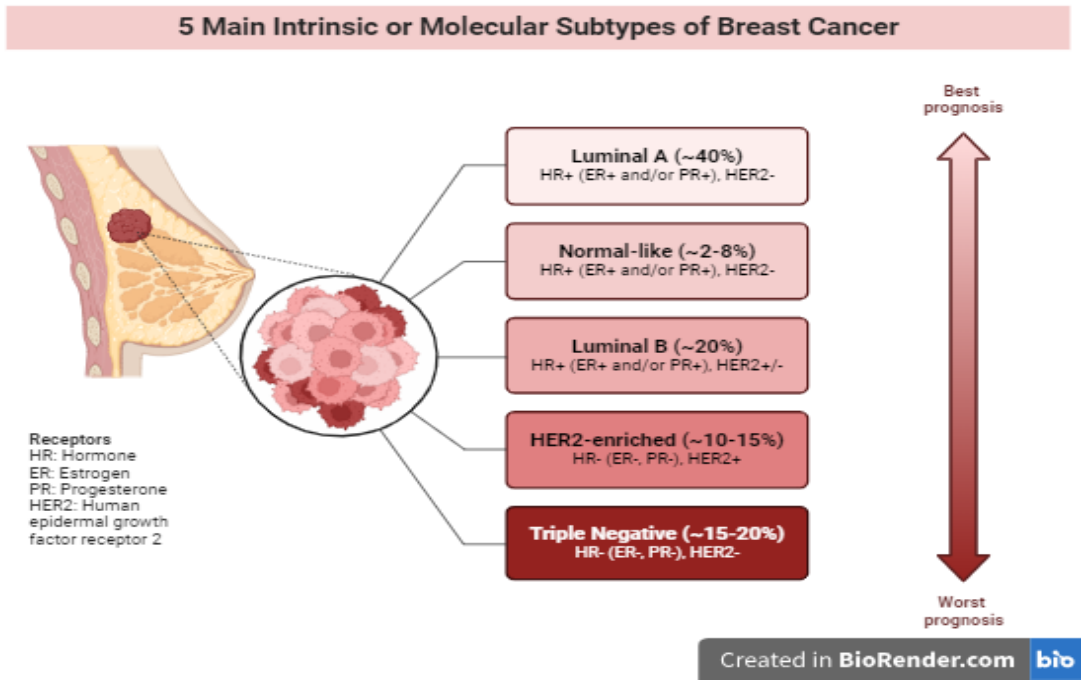


Figure 4. Illustration showing BC 5 intrinsic subtypes adapted and modified from BioRender

1.1.5. Breast Cancer Diagnosis and Treatment Strategies

Early detection of diseases, including BC, is always the best strategy to decrease the aggressiveness and mortality rates. Therefore, early-stage screening methods are being used in the diagnosis of BC cases. These methods include mammography, ultrasound, and magnetic resonance imaging (MRI). Screening mammography reduced mortality rate by 19% especially for women in their 60s [34]. However, it is not suitable for patients having dense breasts. Ultrasound can be recommended as a supplement for patients that cannot have mammography, but it sometimes fails in detecting tumors due to the similarities between healthy and cancerous cells. MRI is recommended for patients at high risk of developing BC, but it is expensive and results in many false positive results [35]. Proteomic and gene biomarkers can also be used in monitoring tumor growth. However, these biomarkers are present at low concentration in early stages of BC, so they cannot be used for early detection [35, 36]. Therefore, new diagnostic tools that have minor disadvantages should be investigated to have more accurate diagnosis of BC.

Treating local BC tumors can be done by surgical removal of the tumor. Hormonal therapy can be used for ER+ and PR+ tumors targeting hormonal receptors. For HER2 positive tumors, targeted therapy using HER2 antibodies can serve as a possible treatment strategy. The most aggressive subtype is TNBC and is characterized by large tumor size, involvement of lymph nodes, high rates of recurrence, and poor prognosis [37, 38]. Therefore, TNBC patients are being subjected to chemotherapy in most of the cases. Radiation can also serve as a treatment strategy to decrease recurrence [39].

The difficulty of early diagnosis, treatment selection, and prognosis prediction increases due to the heterogeneity of BC [40]. For example, TNBC patients are developing therapeutic resistance to chemotherapy resulting in cancer metastasis and recurrence [40, 41]. Tumors considered as TNBC have shown diverse histologic patterns [42]. Thus, TNBC is considered a heterogenous group that needs to be further subtyped. Accurately classifying and understanding these subtypes, can help in developing accurate diagnostic tools and more effective treatment strategies.

1.1.6. Breast Cancer heterogeneity: ER- Breast Cancers

In 2012, Guedj et al. divided BC into six subgroups based on transcriptomic gene cluster. They had very significant differences in tumor grade, metastatic sites, or response to chemotherapy. Four of the subgroups were (ER+ /PR+ /AR+). The other 2 were both ER-; one was (ER-/PR-/AR+) and the other was triple negative (ER-/PR-/AR-) [43]. HER2 expression was amplified among a subgroup of ER+ tumors and a subgroup of ER- (ER-/PR-/AR+) tumors [43].

1.1.6.1. Triple Negative Breast Cancers

TNBCs are clinically, pathologically, and molecularly distinct, resulting in several different behaviors and treatment responses. Additionally, TNBCs have higher risks of recurrence and metastasis to the lungs and brain compared to other BC subtypes [44]. This metastasis is rapid, appearing during the first 3 years of diagnosis, tending to poor prognosis [44]. Therefore, due the diverse differences between TNBCs and their aggressive behavior, it is crucial to better classify TNBCs for having finer treatment strategies. Initially, Lehmann et al subdivided TNBC into six subtypes: basal-like 1

(BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal like (MES), mesenchymal stem like (MSL), and luminal androgen receptor (LAR). These subtypes were determined by cluster analysis of the gene expression of TNBC cases reported in 21 publicly available data sets [45]. In 2016, Lehmann et al refined TNBC subtypes from 6 subtypes to 4 (BL1, BL2, MES, and LAR) by using histological evaluation, isolation of homogenous cell populations, and gene expression analysis on a panel of TNBC tumors. They proved that IM and MSL had distinct profiles only due to the infiltration of lymphocytes and tumor associated mesenchymal cells respectively, and not because they are distinct subtypes [46]. These subtypes differ in response to chemotherapy, yet they are not used in clinical practice [44]. Considering LAR subtype, it is the most distinct among TNBC subtypes. Although it is ER-, it is highly regulated by hormonal pathways due to the high expression of androgen receptor (AR), and downstream AR targets and coactivators [45].

1.1.6.2. Molecular Apocrine Breast Cancer

Initially, apocrine breast carcinomas were classified based on their specific histological and morphological features. These features include abundant granular eosinophilic cytoplasm, nuclei located centrally to eccentrically, prominent nucleoli, and distinctive cell borders [47]. Apocrine breast carcinomas are corelated with high-risk recurrence and poor prognosis, but they have poor gene signature that describes them molecularly [47, 48].

Using transcriptomic profiling on BC, Farmer et al. classified BC into three main subtypes. Based on ER and AR expression in BC tumors the subtypes are: Luminal (ER+ and AR+), basal (ER- and AR-), and molecular apocrine breast cancer (ER- and

AR+). The newly discovered subtype, molecular apocrine breast cancer (MABC), was given the term “apocrine” due to the presence of apocrine features [49]. Another group identified MABC as a separate entity by using other BC datasets [50]. By observing gene expression in ER- tumors, a differential cluster having AR expression was identified [50]. Moreover, this new subtype coincides with Lehmann TNBC LAR subtype (both ER- and AR+). However, Guo et al. found that more than 50% of MABC are considered as HER2+ [51]. Knowing that LAR tumors are TNBC tumors, these tumors are considered as HER2- negative. Therefore, a confusion arises due to the overlap between TNBC LAR subtype (ER-, HER2-, AR+) and HER2- MABC (Figure 5). Thus, MABC/LAR tumors should be considered as a new entity distinct from other BC subtypes. However, it is still difficult to identify this subtype because the extent to which it has been studied is insufficient. Therefore, accurate methods of identification are required to improve its diagnosis, prognosis, and treatment. Novel biomarkers like microRNA (miRNA) could serve as a helpful tool for better classifying and understanding the tumorigenesis of MABC/LAR tumors.

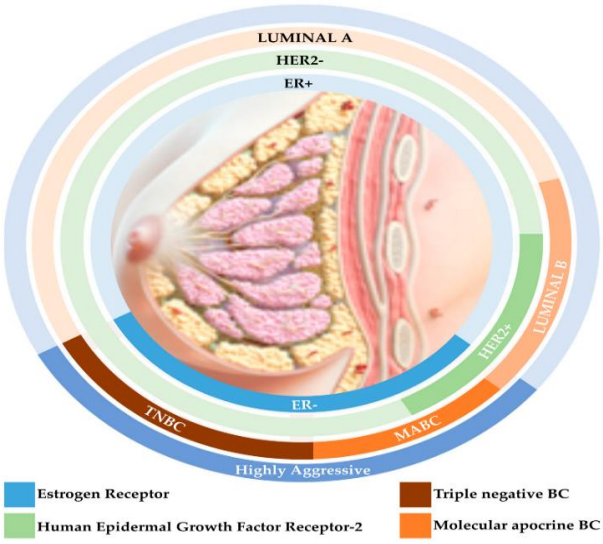


Figure 5. Hormonal receptors expression and aggressiveness of BC subtypes. Adapted from [44].

1.2. Overview on microRNAs

1.2.1. miRNAs Definition

MicroRNAs (miRNAs) are small noncoding RNA sequences of 17-25 nucleotides [52]. According to Bartel, miRNAs account for 1-3% of the human genome and have a role in regulating physiological processes like cellular development, proliferation, metabolism, differentiation, and homeostasis [53, 54]. In 1993, Lee et al, at Harvard University, discovered *lin-4*, the first miRNA in *Caenorhabditis elegans* nematode [55]. They found that *lin-4* gene does not code for any protein, but it produces small RNAs. These RNAs have an antisense complementary strand to multiple sites in the 3' untranslated region (3'UTR) of the *lin-14* gene. This region was previously shown to mediate the repression of the *lin-14* gene [56]. Therefore, they concluded that *lin-4* reduces the translation of *lin-14* mRNA into LIN-14 protein [53, 55]. After which,

several studies discovered hundreds of miRNAs found in humans that are coded within protein coding genes and non-coding RNA units [57].

1.2.2. miRNAs Biogenesis and Mechanism of Action

miRNA genes are either intergenic (within genome exons) or intronic (within genome introns). Intronic miRNAs are transcribed by the same promoter that transcribes the host gene which they are located within. However, intergenic miRNAs rely on their own promoters [58].

Biogenesis and maturation of miRNAs is considered a two-step process that starts in the nucleus and ends in the cytoplasm. In the nucleus, RNA Pol II transcribes miRNA genes into primary miRNAs (pri-miRNAs) [59]. Then the biogenesis continues in either of the two pathways: canonical or non-canonical [59] (Figure 6).

1.2.2.1. The Canonical Pathway

It is the dominant pathway by which most of the miRNAs are processed [59]. In this pathway, a microprocessor complex composed of an RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and a ribonuclease III enzyme (Drosha), cleaves pri-miRNA into a hairpin shaped precursor miRNA (pre-miRNA) of 60-80 nucleotides in length [60]. Then these pre-miRNAs are exported to the cytoplasm by an exportin 5 (XPO5)/RanGTP complex. In the cytoplasm, an RNase III endonuclease (Dicer) and its cofactor transactivation-responsive RNA binding protein (TRBP) continue the processing of pre-miRNAs by cleaving its terminal loop, resulting in a mature miRNA duplex [60-62]. The duplex now has two strands: the “guide” which has a relatively unstable 5’ end or that has a 5’ Uracil at nucleotide position 1, and the “passenger”

strand [58]. In an ATP dependent manner, both strands are loaded into the Argonaute protein family (AGO) forming the RNA-Induced Silencing Complex (RISC) [63].

1.2.2.2. The Non-canonical Pathway

This pathway is divided into Drosha/DGCR8-independent and Dicer-independent pathways. In the Drosha-independent pathway, Mirtrons are generated from the cleavage of short introns. Then they are exported to the cytoplasm by Exportin 5, to be processed by Dicer and loaded onto AGO protein. However, in the Dicer-independent pathway, pre-miRNAs are generated from short hairpin RNA (shRNA) molecules then loaded onto AGO2 protein to be processed further [64-66].

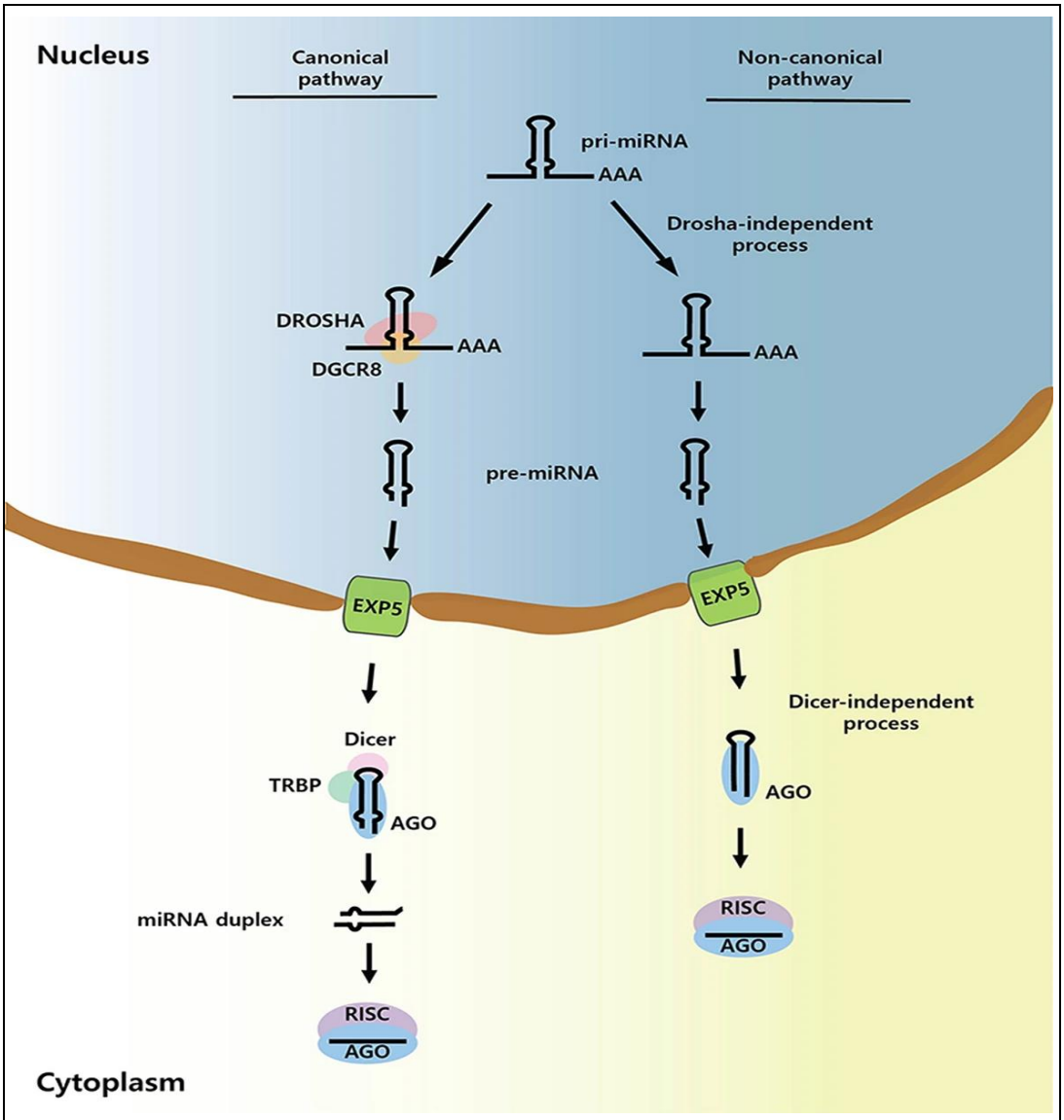


Figure 6. Canonical and non-canonical pathways of miRNA biogenesis.

In the canonical pathway of miRNA biogenesis, pri-miRNAs are transcribed from miRNA genes by RNA polymerase II and are then cleaved by Microprocessor (Drosha+DGCR8) to form pre-miRNAs. Pre-miRNAs are then exported to the cytoplasm, where they are once again cleaved by Dicer, forming miRNA duplexes. One strand of this miRNA duplex is loaded into Argonaute protein (AGO) to form RISC, which takes part in mRNA regulation. In the non-canonical pathway of miRNA biogenesis, miRNAs bypass the Drosha- or Dicer-dependent cleavage step, and this step is replaced by another cleavage reaction carried out by different proteins. Adapted from [67]

In both pathways, RISC functions as a guide to direct the silencing of target mRNA [68]. Mature miRNAs are denoted with a -5p or -3p suffix based on the precursor arm they originate from, either 5' or 3' respectively [69]. It was previously thought that only one pair is active, but recent studies showed that both pairs can be active and target common genes [70]. miRNAs repress gene expression by interfering in posttranscriptional modifications by two main mechanisms [71] (Figure 7). They can bind to the complementary mRNA, preventing the mRNA from being translated, thus decreasing gene expression [72]. Another way miRNAs can repress gene expression is by deadenylation, and exonucleolytic digestion of the mRNA, thus degrading it [73-77].

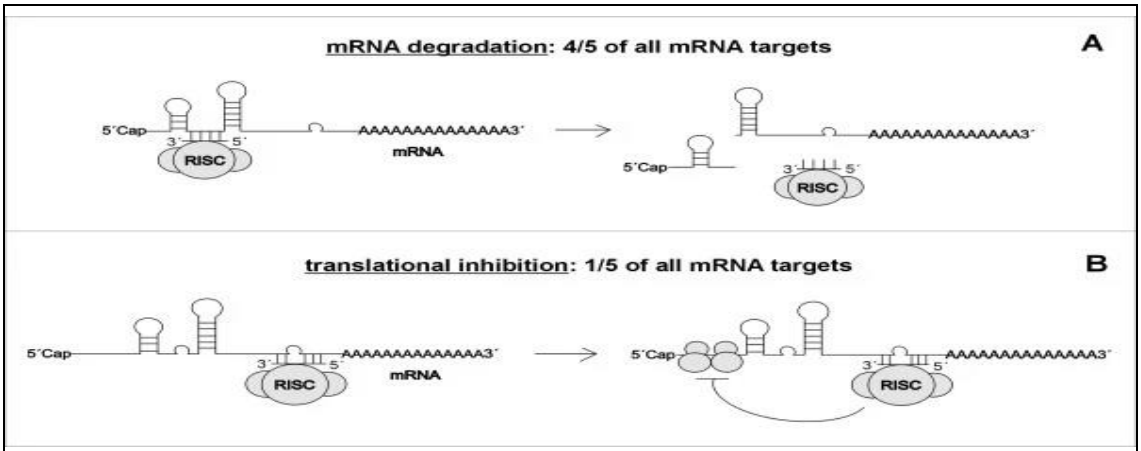


Figure 7. Mechanism of miRNA action on target mRNA.

According to the complementarity between miRNA and 3' untranslated region (UTR) of target mRNA, there are two mechanisms of miRNA action: (A) when miRNA is near-perfectly complementary with target mRNA, deadenylation and subsequent degradation of the target mRNA occurs (major mechanism of miRNA action); (B) when miRNA is only partially complementary to its target mRNA, translational inhibition occurs. Adapted from [78].

1.2.3. miRNAs in Biological Processes

miRNAs play several roles in various biological processes such as cell proliferation, differentiation, survival, development, and homeostasis [79]. For example, miR-96 causes developmental defects in humans when mutated [80]. Also, miRNAs play a critical role in the regulation of the immune system. Thus, their dysregulation results in severe defects in immune cell development leading to autoimmune disorders. For instance, in monocytes, miR-132, miR-146, and miR-132 regulate immune responses after pathogen recognition [81]. Moreover, miRNAs play a role in the development and differentiation of cardiac and skeletal muscles. It has been validated that miR-1 is abundantly expressed in muscle progenitor cells and differentiating muscle cells [82].

1.2.4. miRNAs Role in Cancer

miRNA dysregulation was suspected to have a role in tumorigenesis. The first study that proved the correlation between miRNA dysregulation and cancer was done by Dr. Croce's group in 2002 [83]. They were interested in investigating the role of the genes in the 13q14 chromosomal region which is frequently deleted in Chronic Lymphocytic Leukemia (CLL) cases [84]. The results showed that 2 miRNA genes, miRNA 15-a and miRNA 16-1, were found in this region which is deleted or downregulated in majority of B-cell CLL. These miRNAs were found then to act as tumor suppressors that induce apoptosis by repressing the expression of B-cell lymphoma 2 (BCL2) gene, a gene that enhances cell survival and inhibits apoptosis [85, 86]. Another study reported that miR-17-92 gene cluster was amplified in B-cell lymphomas and lung cancer resulting in an over-expression of these miRNAs [87, 88].

Therefore, based on their role in cancer and the genes they target, miRNAs are classified as either oncomiRs or tumor suppressor miRNAs (Figure 8) [89]. In lung cancer, miRNA let-7, which acts as a tumor suppressor, was found to be involved in the pathogenesis of the disease due its reduced expression in both *in vivo* and *in vitro* models what caused shortened postoperative survival [90] . In addition, let-7 was found to target and repress the translation of two oncogenes RAS and MYC which are significantly overexpressed in lung tumor tissues [91]. However, miR-17-92 cluster is highly expressed in lung cancer and it enhances tumor growth [87]. Therefore, miR17-9 acts as an oncomiR by repressing the expression of two predicted tumor suppressing genes, PTEN and RB2 [92]. Also, miR-221, miR-222, and miR-146 are overexpressed in thyroid tumors and are considered as oncomiRs that are predicted to target KIT mRNA which is downregulated in thyroid carcinoma [93]. Interestingly, some miRNAs target both oncogenic and tumor suppressor factors thus the net effect determines whether the miRNA acts as an oncomiR or tumor suppressor based on the ratio of the targeted oncogenic/tumor suppressor genes expression [94]. To illustrate, miR-125b acts as an oncomiR by targeting proapoptotic factors (TP53, BAK1, BMF, BBC3, and MAPK14), and metastasis inhibitors (STARD13, TP53INP1, and TP53) and acts as tumor suppressor by targeting pro-proliferative factors (JUN, STAT3, E2F3, IL6R, and ERBB2/3), metastasis promoters (MMP13, LIN28B, and ARID3B) [95, 96]. The net effect of miR-125b is tumor suppressing since it targets BCL6 gene, the hallmark of CLL [97, 98]. Another interesting miRNA is miR-155, which acts as an oncomiR or tumor suppressor based on the type of cancer [94]. It is considered as oncomiR due to its oncogenic properties in a large number of solid and hematologic malignancies [99, 100]. However, experiments showed that miR-155 inhibited proliferation in melanoma

cell lines by targeting SKI, an oncogene overexpressed in melanoma [101]. Other examples include the experiment done on gastric cancer (GC) and ovarian cancer (OC) that showed the tumor suppressive role of miR-155 by targeting SMAD2 and CLDN1 respectively [102, 103].

Moreover, studies validated that miRNAs genes are dysregulated in cancer due to their location in fragile sites or cancer-associated genomic region (52.5% of the genes are located in these sites) that are susceptible to chromosomal abnormalities (deletion, translocation, duplication) suggesting an important role in tumorigenesis [104]. In addition, epigenetic factors like DNA hypermethylation of the promoter region of miRNA can play a role in downregulating its expression like miR-9-1 downregulated expression in BC [105, 106]. On the other hand, miRNAs can be upregulated through histone acetylation of their genes. A study done on hepatocellular carcinoma (HCC) showed that miR-224 is upregulated due to increased histone acetylation and downregulated with the addition of a histone deacetylase [107]. Additionally, p53, a tumor suppressor factor, has shown a role in altering miRNAs expression by regulating their transcriptional process. In fact, miRNAs are important factors in the pathway p53 follows to suppress tumorigenesis. We can see this with miR-145, whose expression is induced by p53, negatively regulating oncogene c-Myc [108, 109]. Posttranscriptional modifications can also be regulated by p53. For instance, posttranscriptional maturation of miRNAs that have growth suppressive functions like miR-16-1, miR-143, and miR-145 were enhanced by p53 [110]. Moreover, downregulation of factors involved in processing miRNA like DROSHA and DICER as reported in breast cancer, lung cancer, and ovarian cancer can have a role in the dysregulation of miRNAs [111, 112]. Interestingly, miRNAs' profiles have shown a difference between subtypes of a cancer.

For example, miR-205 expression is used to discriminate between squamous and non-squamous non-small cell lung carcinoma [113-115]. The early diagnosis of the tumor provides better survival in cancer patients. Several reports proved that miRNAs can serve as a new potential early diagnostic marker. In pancreatic ductal adenocarcinoma, miR-205 and miR-21 are overexpressed preceding any phenotypic changes in the ducts, suggesting possible use of these miRNAs in early detection of this type of cancer [115, 116]. Another possible use in determining survival time, low miR-191 and high of miR-193 was shown to be associated with shorter survival time in melanoma [115, 117]. Considering the use of miRNA as a prognostic biomarker, it was shown that determining the levels of miR-26 does not serve as an indicator of survival in HCC. However, patients with low levels of miR-26 had improved survival by responding well to interferon-alpha treatment. Therefore, quantifying miRNAs can serve as a marker to differentiate between patients in giving treatment based on how well they are going to respond and benefit [115, 118].

We can conclude that miRNAs action is tissue and context specific. Eventually, miRNA profiling can be used in accurately discriminating between different types of cancer, between normal and cancerous tissue, and identifying subtypes of a cancer [115, 119, 120].

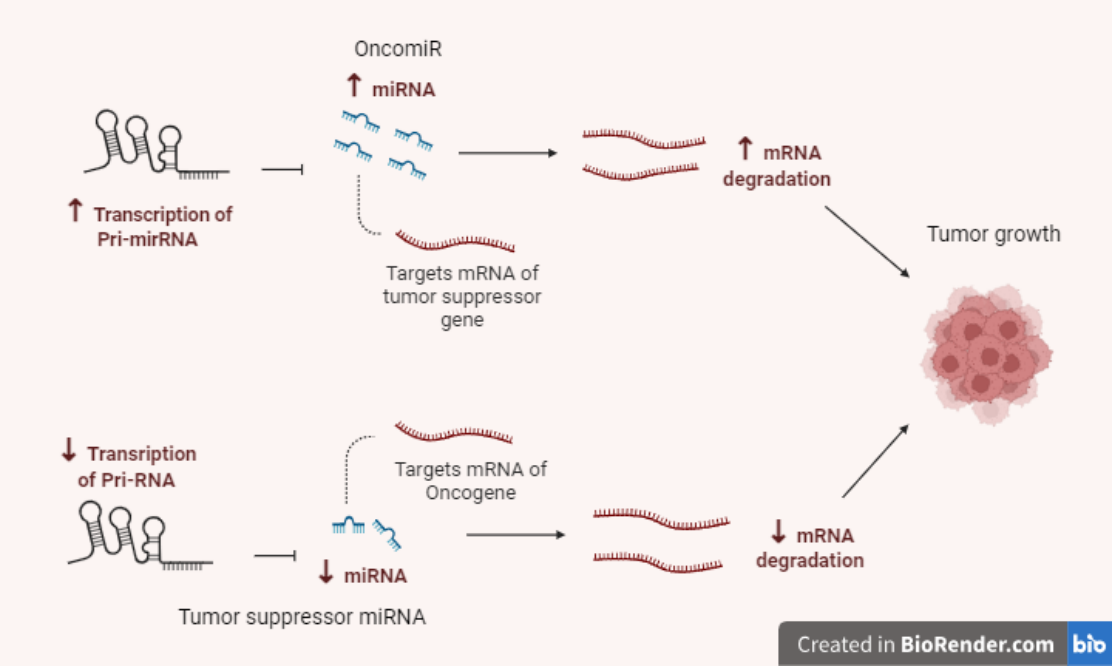


Figure 8. miRNA dysregulation in Cancer and acting as either oncomiRs or tumor suppressor miRNAs, created by BioRender.

1.2.5. miRNAs Role in Breast Cancer

Dysregulation of miRNA in BC was first discovered by Iorio et al in 2005. They found different miRNAs profile between breast tumor tissue and normal tissue [121]. Several miRNAs are consistently dysregulated in all BC types. For example, miR-21 and miR-155 are upregulated and miR-125b and miR-145 are down regulated in all BC types compared normal tissue [122]. However, a study done by Blenkiron et al analyzed miRNA profiles in BC tumors which demonstrated a distant miRNA profile between molecular subtypes of BC (luminal A, luminal B, basal-like, HER2+ and normal-like) [123]. Therefore, due to the heterogeneity of BC, miRNAs can be considered as a potential marker in diagnosis and subtyping BC tumors. A study showed that several miRNAs are overexpressed in luminal tumors (ER+ tumors) but not ER- tumors like miR-26, miR-5681a, miR-5695, miR-887, miR-149, miR-375, miR-342, miR-190b,

miR-29c, and miR-29b. Also, some miRNAs like miR-455-3p, miR-934, miR-135b and miR-577 are downregulated among ER+ samples compared to normal tissue. However, basal like tumors are characterized by high expression of miR-584, miR-138, miR-135b, miR-455, miR-577, and miR-934. Interestingly, they have a low expression of some miRNAs that are upregulated in ER+ tumors like miR-29c, and miR-190b. In HER2-enriched tumors, miR-34a, miR-2115, miR-4728, and miR-7158 have a high expression with miR-7158 being strongly associated with HER2-enriched tumors. Moreover, the difference in expression of miR-99a, let-7c, miR-125b helped in differentiating between luminal A and B tumors [124].

As discussed before, miRNAs act as either oncomiRs or tumor suppressor miRNAs. Therefore, they affect BC tumorigenesis by regulating oncogenic and tumor suppressor pathways that regulate apoptosis, invasion, metastasis, angiogenesis, and epithelial to mesenchymal transition (EMT) [125].

miR-125a promotes apoptosis by targeting gene coding for HuR protein (Human antigen R). Therefore, the downregulation of miR-125a in human breast tissue contributed to breast carcinogenesis [126, 127]. Also, it was confirmed by a study that the overexpression of miR-21 in BC tissues stimulates cell proliferation by targeting and inhibiting a tumor suppressor factor PDCD4 (programmed cell death protein 4) [128, 129].

Moreover, miR-205 is downregulated in MDA-MB-231 (TNBC model) and MCF-7 (ER+ BC model) BC cell lines when compared to MCF-10A (non-cancerous model of human mammary epithelial cells) cell line. To investigate its role, miR-205 was upregulated in MDA-MB-231 cells what suppressed their invasive ability [130].

Tavazoie et al. concluded after a study done on miR-126 and miR-335, that these two miRNAs have low expression in BC tissues compared to normal ones. In the study they found that miR-126 reduces tumor growth and proliferation while miR-335 inhibits metastatic cell invasion by targeting factors that promote metastasis like cell transcription factor SOX4 and extracellular matrix component tenascin C [131]. Another study quantified miRNAs that are associated with metastatic BC. Results proved that 15 miRNAs are overexpressed and 17 miRNAs are downregulated in metastatic BC tissues compared to non-metastatic BC tissues. These results suggest that the role of miRNAs involved in metastasis is variable between being an oncomiR or tumor suppressor miRNA [132].

Dysregulation of miRNA in cancer plays a role also in angiogenesis. Cancer cells utilize maximally nutrients and oxygen to help in their rapid growth, so they need more blood supply, initiating angiogenesis. This process can be accomplished due to the downregulation of miR-205 in BC [130]. This miRNA inhibits angiogenesis by suppressing angiogenesis promoters like HER and VEGF-A (vascular endothelial growth factor A) [130].

EMT is an important process in the formation of the body, by inducing the differentiation of tissues and organs, and its involvement in tissue repair. However, EMT dysregulation may promote carcinoma by giving cancer cells the ability to invade, migrate, and survive [133]. It was first discovered in 1989 by Boyer and colleagues that cancer cells can transform into mesenchymal cells prior to metastasis by losing cell-to-cell adhesions [134]. EMT can be measured and detected by certain markers like E-cadherin (expressed in epithelial phenotype), N-cadherin (expressed in mesenchymal phenotype), and transforming growth factor-beta (TGF-β), which is a cytokine that

induces EMT (Figure 9). After reporting EMT in BC, the downregulation of some miRNAs like miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) and miR-205 promoted EMT. Experiments showed that these miRNAs inhibited EMT by targeting ZEB1 and ZEB2 (Zinc finger E-box binding homeobox) genes. These two genes promote EMT by repressing E-cadherin, thus detaching cells from each other and giving them migratory freedom [135-138].

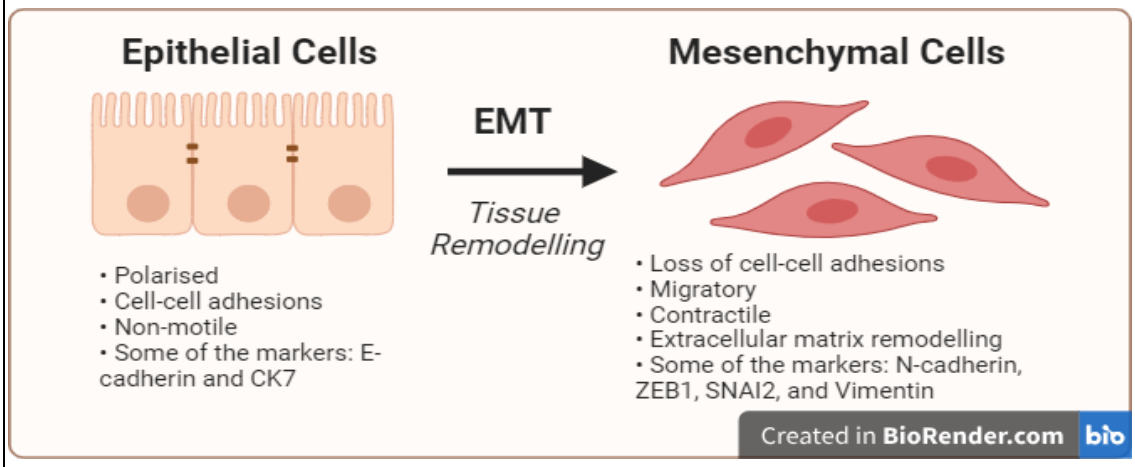


Figure 9. EMT illustration showing epithelial and mesenchymal different characteristics and markers Created by BioRender

1.2.6. miRNAs Role in AR+ tumors

Studies proved that there is an interplay between miRNAs and AR expression. The first correlation was proved in prostate cancer (PCa). Fletcher et al. suggested that in PCa, AR expression modulated the levels of an oncomiR miR-27a. This miRNA suppressed the expression of Prohibitin (an AR repressor). Thus, by positive feedback loop miR-27a and AR expression promoted PCa cell growth and maintained the oncogenic phenotype [139]. Considering the interaction between AR expression and

miRNAs in BC, a study proved that miRNAs are differentially expressed between AR+ and AR- cell lines [140]. Therefore, it is important to investigate if there is any crucial role for miRNAs in AR function.

No studies have been done yet to determine the interaction between miRNAs and MABC/LAR tumors. Knowing that the main feature of these tumors is the high AR expression, miRNAs could have a role in their tumorigenesis. Lyu et al. validated the role of let-7a in inhibiting cell proliferation of MDA-MB-453 (MABC cellular model, ER-/PR-/AR+). Also, this miRNA is upregulated due to the androgen induced AR activating signal [141]. Thus, the relation between miRNAs and MABC/LAR tumors should be further studied and investigated.

Using publicly available data on The Cancer Genome Atlas Breast Invasive Carcinoma Collection (TCGA-BRCA) and in silico analysis, our lab was able to classify the samples into their corresponding molecular subtypes. Then differential expression analysis identified dysregulated miRNAs in MABC samples compared to non-MABC samples. These findings were further validated in a cohort of French BC samples. The cohort was composed of 111 ER-negative samples characterized into 68 MABC samples and 43 non-MABC samples. Characterization criteria included AR, FOXA1, and AR-related gene expressions. After that, using miRNA PCR assay was done for a panel of differentially expressed miRNAs. Interestingly, miR-187-3p had a significant upregulation in MABC samples compared to non-MABC samples (Figure 10). These results raised our interests in investigating the role of miR-187-3p in MABC tumorigenesis.

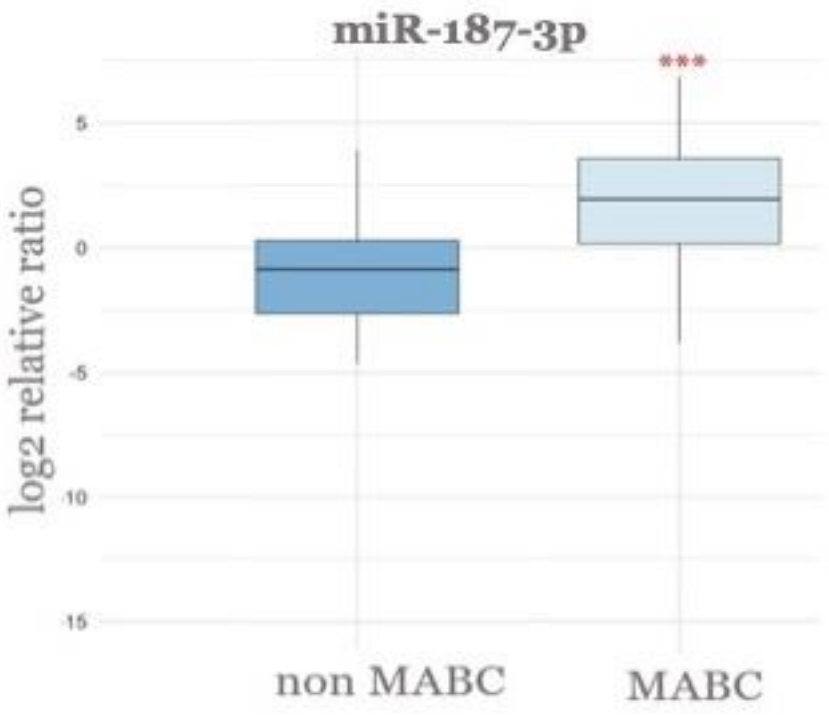


Figure 10. miR-187-3p expression in non-MABC and MABC tissue samples using miRCURY kit.

Box plot representing the log2 relative ratio of miR-187-3p expression (delta CT to mean of sno48 and sno49) non-MABC (n=43) and MABC (n=68). *** represents p-value<0.001

1.2.7. miR-187-3p Dysregulation in Cancer and its Role in Tumorigenesis

miR-187-3p is being studied more frequently in recent years. The gene coding miR-187-3p is located at the 18q12.2 chromosome of the human genome [142]. miR-187-3p is downregulated in several types of tumors and being considered as a tumor suppressor. Considering male genitourinary tumors, miR-187-3p showed a reduced expression in PCa compared to benign hyperplasia [143]. In cancers occurring in the digestive system like HCC and colorectal cancer (CRC), miR-187-3p also showed a reduced expression [144, 145]. The downregulation of miR-187-3p in HCC is due to hypoxia. S100A4 was validated to be a downstream target of miR-187-3p. S100A4 is a

gene that promotes proliferation and metastasis [146]. Thus decreasing the expression of S100A4, inhibited EMT progression and the promoting effects of hypoxia on metastasis [147]. In CRC results showed that miR-187-3p impaired cell migration and invasion through repressing its target gene Sprouty RTK Signaling Antagonist 1 (SPRY1). However, high levels of miR-187-3p in CRC tumors was correlated with inflammatory conditions resulting in poor prognosis [145]. In non-small cell lung cancer (NSCLC), miR-187-3p, was downregulated in tumor tissues compared to normal tissues and very low in NSCLC cell lines. Functional assays done showed that miR-187-3p inhibited cell proliferation, migration, invasion, and promoted apoptosis by targeting oncogenic BCL6. Moreover, measuring the expression of miR-187-3p can help in determining the prognosis of cancer where it is dysregulated. It was determined that metastatic HCC had a lower expression of miR-187-3p than non-metastatic HCC tumors. Therefore, low level of this miRNA is associated with metastasis and cancer progression [147]. In clear cell renal cell carcinoma (ccRCC), miR-187-3p is downregulated compared to normal tissue and is corelated with poor prognosis. That is due to the ability of miR-187-3p in restraining cell proliferation, migration and invasion capacities by targeting LRFN1 gene [148].

1.2.8. miR-187-3p Dysregulation in BC and its Role in Tumorigenesis

In BC, miR-187-3p has not been studied much and is poorly investigated. However, available data reveals that miR-187-3p is dysregulated in BC tumors [149]. Therefore, miR-187-3p should be further studied in BC since it might be used in differentiating BC subtypes, determining tumor progression, and affecting drugs sensitivity. A study compared the expression of miR-187-3p in monolayer culture and

multicellular tumor spheroids (MCTS) BC models of MCF-7 cells. The results revealed that miR-187-3p is upregulated in MCTS BC model compared to monolayer BC model. Therefore, it can be concluded that miR-187-3p has a role in promoting aggressive and invasive tumor ability. Thus, determining the expression of miR-187-3p can help in differentiating between high-risk tumors (high levels of miR-187-3p) and low risk tumors (low levels of miR-187-3p) [142, 150]. However, another study proved that miR-187-3p is downregulated in BC by comparing its expression in 30 pairs of BC tumors and corresponding non-tumor tissue samples. Then using MDA-MB 231 cell line as a model, the authors were able to determine the role of miR-187-3p in tumorigenesis by upregulating its expression. Overexpression of miR-187-3p reduced cell viability and increased apoptosis when compared to untreated cells. For that reason, the authors concluded that miR-187-3p reduces cancer progression by inducing apoptosis, opposing the results of previous study mentioned earlier [149]. Other than using miR-187-3p as a tumor progression marker, it can be used in predicting drug response and sensitivity. Differentially expressed gene elimination strategy (DEGES) analysis was done between patients having metastatic BC that are considered as responders to drugs and non-responders to determine miRNAs associated with systemic treatment. The analysis revealed 12 miRNAs as oppositely regulated between responders and non-responders. Of which miR-187-3p exhibited a significant upregulation in responders compared to non-responders. Moreover, miR-187-3p upregulation has shown association with increased overall survival (OS) and progression free survival (PFS) because it regulates Mitogen-activated protein kinase (MAPK) signaling pathways. Therefore, miR-187-3p can be used as a marker to determine the response of patients having metastatic BC to treatment [151-153].

Considering drug sensitivity, miR-187-3p has shown a role in regulating the sensitivity of chemotherapy drugs such as gemcitabine. In the experiment previously discussed, the overexpression of miR-187-3p in MDA-MB-231 cells increased the sensitivity to gemcitabine by enhancing its anti-proliferative and pro-apoptotic effects. Thus, this study revealed an important potential use of miR-187-3p as a therapeutic target in BC [149]. In conclusion, miR-187-3p is dysregulated in BC and has a role in tumorigenesis. However, its expression and role in MABC is not yet studied and should be further investigated.

1.3. Aim of the Study

The heterogeneity of BC renders its classification, prognosis, diagnosis, and treatment as challenging. Specifically, MABC tumors that are not well studied and are being misdiagnosed as TNBC tumors. Therefore, the main aim of our work is to find markers that can aid in refining the classification criteria. Since miRNAs have been reported to be dysregulated and to have a role in tumorigenesis, we were interested in investigating the role of differentially expressed miRNAs between MABC and non-MABC tumors. One of the miRNAs as per previous data at our lab, was reported to be upregulated in MABC samples compared to non-MABC samples. This miRNA is miR-187-3p that is reported to be implicated in several cancer related pathways altering cancer proliferation, invasion, migration, and aggressiveness. These findings were described in other cancers or BC subtypes but not in MABC. Thus, in this study we aim to investigate the role miR-187-3p in MABC tumorigenesis. For this purpose, we will first try to find the best in vitro cellular models that resemble MABC and non-MABC by determining AR and miR-187-3p basal expressions. Then miR-187-3p will be

upregulated or downregulated in the chosen cell lines. Invasion and proliferation assays will be done to determine the role of miR-187-3p in tumorigenesis. By using in silico analysis by miRtargterlink 2.0 database and literature review, potential targets of miR-187-3p will be chosen. Further selection will be performed on target genes where the ones downregulated in MABC compared to non-MABC based on TCGA-BRCA data will be chosen. After that the effect of the modulated expression of miR-187-3p on the selected potential targets will be assessed by RT-qPCR.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bioinformatics Analysis: In Silico miRNA-mRNA integration analysis

miRtargetlink 2.0 was used as a database to search for predicted and weakly validated targets of miR-187-3p. This database reported predicted targets that are mentioned in all databases. Therefore, 34 predicted targets, mentioned in at least 2 databases, were selected. Also, 16 weakly validated targets were reported in miRtargetlink 2.0, thus selected. Based on literature review, 7 targets were selected as being reported to be miR-187-3p targets. Further selection depended on the targets' significant downregulation in MABC samples compared to non-MABC samples based on TCGA-BRCA database. PubMed was used as a searching engine to determine miRNA-mRNA interaction and the role of selected targets in cancer as follows: ("breast neoplasms"[MeSH Terms] OR ("breast"[All Fields] AND "neoplasms"[All Fields]) OR "breast neoplasms"[All Fields] OR ("breast"[All Fields] AND "cancer"[All Fields]) OR "breast cancer"[All Fields]) AND "mir-187-3p"[All Fields], "mir-187-3p"[All Fields] AND ("cancer s"[All Fields] OR "cancerated"[All Fields] OR "canceration"[All Fields] OR "cancerization"[All Fields] OR "cancerized"[All Fields] OR "cancerous"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "cancer"[All Fields] OR "cancers"[All Fields]), "mir-187-3p"[All Fields] AND ("target"[All Fields] OR "targetability"[All Fields] OR "targetable"[All Fields] OR "targeted"[All Fields] OR "targeting"[All Fields] OR "targetings"[All Fields] OR "targets"[All Fields] OR "targetted"[All Fields] OR "targetting"[All Fields]), and "name of the target"[All Fields] AND ("cancer s"[All Fields] OR "cancerated"[All Fields] OR "canceration"[All Fields]

OR "cancerization"[All Fields] OR "cancerized"[All Fields] OR "cancerous"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields] OR "cancer"[All Fields] OR "cancers"[All Fields]).

2.2. Cell Culture

MDA-MB-231 (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. 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 1).

Table 1. Characteristics of the Used BC Cell Lines (ATCC)

Cell line	Organism and Age	Origin	Primary tumor	Receptors
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	AR + ER- PR- HER2-
MCF-10A	Human, 36 years old	Mammary gland, Breast	N/A	N/A

2.3. 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. The process is illustrated in Figure 11.

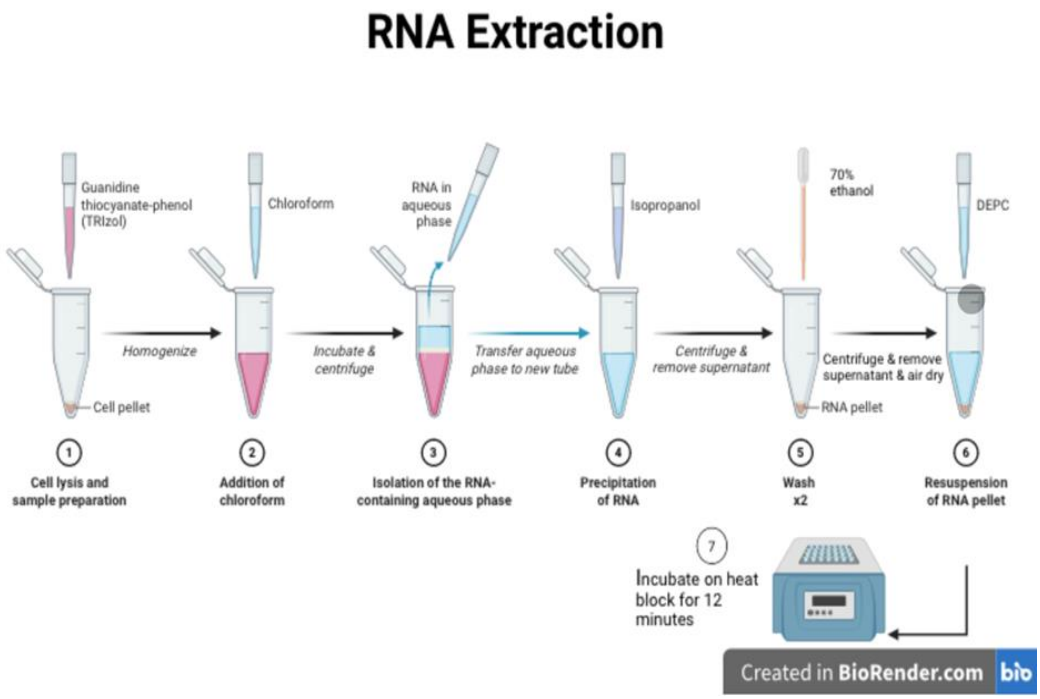


Figure 11. Illustration showing RNA extraction steps. Adapted and modified from BioRender

2.4. 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(ID-001093) as an endogenous control and miR-187-3p (ID-241073), 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-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 96 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-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 and NC mimic) transfected cells.

2.5. Transfection of Cells

275,000 cells of MDA-MB-231 and MDA-MB-453, were seeded per well in a 6-well plate to reach 60-80% confluency for RNA extraction. Cells were seeded in duplicates for each of the 3 conditions: control cells (untransfected), cells transfected with miR-187-3p mimic to upregulate miR-187-3p or miR-187-3p inhibitor to downregulate miR-187-3p, and cells transfected with negative control (NC) mimic or NC inhibitor respectively to using mimics or inhibitors (GenePharma, Shanghai), all of which are FAM-labeled. 5,000 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. Finally, for the invasion assay, 55,000 cells were seeded per well in 24 well plates and incubated overnight. Media was replaced with antibiotic-free media two hours prior to transfection for all assays. 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. Lipofectamine and miR-187-3p mimic/NC or inhibitor/NC were diluted in opti-MEM to reach the final concentration of 12nM and 100 nM respectively. Then, the mix is incubated for 5 minutes and then transferred to the diluted lipofectamine in a 1:1 ration and incubated for 10 minutes at RT. 100 µl of the complex was added to the plates in a circular motion to ensure proper dispersion of the liquid. After 24 hours, the cells are processed or stored at -80°C. However, the 96 well plates were incubated for 24, 48, and 72 hours to perform MTT assay.

2.6. 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.

2.7. MTT Assay

MDA-MB-231 and MDA-MB-453 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.

2.8. Invasion Assay

To perform the invasion assays, 24-well tissue culture plates were fitted with inserts (8 µm pore size, BD Falcon) coated with growth factor-reduced Matrigel (1:1 dilution with serum- free medium). MDA-MB-231, MDA-MB-453, and MCF-10A each with the densities mentioned earlier were then seeded on top of the matrigel and left at

37°C until they reach confluency. Then cells were transfected with mimics or inhibitors and their corresponding NC as per manufacturer's instructions detailed earlier. After 24 hours, cells were labeled with Calcein-AM, and incubated for 1 hour at 37°C after which inserts were fixed with 4% paraformaldehyde (PFA). Next, cells were counterstained with DAPI. Fluorescent cells, which successfully invaded through the matrigel, were photographed and counted at 40X magnification using laser scanning microscope (LSM) 710 confocal microscope (Figure12). At least five fields were counted in z-stacks and reported as percentage of invaded cells as compared to the control, cells transfected with NC.

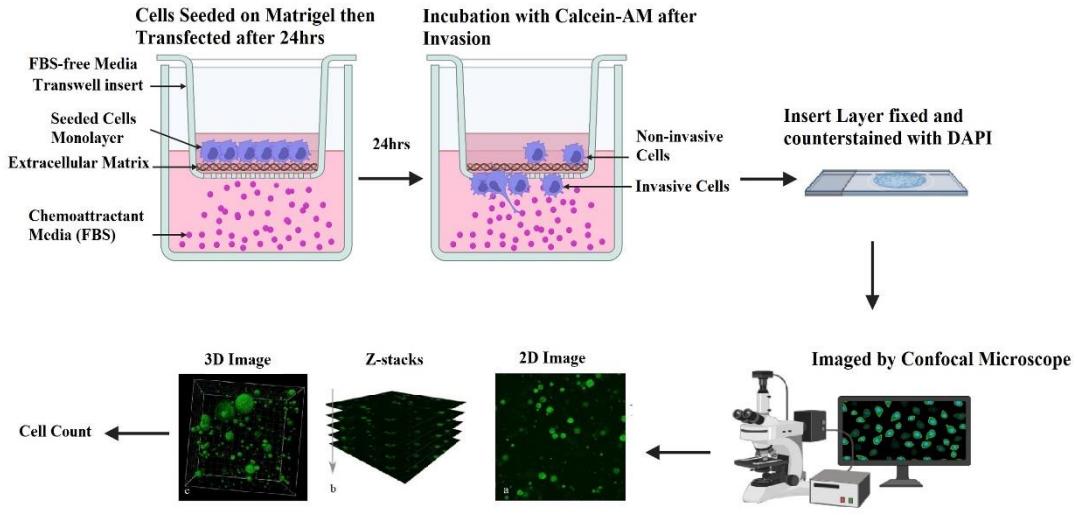


Figure 12. Matrigel Invasion Assay

Cells are allowed to invade through a matrigel where they have been seeded onto a semi-permeable membrane cell culture insert with chemoattractant added below the membrane. Invaded cells can then be quantified by staining cells with Calcein-AM

2.9. Primers Optimization of the Selected mRNA Targets

Primers of the selected mRNA targeted genes by 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 un-transfected MDA-MB-231 or MDA-MB-453 cells depending on the expression of each gene in a specific cell line based on The Human Protein Atlas.

2.9.1. 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.

2.9.2. Primers Optimization by RT-qPCR

Different gradients of temperature were used for the optimization of primers. The master mixes were prepared by mixing 5 µl of SYBER, 0.2 µl of each primer (forward and reverse), and 3.6 of diethyl pyrocarbonate (DEPC). 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. The concentration of the forward and reverse primers of all

the genes was 10 µM for each primer. The plate was briefly centrifuged at 2,400xg for 1 minute to recover the full volume that needs to be in the bottom of the well. Then, the plate was loaded into the Real-time PCR Biorad CFX96 machine, and the following protocol was followed: 10 minutes hold at 94°C, 40 cycles of 15 seconds at 94°C. Then a varying gradient for each gene: a gradient of 60.8-64.9 for CENPA, MBNL1, AGO1, SOX4, INPP5B, and ITPR2 genes and a gradient of 61.3-67 for AR, BCL2, C1S and SPRY1 genes for 1 minute (Table 2). In addition to 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.

Table 2. Primer Optimization Performed on Different Temperatures

Primer	Temperature Gradient °C
CENPA	60.8-64.9
MBNL1	60.8-64.9
AGO1	60.8-64.9
SOX4	60.8-64.9
INPP5B	60.8-64.9
ITPR2	60.8-64.9
AR	61.3-67
SPRY1	61.3-67
BCL2	61.3-67
C1S	61.3-67
TBP	No Gradient

2.10. 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). Using ΔΔCq, the relative expression of the mRNA targets was determined in miR-187-3p inhibitor and miR-187-3p mimic transfected cells compared to NC inhibitor and NC mimic transfected cells, respectively. Protocol was followed according to manufacturer's constructions. TBP was used as an endogenous control.

2.11. Statistical Analysis

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 3

RESULTS

3.1. AR expression in BC cell lines

In order to determine if the cellular models we are using resemble MABC and non-MABC samples, AR expression in MCF-10A, MDA-MB-231, and MDA-MB-453 was determined by performing RT-qPCR and using TBP as an endogenous control. The results revealed overexpression of AR in MDA-MB-453 cell line compared to MCF-10A and MDA-MB-231 cell lines (Figure 13).

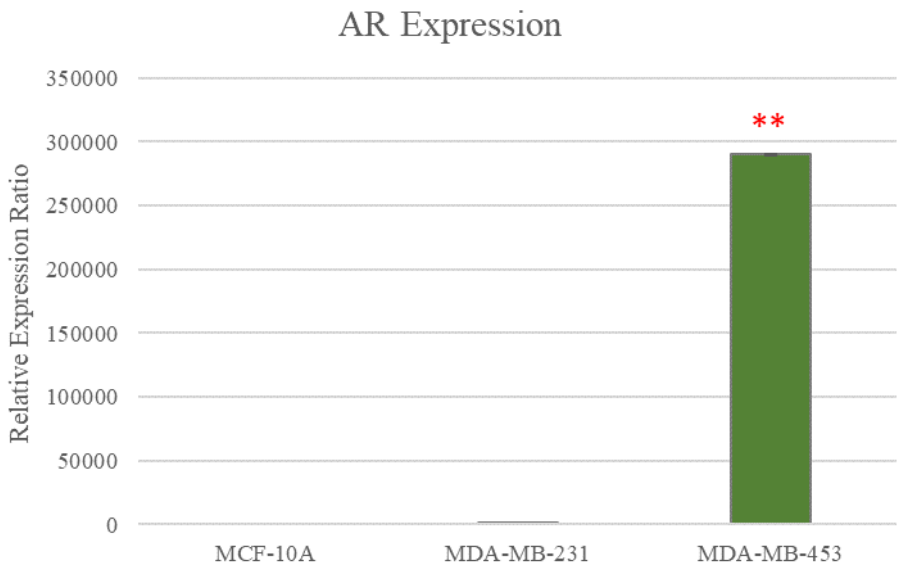


Figure 13. Relative Expression Ratio of AR in MDA-MB-231 and MDA-MB-453 Compared to MCF-10A.

TBP was used as an endogenous control. Error bars represent SEM (n=3). ** denotes $p<0.01$

3.2. miR-187-3p Basal Expression in BC cell lines

The expression of miR-187-3p in 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 proved that miR-187-3p is significantly upregulated in MDA-MB-453 cells but not in MDA-MB-231 cells (Figure 14). These data were consistent with previous lab work that proved that miR-187-3p is upregulated in MABC compared to non-MABC tissues. Therefore, MDA-MB-453 was chosen as an MABC in vitro model and MDA-MB-231 as a non-MABC in vitro model.

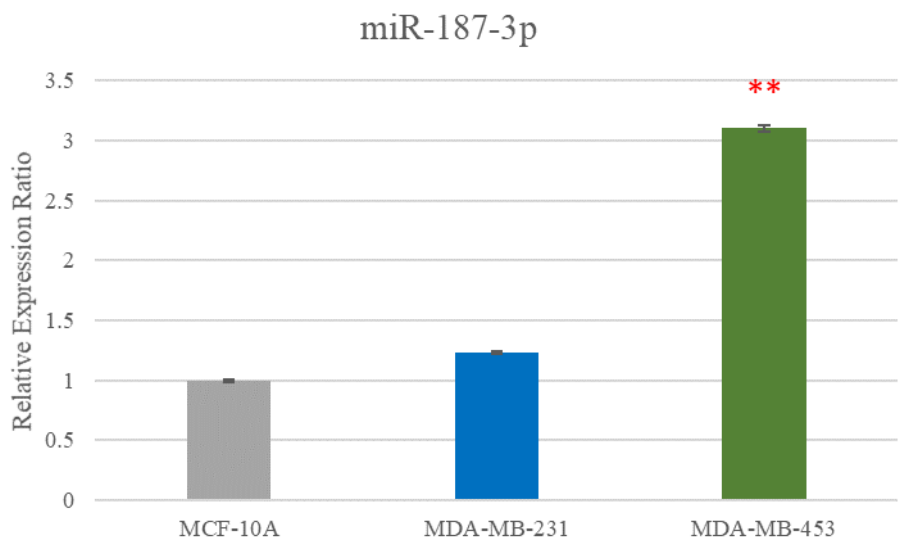


Figure 14. Relative Expression Ratio of miR-187-3p in MDA-MB-231 and MDA-MB-453 Compared to MCF-10A.

RNU6B was used as an endogenous control. Error bars represent SEM (n=3). ** denotes $p<0.01$

3.3. miR-187-3p Downregulation in MDA-MB-453 and Upregulation in MDA-MB-231 upon Transfection with miR-187-3p FAM-labeled Inhibitor and Mimic, respectively.

To unveil the role of miR-187-3p in BC models, transfection of MDA-MB-453 cells was performed with miR-187-3p inhibitor and negative control (NC) inhibitor while MDA-MB-231 cells were transfected with miR-187-3p mimic and NC mimic. Transfection efficiency for both conditions and for NC conditions was validated by flow cytometry compared to un-transfected cells, 24 hours post transfection (Figure 15A and 16A). MDA-MB-453 cell line was significantly transfected with miR-187-3p inhibitor (98.3%) and NC inhibitor (99.2%) (Figure 15B) compared to un-transfected cells. Also, MDA-MB-231 cell line was significantly transfected with miR-187-3p mimic (78.8%) and NC mimic (82.2%) (Figure 16B) compared to un-transfected cells. In addition, the levels of miR-187-3p were detected as compared to the corresponding NC in both transfected cell lines by RT-qPCR and using RNU6B as an endogenous control. The downregulation of miR-187-3p was significant in miR-187-3p inhibitor-transfected MDA-MB-453 cells as compared to NC inhibitor (Figure 15C). miR-187-3p was significantly overexpressed in MDA-MB-231 cells transfected with miR-187-3p mimic as compared to NC mimic (Figure 16C).

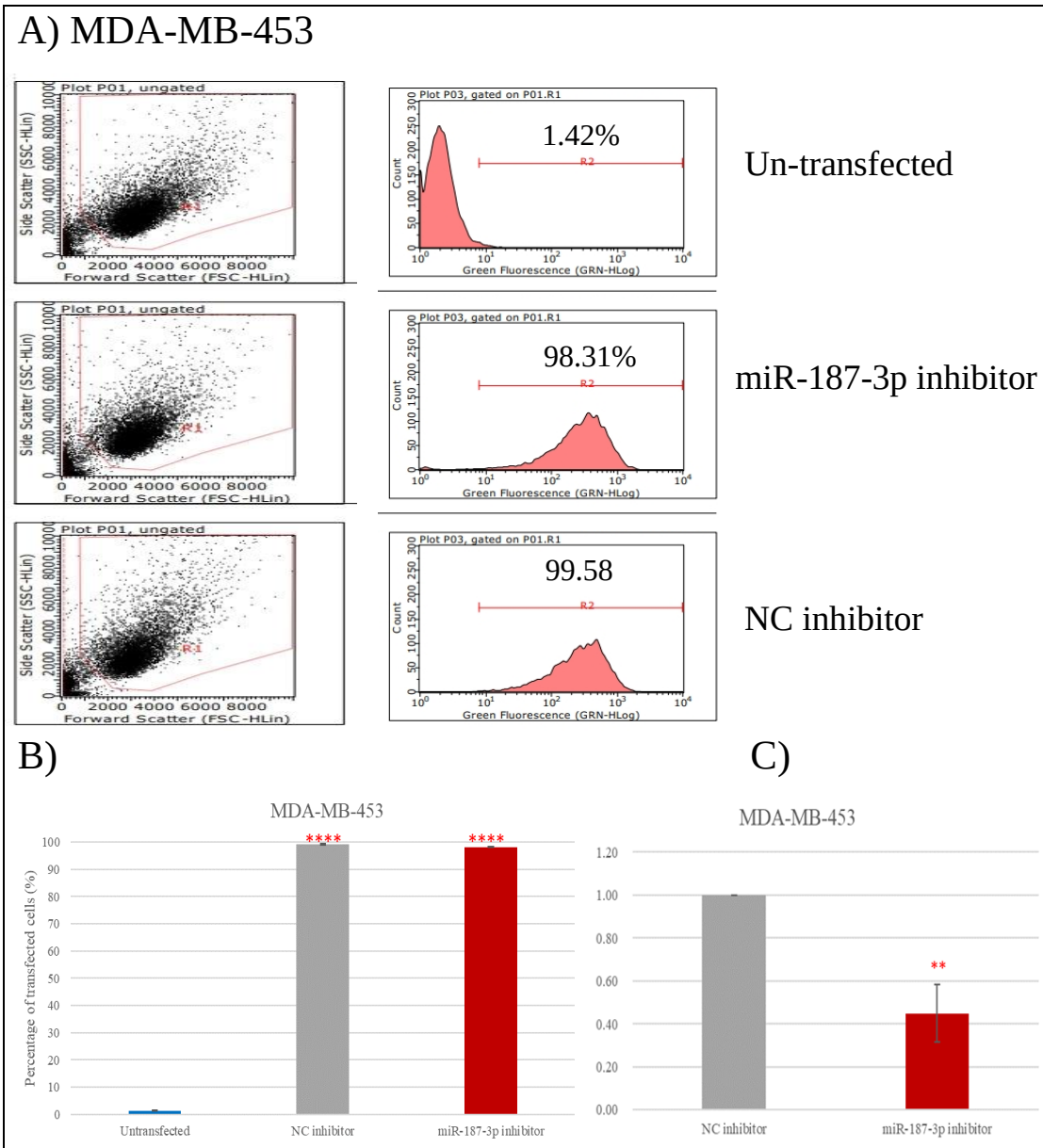


Figure 15. Transfection Efficiency measured by Flow Cytometry and RT-qPCR in MDA-MB-453 transfected with miR-187-3p inhibitor and NC inhibitor.

(A) Representative figure showing the shift in mean fluorescence intensity upon transfection of MDA-MB-453 with FAM-labelled miR-187-3p inhibitor and NC inhibitor as compared to un-transfected cells. (B) Transfection Efficiency was measured by Flow Cytometry and the graph shows percentage of FAM-labelled miR-187-3p inhibitor and NC inhibitor transfected cells as compared to un-transfected cells. (C) RT-qPCR analysis of miR-187-3p levels in miR-187-3p inhibitor transfected cells as compared to NC inhibitor. RNU6B was used as an endogenous control. Error bars represent SEM (n=4, n=3 respectively). ** Denotes $p<0.01$, **** Denotes $p<0.0001$

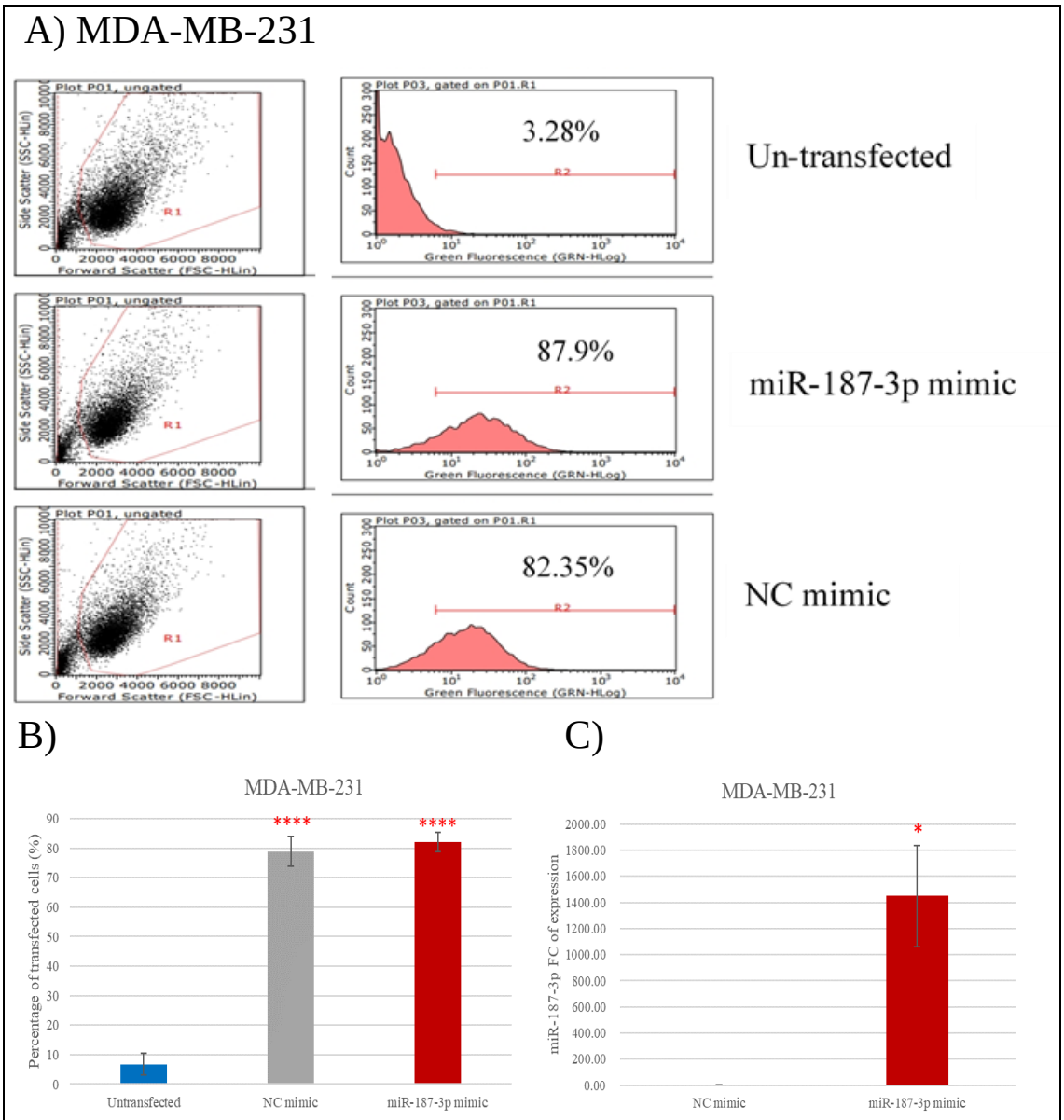


Figure 16. Transfection Efficiency measured by Flow Cytometry and RT-qPCR in MDA-MB-231 transfected with miR-187-3p mimic and NC mimic.

(A) Representative figure showing the shift in mean fluorescence intensity upon transfection of MDA-MB-231 with FAM-labelled miR-187-3p mimic and NC mimic as compared to un-transfected cells. (B) Transfection Efficiency was measured by Flow Cytometry and the graph shows the percentage of FAM-labelled miR-187-3p mimic and NC mimic transfected cells as compared to un-transfected cells. (C) RT-qPCR analysis of miR-187-3p levels in miR-187-3p mimic transfected cells as compared to NC mimic. RNU6B was used as an endogenous control. Error bars represent SEM (n=4, n=3 respectively). * Denotes $p<0.05$, **** Denotes $p<0.0001$

3.4. Downregulation of miR-187-3p inhibits MDA-MB-453 Cells Proliferation

To determine the effect of miR-187-3p on MDA-MB-453 proliferation, MTT assay was done. Also, transfection efficiency of cells seeded in 96-well plates was determined by Flow Cytometry. Results show that MDA-MB-453 cells were significantly transfected with miR-187-3p inhibitor and its respective NC inhibitor (Figure 17). Upon the downregulation of miR-187-3p, proliferation of MDA-MB-453 cells decreased significantly at 48 ($p=0.05$) and 72 ($p=0.008$) hours but not at 24 hours compared to cells transfected with NC inhibitor (Figure 18).

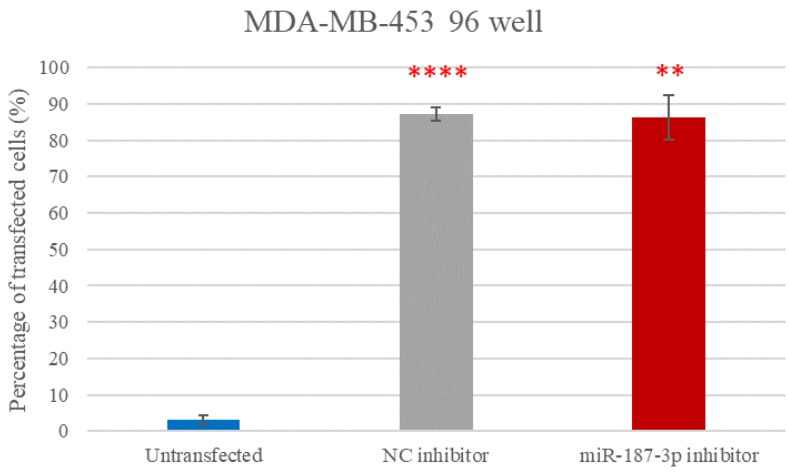


Figure 17. Transfection Efficiency measured by Flow Cytometry in MDA-MB-453 in cells seeded in 96 well plates and transfected with miR-187-3p inhibitor and its corresponding NC inhibitor.

Percentage of FAM-labelled miR-187-3p inhibitor and NC inhibitor MDA-MB-453 transfected cells as compared to un-transfected cells. Error bars represent SEM (n=3). ** Denotes $p<0.01$, **** Denotes $p<0.0001$

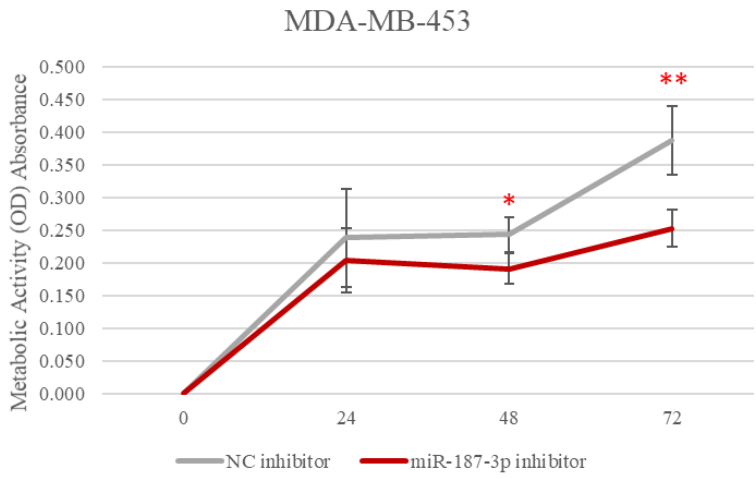


Figure 18. Proliferation of MDA-MB-453 upon the downregulation of miR-187-3p as compared NC inhibitor and NC mimic 24, 48, and 72 hours post transfection

Line graph representing metabolic activity for 72 hours of MDA-MB-453 upon the downregulation of miR-187-3p compared to NC inhibitor. Error bars represent SEM (n=3). * Denotes $p<0.05$, ** denotes $p<0.01$.

3.5. miR-187-3p promotes MDA-MB-231 Cells Proliferation

To determine the effect of miR-187-3p on MDA-MB-231 proliferation, MTT assay was done. Also, transfection efficiency of cells seeded in 96-well plates was determined by Flow Cytometry. Results show that MDA-MB-231 cells were significantly transfected with miR-187-3p mimic and its respective NC mimic (Figure 19). Upregulation of miR-187-3p in MDA-MB-231 cells increased cell proliferation significantly at 24 ($p=0.022$) and 48 ($p=0.03$) hours but not at 72 hours compared to NC mimic transfected cells (Figure 20).

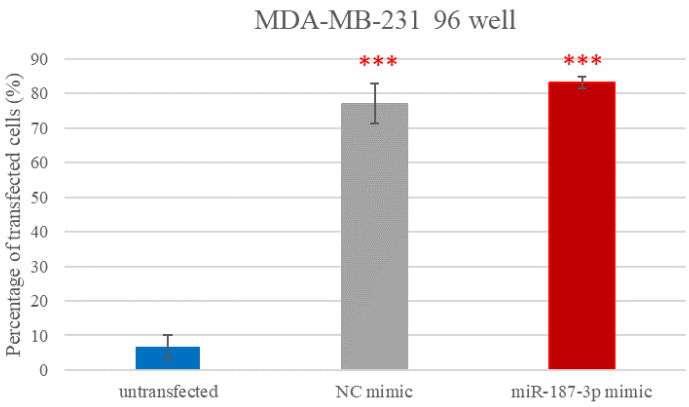


Figure 19. Transfection Efficiency measured by Flow Cytometry in MDA-MB-231 seeded in 96 well plates transfected with miR-187-3p mimic and its corresponding NC mimic.

Percentage of FAM-labelled miR-187-3p mimic and NC mimic MDA-MB-231 transfected cells as compared to un-transfected cells. Error bars represent SEM (n=3). *** Denotes $p<0.001$

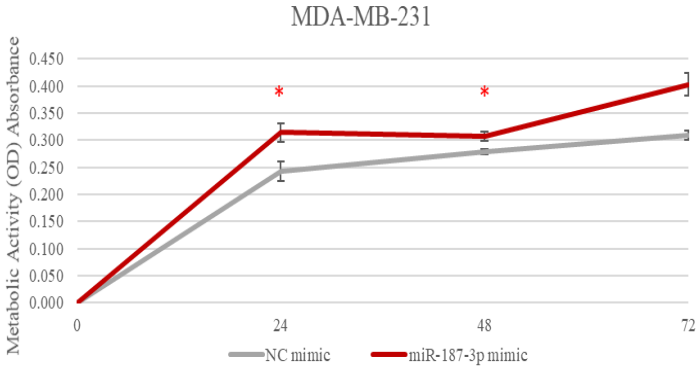


Figure 20. Proliferation of MDA-MB-231 upon the upregulation of miR-187-3p as compared NC mimic transfected cells 24, 48, and 72 hours post transfection

Line graph representing metabolic activity for 72 hours of MDA-MB-231 upon the upregulation of miR-187-3p compared to NC mimic. Error bars represent SEM (n=3). * Denotes $p<0.05$.

3.6. miR-187-3p promotes the invasive ability of MCF-10A, MDA-MB-453, and MDA-MB-231 cells.

To determine the effect of miR-187-3p on the invasive ability of MCF-10A, MDA-MB-453, and MDA-MB-231, Matrigel invasion assay was done. Upon the upregulation of miR-187-3p in MCF-10A and MDA-MB-231, the invasive ability of transfected cells increased significantly as compared to NC mimic transfected cells (Figures 21 and 22 respectively). Moreover, the downregulation of miR-187-3p in MDA-MB-453 cells decreased their invasive ability as compared to cell transfected with NC inhibitor (Figure 23).

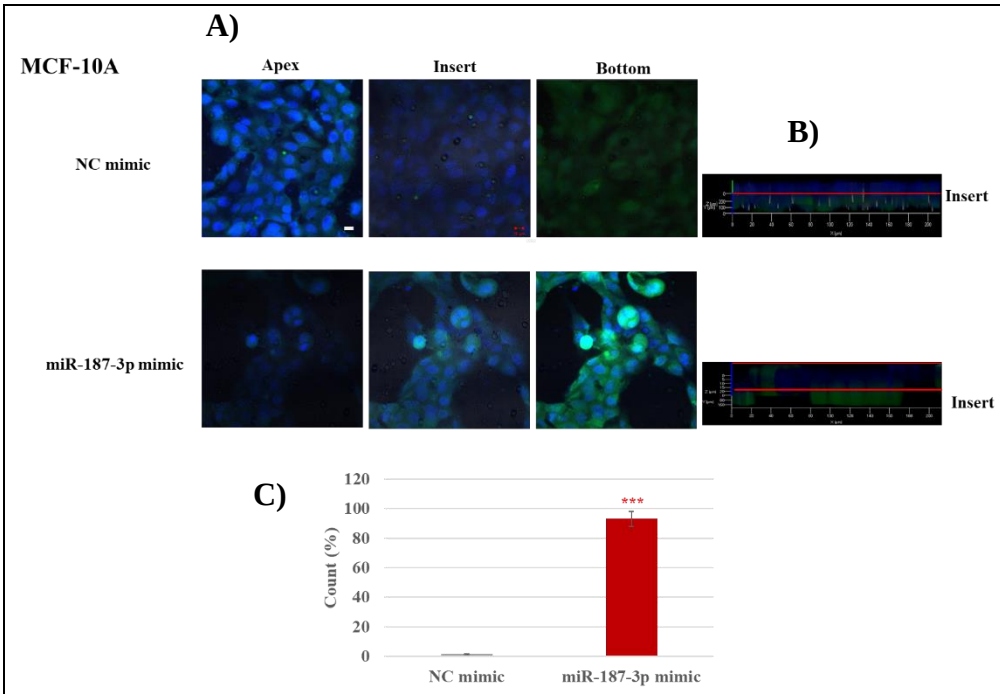


Figure 21. Invasion of MCF-10A cells upon the upregulation of miR-187-3p as compared to NC mimic transfected cells 24 hours post transfection.

(A) Representative immunofluorescence z-stack images of NC, or miR-187-3p mimic, transfected cells stained with Calcein (Green) and counterstained with DAPI (Blue). Micro-graphs are representative of at least 5 regions. Scale represents 10 µm. (B) Representative depth analysis of z-stack images reconstructed to a 3-D image using Zen software. (C) Bar graphs representing % of invading cells in NC, or miR-187-3p mimic as compared to initially seeded cells. Error bars represent SEM (n=3). *** denotes $p<0.001$

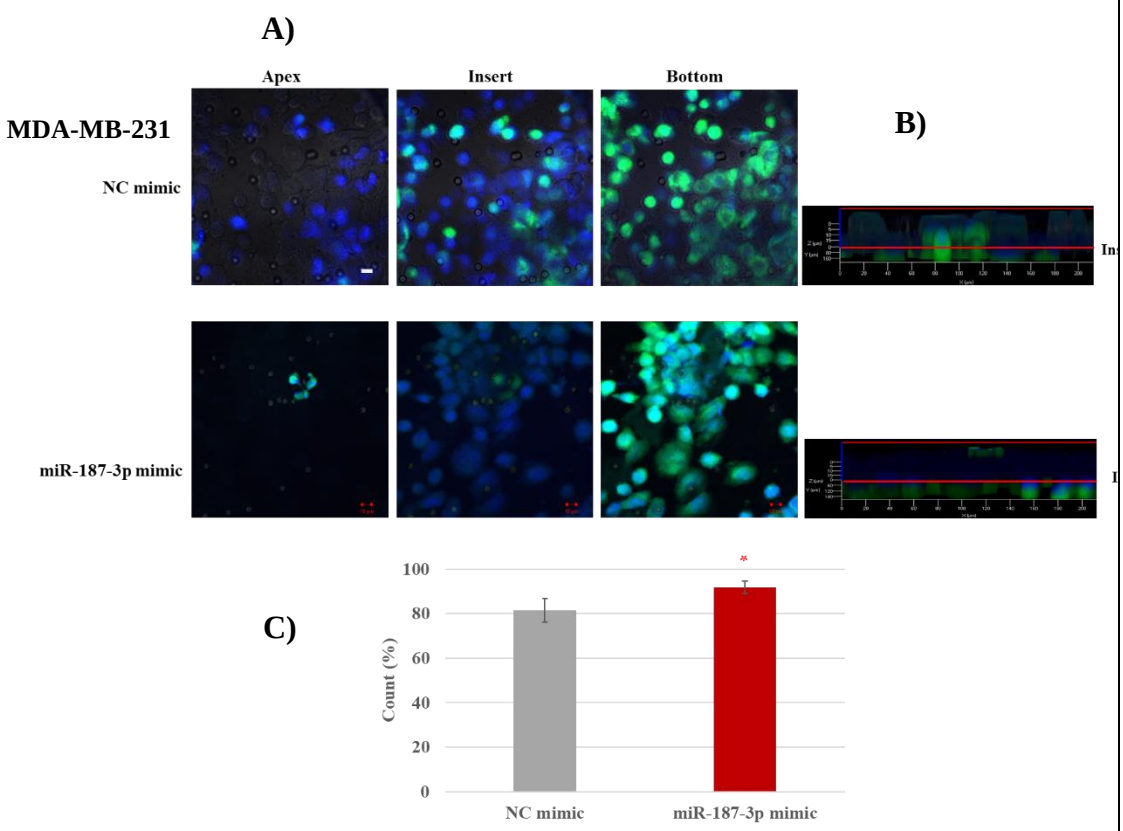


Figure 22. Invasion of MDA-MB-231 cells upon the upregulation of miR-187-3p as compared to NC mimic 24 hours post transfection.

(A) Representative immunofluorescence z-stack images of NC, or miR-187-3p mimic, transfected cells stained with Calcein (Green) and counterstained with DAPI (Blue). Micro-graphs are representative of at least 5 regions. Scale represents 10 µm. (B) Representative depth analysis of z-stack images reconstructed to a 3-D image using Zen software. (C) Bar graphs representing % of invading cells in NC, or miR-187-3p mimic as compared to initially seeded cells. Error bars represent SEM (n=3). * Denotes $p<0.05$

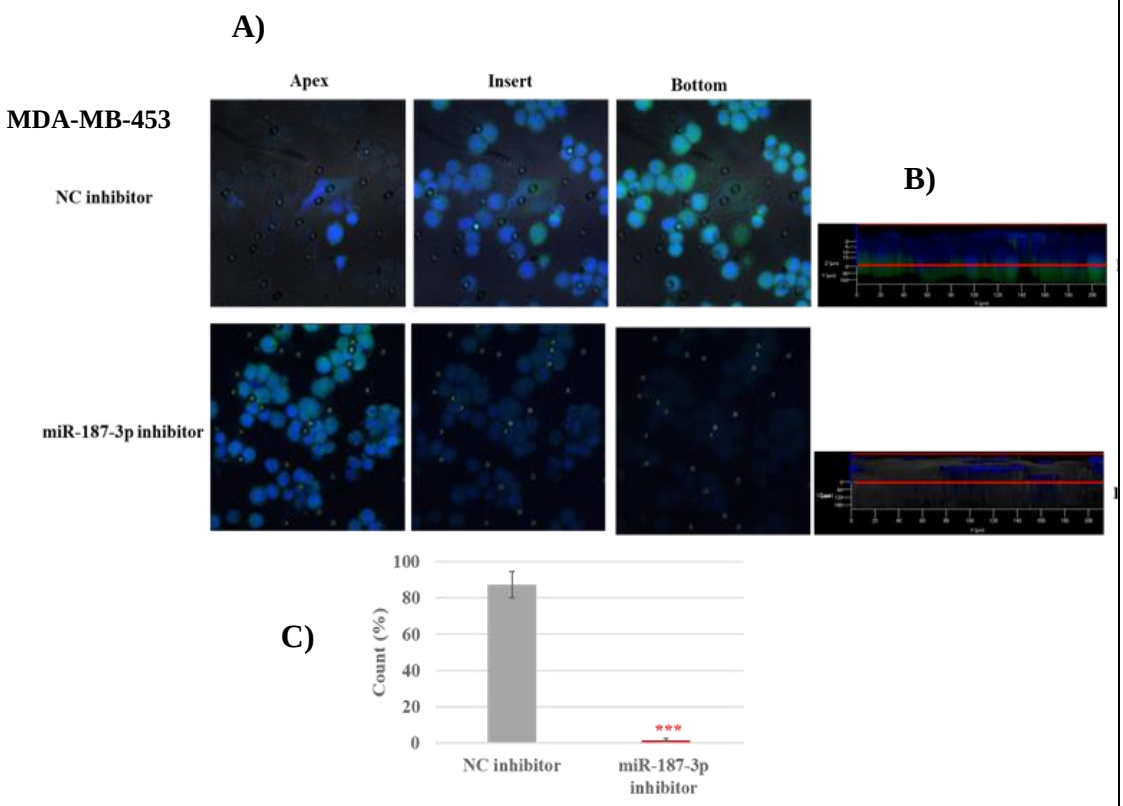


Figure 23. Invasion of MDA-MB-453 cells upon the downregulation of miR-187-3p as compared to NC inhibitor 24 hours post transfection.

(A) Representative immunofluorescence z-stack images of NC, or miR-187-3p inhibitor, transfected cells stained with Calcein (Green) and counterstained with DAPI (Blue). Micro-graphs are representative of at least 5 regions. Scale represents 10 µm. (B) Representative depth analysis of z-stack images reconstructed to a 3-D image using Zen software. (C) Bar graphs representing % of invading cells in NC, or miR-187-3p inhibitor as compared to initially seeded cells. Error bars represent SEM (n=3). *** denotes $p<0.001$

3.7. Selection of mRNA targets of miR-187-3p

miR-187-3p predicted and weakly validated targets were selected through miRtargetlink 2.0 database. PubMed search was used to determine validated targets that are mentioned in literature but not in miRNA-mRNA interactions databases. Then the expression of the targets was checked in TCGA-BRCA data. Targets whose expression

is significantly decreased in MABC samples (mApo) compared to non-MABC samples (basL) were selected (Figure 24). Then PubMed search was done to determine the role of selected targets in cancer (Table 3). As such the following targets were selected: SOX4, INNP5B, ITPR2, MBNL1, C1S, AGO1, BCL2, and CENPA.

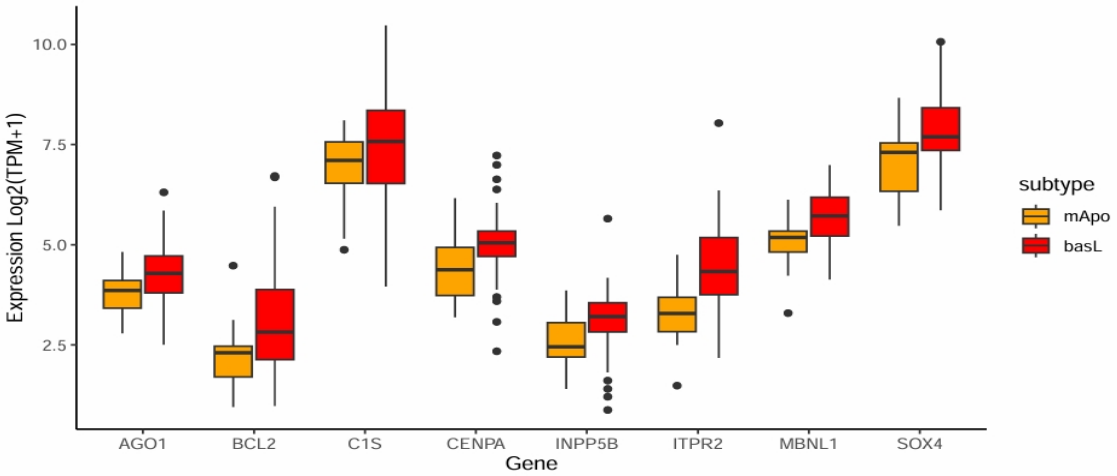


Figure 24. Differentially expressed target genes of miR-187-3p in TCGA-BRCA samples.

Box plots of the log2(TPM+1) normalized expression for the target genes were plotted using ggplot2 R package p-value<0.05 were considered as significantly dysregulated genes.

Table 3. miR-187-3p selected targets and their role in BC or other types of Cancers

Suffix	Targets	Role in BC or other cancers	Reference
3p	AGO1	promotes proliferation, migration, and invasion of lung cancer cells	[154]
3p	C1S	increased proliferation and viability of clear cell renal cell carcinoma	[155]
3p	MBNL1	suppresses invasion, metastasis and transendothelial migration	[156]
3p	INPP5B	inhibits the proliferation and metastasis of lung adenocarcinoma	[157]
3p	ITPR2	regulate the expressions of some cancer-related genes	[158]
3p	CENPA	promotes cell proliferation and migration of BC	[159]
NA	SOX4	increased BC cell viability, migration, invasion	[160],[161]
NA	BCL2	Better survival of patients with (BCL2+)	[162]

3.8. Expression levels of miR-187-3p targets in MDA-MB-453 cells

Primers of the selected target genes were designed by National Center for Biotechnology Information (NCBI) and checked on Primer Blast. The respective melting temperature and sequences of the primers are showed in Table 4.

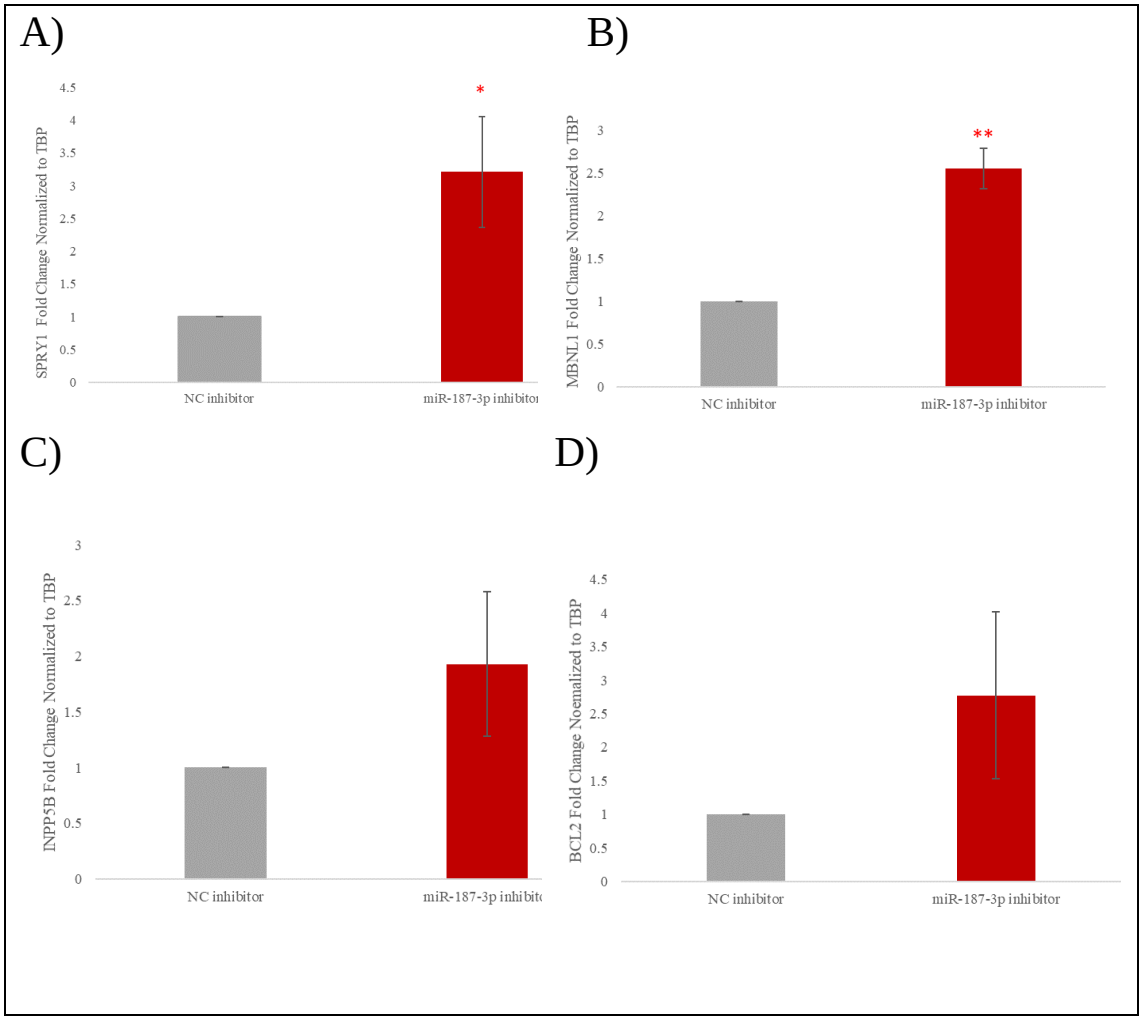
AR primer was chosen to determine its expression in the chosen cell lines. SPRY1 primer was chosen as a control to determine miRNA-mRNA interaction.

The annealing temperatures of the selected target genes were first optimized by RT-qPCR. Analysis showed that all target genes functioned at 10 µM. The optimal temperature is 60.8°C for MBNL1, CENPA, AGO1, INPP5B, ITPR2, and SOX4. 62.9°C for SPRY1, 63.4°C for BCL2, 63.9°C for C1S and 63.1°C for AR.

Table 4. The Sequence and Melting Temperature (Tm) of Primers of TBP, SPRY1, AR, and Predicted miR-187-3p targets Designed on NCBI

Gene	Primer	Sequence	Tm (°C)	Optimized Tm (°C)
TBP	TBP-F	5'-TGTATCCACAGTGAATCTTGGTTG-3'	61.9	60
	TBP-R	5'-GGTTCGTGGCTCTCTTATCCTC-3'	63.7	
AGO1	AGO1-F	5'-ACAGTGTCGAGAAGAGGTGCTC-3'	63.4	60.8
	AGO1-R	5'-GAGTAGGTGTTCTTGAGATBCCG-3'	62.1	
INPP5B	INPP5B-F	5'-ATGATACGGGCTCTGACGACTG-3'	63.2	60.8
	INPP5B-R	5'-CATGTGGCTCTGGTAACTCAGC-3'	62.3	
ITPR2	ITPR2-F	5'-GCACCTTGGGGTTAGTGGAT-3'	62.9	60.8
	ITPR2-R	5'-GTGTGGTTCCCTTGTTTGGC-3'	62.6	
CENPA	CENPA-F	5'-CACTCGTGGTGTGGACTTCA-3'	62.6	60.8
	CENPA-R	5'-GCCAGTTGCACATCCTTTGG-3'	62	
C1S	C1S-F	5'-GGAGAGAGGGAACTGACCCA-3'	66.1	63.9
	C1S-R	5'- CTGCCTGTCTCTTGGTCCTG-3'	63.9	
SOX4	SOX4-F	5'- CCCAGCAAGAAGGCGAGTTA-3'	62.3	60.8
	SOX4-R	5'- CATCGGCCAAATTCGTCACC-3'	62	
MBNL1	MBNL1-F	5'-CCCATGACAAGGAGCTGACA-3'	62.8	60.8
	MBNL1-R	5'- GAGACGTCAGGAAAGGCACT-3'	62.1	
BCL2	BCL2-F	5'- CATGTGTGTGGAGAGCGTCA-3'	62.1	63.4
	BCL2-R	5'- AGCCCAGACTCACATCACCA-3'	63.9	
SPRY1	SPRY1-F	5'-GAAAGAGGACCTGACACAGCAC-3'	63	62.9
	SPRY1-R	5'-CTCTCAGCAGAGCAAAGGCACT-3'	63.6	
AR	AR-F	5'-ATGGTGAGCAGAGTGCCCTATC-3'	59	63.1
	AR-R	5'- ATGGTCCCTGGCAGTCTCCAAA-3'	60.2	

To discover if miR-187-3p targets SOX4, INNP5B, ITPR2, MBNL1, C1S, AGO1, BCL2, and CENPA, RT-qPCR was done on transfected cells harvested 24 hours post transfection using TBP as an endogenous control. A previously validated target SPRY1 of miR-187-3p was used as a control to validate miRNA-mRNA interaction [145]. Upregulation of SPRY1, AGO1 and MBNL1 expression was observed in MDA-MB-453 cells upon the downregulation of miR-187-3p when compared to NC inhibitor transfected cells. This indicates that these targets may be potential targets for miR-187-3p. Other genes had no significant change in expression upon the downregulation of miR-187-3p (Figure 25).



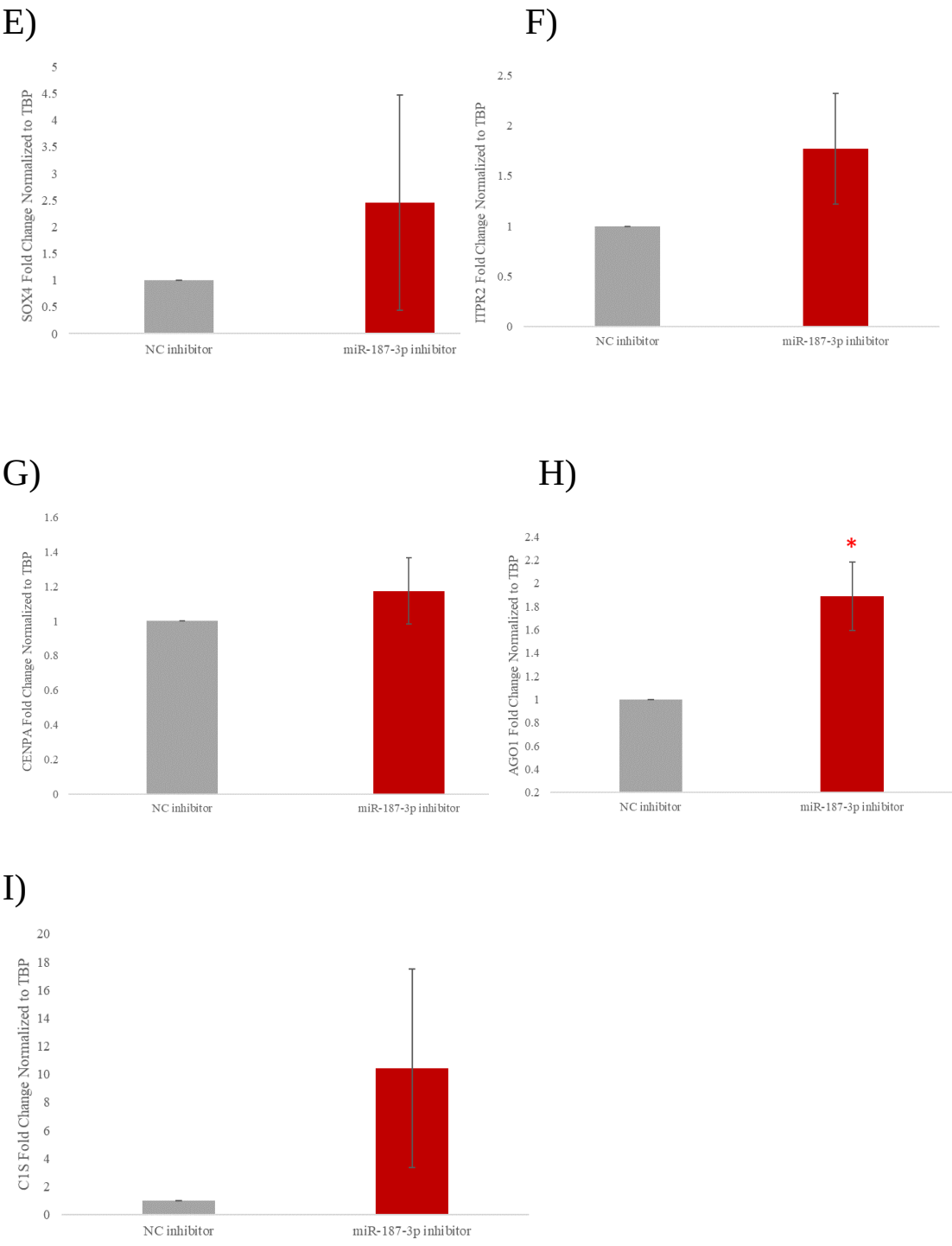
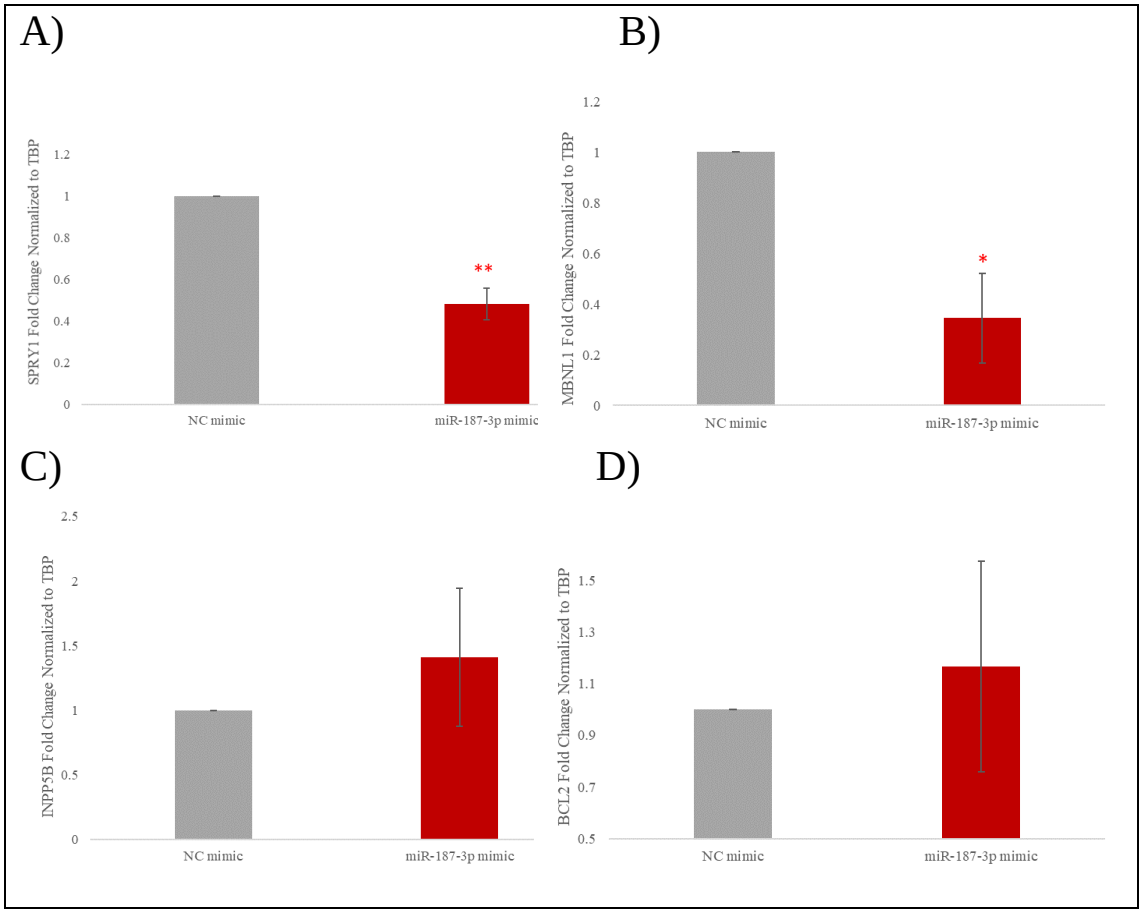


Figure 25. Expression Levels of SPRY1 and miR-187-3p potential targets upon the downregulation of miR-187-3p in MDA-MB-453 cells as compared to NC inhibitors transfected cells, 24 hours post transfection by RT-qPCR.

(A) SPRY1, (B) MBNL1, (C) INPP5B, (D) BCL2, (E) SOX4, (F) ITPR2, (G) CENPA, (H) AGO1, (I) C1S. Error bars represent SEM (n=3). * Denotes $p<0.05$, ** Denotes $p<0.01$

3.9 Expression levels of miR-187-3p targets in MDA-MB-231 cells

To discover if miR-187-3p targets SOX4, INNP5B, ITPR2, MBNL1, C1S, AGO1, BCL2, and CENPA, RT-qPCR was done on transfected cells with miR-187-3p mimics and NC mimic transfected cells harvested 24 hours post transfection using TBP as an endogenous control. A previously validated target SPRY1 of miR-187-3p was used as a control to validate miRNA-mRNA interaction [145]. Downregulation of SPRY1, AGO1 and MBNL1 expression was observed in MDA-MB-231 cells upon the upregulation of miR-187-3p when compared to NC mimic. This indicates that these targets may be potential targets for miR-187-3p. Other genes had no significant change in expression upon the downregulation of miR-187-3p (Figure 26).



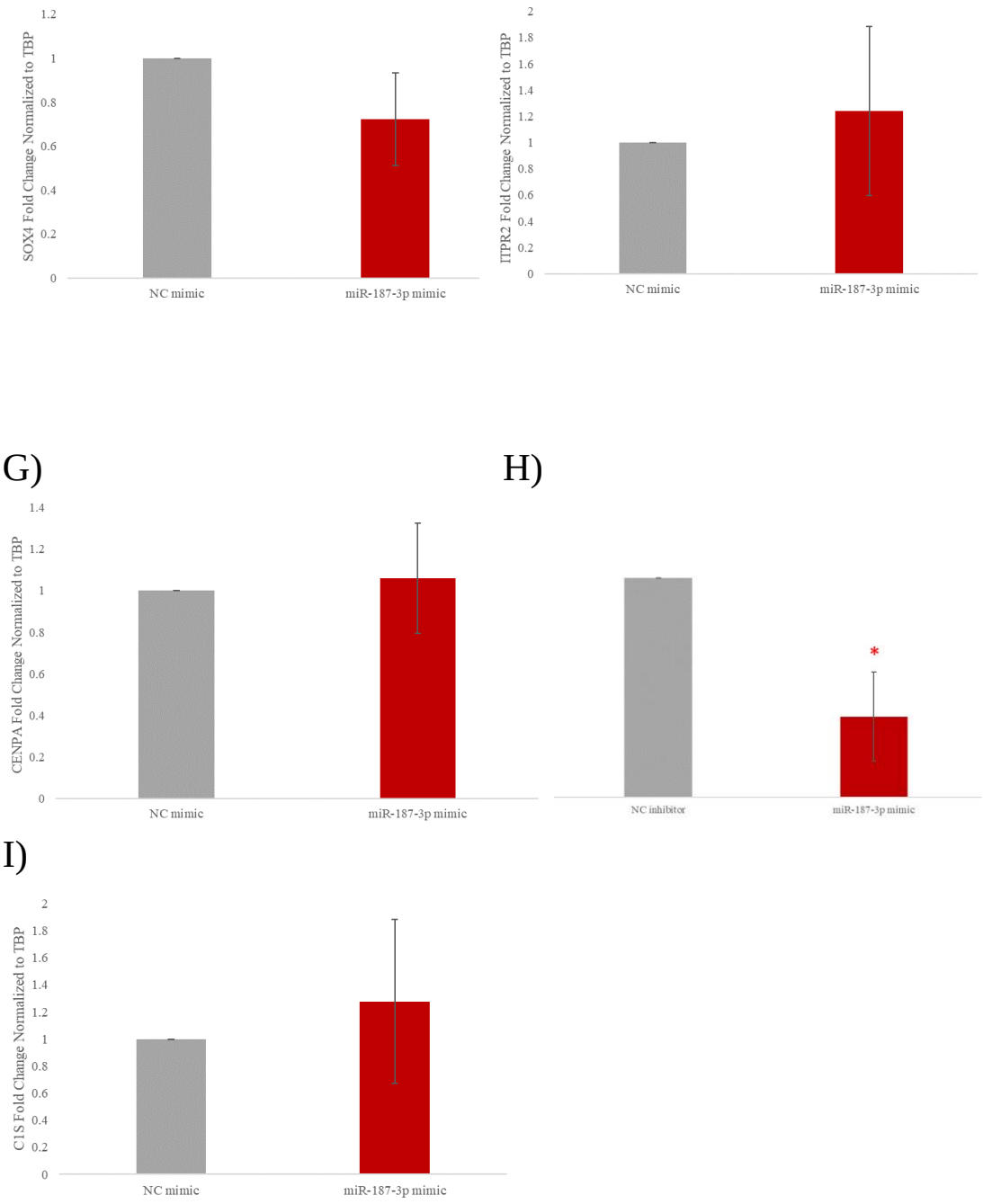


Figure 26. Expression Levels of SPRY1 and miR-187-3p potential targets upon the upregulation of miR-187-3p in MDA-MB-231 cells as compared to NC mimic, 24 hours post transfection by RT-qPCR.

(A) SPRY1, (B) MBNL1, (C) INPP5B, (D) BCL2, (E) SOX4, (F) ITPR2, (G) CENPA, (H) AGO1, (I) C1S. Error bars represent SEM (n=3). * Denotes $p<0.05$, ** Denotes $p<0$.

CHAPTER 4

DISCUSSION

BC is the most common and deadliest cancer among females worldwide and in Lebanon [1]. It has been difficult to diagnose and treat BC cases due to its heterogeneity. Several studies tried to better classify BC, especially the ER- tumors. This led to the identification of MABC and LAR tumors, which overlap [43] [46] [49]. Therefore, novel biomarkers could serve as a tool to classify MABC as a distinct entity. Recent studies proved that several miRNAs are dysregulated in cancer and act as oncomiRs or tumor suppressor miRNAs. In BC, miRNAs play a critical role in carcinogenesis by altering several pathways involved in cancer initiation and/or progression like apoptosis, angiogenesis, metastasis, and other pathways and were shown to potentially classify BC into the different molecular subtypes [123, 125]. Therefore, our lab is interested in using miRNAs as diagnostic tool to classify MABC from non-MABC tumors thus yielding better prognostic outcomes. Therefore, based on publicly available data, an in-silico analysis revealed miR-187-3p as upregulated in MABC samples, compared to non-MABC samples. This was further validated in a cohort of French ER- tumor samples. miR-187-3p was reported in literature as a tumor suppressor miRNA based on its role in several types of cancers. miR-187-3p was also reported to be downregulated in BC tumor samples compared to non-tumor samples. Also, its role was proved in reducing cell viability and promoting apoptosis of MDA-MB-231 cells [149]. However, another study corelated miR-187-3p with aggressiveness of MCF-7 cells [142, 150]. Knowing that miRNAs are tissue and context specific, we wanted to investigate the role of miR-187-3p in MABC.

We started our study by checking BC cellular models that resemble MABC and non-MABC. Therefore, we determined the expression of AR and confirmed that it is overexpressed in MDA-MB-453 compared to MDA-MB-231 and MCF-10A cell lines. These results were in accordance with the description of cell lines provided by ATCC (Table 1) [163]. Then we checked the basal expression of miR-187-3p in MDA-MB-453 and MDA-MB-231 and compared them to MCF-10A cell line. Results proved the upregulation of miR-187-3p in MDA-MB-453 as compared to MCF-10A and MDA-MB-231, and this is in accordance with our findings in BC patients. This led to the selection of MDA-MB-453 as a MABC model and MDA-MB-231 as a non-MABC model.

After choosing our cellular models, we decided to modulate the expression of miR-187-3p by transfecting the models with either mimic or inhibitors. Therefore, MDA-MB-453 was transfected with miR-187-3p inhibitor while MDA-MB-231 was transfected with miR-187-3p mimic. Flow cytometric results showed that cells were significantly transfected with mimics or inhibitors and their respective NC mimics or inhibitors. Also, transfection validation was done by RT-qPCR. miR-187-3p was overexpressed or downregulated in cells transfected with mimic or inhibitor compared to cells transfected with NC mimic or NC inhibitor respectively.

Considering the functional assays, we did MTT assay to determine the effect of modulated expression of miR-187-3p on the cells' proliferative ability. After confirming the transfection efficiency, upon the downregulation of miR-187-3p in MDA-MB-453, we found that the proliferative ability of cells was reduced compared to NC inhibitor transfected cells. Our group was the first to determine the effect of miR-187-3p on the proliferative ability of MDA-MB-453 cells. Regarding MDA-MB-231

cells, the upregulation of miR-187-3p increased cells' proliferative ability. However, by checking literature review, our results contrasted with the results of a study that concluded that miR-187-3p reduced cell viability after 100 hours using 50nM concentration of mimics and promoted apoptosis upon its upregulation in MDA-MB-231 cells [149].

To determine the effect of modulated expression of miR-187-3p on the invasive ability of BC cell, Matrigel invasion assay was done. Knowing that both MDA-MB-231 and MDA-MB-453 are invasive, we did the assay also on MCF-10A cell line. The upregulation of miR-187-3p in MCF-10A cells induced an invasive ability to the cells. Moreover, its downregulation significantly reduced the invasive ability of MDA-MB-453 cells compared to NC inhibitor. Although MDA-MB-231 cells have a high invasive ability, upon the upregulation of miR-187-3p, the invasive ability increased significantly as compared to NC mimic. Moreover, according to a study comparing monolayer (2D model) and multicellular layer (3D model) cellular models, miR-187-3p expression was correlated with inducing invasive ability of MCF-7 BC cells [150]. However, a study done on HCC *in vitro* cellular models, reported miR-187-3p as an invasion suppressor. Also, they validated their result in an *in vivo* model, revealing miR-187-3p as an anti-metastatic miRNA [144]. Other studies done on PCa and CRC *in vitro* cellular models also concluded that miR-187-3p represses cells' invasive ability [44, 145]. Therefore, we conclude that miR-187-3p exerts different invasive role on different types of cancer.

To determine possible miRNA-mRNA interaction between miR-187-3p and its selected potential targets, we first selected targets using miRtargetlink 2.0 tool and

PubMed search. Then we checked their expression in TCGA-BRCA data to select targets downregulated in MABC tumors compared to TNBC tumors.

Finally, we checked the effect of modulating miR-187-3p expression on the expression of selected targets. We first determined the expression of SPRY1, a validated target of miR-187-3p, in our cellular models [145]. SPRY1 was overexpressed upon the downregulation miR-187-3p in MDA-MB-453 cells as compared to NC inhibitor. In addition, a decreased expression of SPRY1 was detected upon the upregulation of miR-187-3p in MDA-MB-231 cell compared to NC mimic. Therefore, we proved the presence of an inverse correlation between miR-187-3p and SPRY1 in our cellular models. Out of the selected targets that we studied, MBNL1 and AGO1 had an overexpression upon the downregulation of miR-187-3p and a reduced expression upon its upregulation showing an inverse correlation between miR-187-3p levels and these two genes, thus suggesting potential miRNA-mRNA interaction. These results are in accordance with the in-silico analysis done on TCGA-BRCA that determined an inverse correlation between miR-187-3p and the two genes. Other targets had no significant change in both conditions. Results are summarized in Figure 27.

MBNL1 (Muscleblind-like 1) is a gene coding for an RNA-binding protein that regulates RNA alternative splicing, localization, and integrity. It has been proved that MBNL1 has a critical role in regulating alternative splicing in the fetal to adult heart transition. Also, it is important for the differentiation process of the brain and skeletal muscles. Downregulation of MBNL1 may result in myotonic dystrophy [164, 165]. Considering its role in cancer, it has been reported that MBNL1 is overexpressed in esophageal cancer, associated with poor prognosis, and promotes migration [166]. However, overexpression of MBNL1 in Skin Squamous Cell Carcinoma suppressed cell

growth, metastasis, and migration. Moreover, overexpression of MBNL1 in *in vivo* BC models reduced the metastatic ability of cancerous cells. Furthermore, knockdown of MBNL1 from MDA-MB-231 cells, significantly increased their invasive ability but had no effect on their proliferative ability. This tumor suppressive role is accomplished by the binding of MBNL1 protein to the 3'UTR two tumor suppressive proteins DBNL1 and TACC1, thus enhancing their stability. To support these findings, analysis of MBNL1 expression in breast tumor samples was performed. Results confirmed a significant association between the expression of MBNL1 and metastasis-free survival [167]. No association between MBNL1 and miR-187-3p was reported in literature. However, our lab results proved possible miRNA-mRNA interaction between miR-187-3p and MBNL1. In addition, upregulation of miR-187-3p which led to decrease in MBNL1 level, promoted the invasive ability of MDA-MB-231 cells. We conclude that our lab findings are in accordance with literature where we suggest that miR-187-3p increases the invasive ability of MDA-MB-231 by targeting MBNL1. Also, the downregulation of miR-187-3p decreased the invasive ability of MDA-MB-453 cells potentially due to the overexpression of MBNL1.

The other potential target is AGO1 (argonaute RISC component 1). This gene encodes a member of the argonaute family of proteins. AGO proteins bind to mature miRNA forming RISC complex that guides miRNA in its mRNA silencing process. It has been reported previously that AGO1 play a role in the transcription of oncogenic genes [168]. Moreover, its role as an oncogene or tumor suppressor gene is controversial. In lung cancer, AGO1 promoted proliferation and migration of cancer cell in both *in vivo* and *in vitro* models [169]. In addition, AGO1 activated TGF-β pathway in HCC and OC, thus promoting cancer metastatic behavior [170, 171]. However, in

neuroblastoma, AGO1 reduced cancer cells migration and proliferation [172]. In BC, patient poor survival has shown an association with low expression of AGO1 [173]. However, the role of AGO1 in BC tumorigenesis was not reported neither an interaction with miR-187-3p. Moreover, our lab results suggest AGO1 as a potential target of miR-187-3p. Also, by this interaction, miR-187-3p is increasing cell proliferation which is in accordance with the results of the study done on neuroblastoma. However, miR-187-3p silencing AGO1 increased the invasive ability of BC cell, which is in contrast with studies done on HCC and OC.

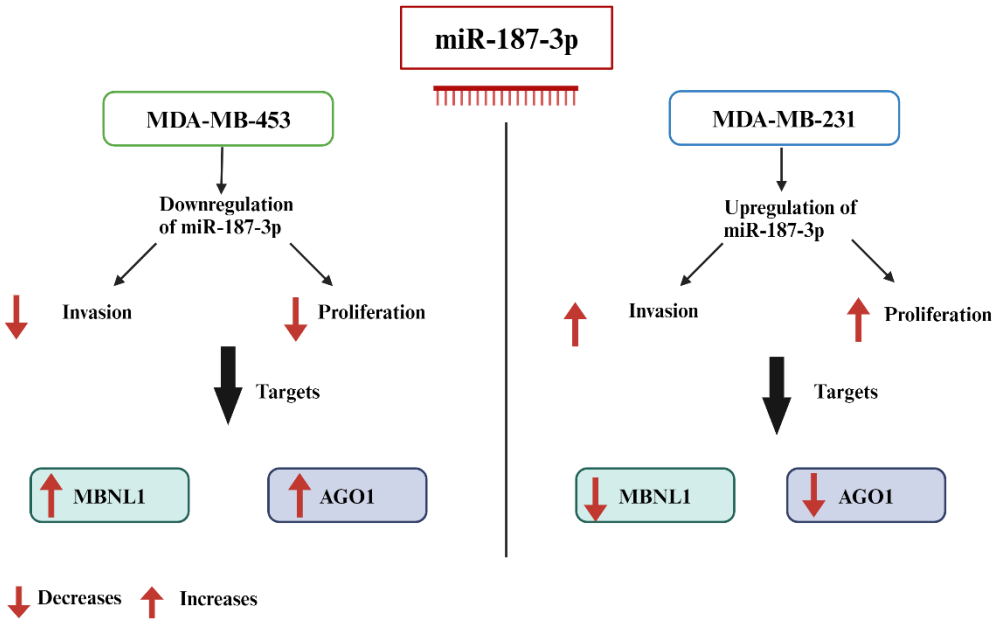


Figure 27. Results Summary

4.1. Limitations and Future Perspectives

In our experiments, cellular models were transfected transiently. In this transfection, miRNA we are transfecting are not integrated into the genome, thus they

are degraded after a short period of time. Therefore, the results we obtained represented short-term effect of miR-187-3p and not long-term effect. For later experiments, we recommend stable transfection of cell lines for us to observe long-term effect of miR-187-3p.

Considering the potential targets MBNL1 and AGO1, we determined an inverse correlation between their expression and the expression of miR-187-3p. however, RT-qPCR is not considered a validation experiment, since it does not determine direct interaction. Therefore, we aim in future experiments to validate the interaction between miR-187-3p and targets using the dual luciferase assay. Moreover, we can perform western blotting to validate the effect at the protein level and to prove if miR-187-3p is targeting these genes and inhibiting their translation. Also, we can modulate the expression of the genes in our cell lines to determine their direct effect on invasion and proliferation.

Although we did two functional assays, we didn't perform an assay to show the effect of modulating miR-187-3p on the migration ability of our cellular models. It is reported in literature that miR-187-3p has a role in the migration ability of cancerous cells. Also, the potential targets are reported as key players in the process. Therefore, its critical to perform a migration assay.

Finally, all our experiments were done on *in vitro* cellular models. Therefore, the next step for our project is to establish an *in vivo* model, that better resembles MABC disease state. Then modulate the levels of miR-187-3p in this model and determine its role in the tumorigenesis of MABC.

In conclusion, miR-187-3p plays an active role promoting the invasive and proliferative ability of MABC by targeting MBNL1 and AGO1. Further studies and experiments should be done to validate these results and prove their significance.

BIBLIOGRAPHY

1. Cancer, I.A.f.R.o. *Cancer Today*. 2022; Available from: https://gco.iarc.fr/today/en/dataviz/pie?mode=cancer&group_populations=1&sexes=2&include_nmsc_other=0&multiple_populations=1&pie_size=3&types=1.
2. Bray, F., et al., *Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA: A Cancer Journal for Clinicians. **n/a**(n/a).
3. Momenimovahed, Z. and H. Salehiniya, *Epidemiological characteristics of and risk factors for breast cancer in the world.* Breast Cancer (Dove Med Press), 2019. **11**: p. 151-164.
4. Fares, M.Y., et al., *Breast Cancer Epidemiology among Lebanese Women: An 11-Year Analysis.* Medicina, 2019. **55**(8): p. 463.
5. Shamseddine, A., et al., *Cancer incidence in postwar Lebanon: findings from the first national population-based registry, 1998∗.* Annals of Epidemiology, 2004. **14**(9): p. 663-668.
6. Sibai, A.M., et al., *Prevalence and covariates of obesity in Lebanon: findings from the first epidemiological study.* Obes Res, 2003. **11**(11): p. 1353-61.
7. Lakkis, N.A., et al., *Breast cancer in Lebanon: Incidence and comparison to regional and Western countries.* Cancer Epidemiology, 2010. **34**(3): p. 221-225.
8. Sadri, G. and H. Mahjub, *Passive or active smoking, which is more relevant to breast cancer.* Saudi Med J, 2007. **28**(2): p. 254-8.
9. El Saghir, N.S., et al., *BRCA1 and BRCA2 mutations in ethnic Lebanese Arab women with high hereditary risk breast cancer.* Oncologist, 2015. **20**(4): p. 357-64.
10. Drew, P., S. Cawthorn, and M. Michell, *Interventional Ultrasound of the Breast*. 1 ed, ed. P. Drew, S. Cawthorn, and M. Michell. 2007, Colorado: CRC Press.
11. SEER Training Modules, M.N.U.S.N.I.o.H., National Cancer Institute, *Mammary Glands.* 2023.
12. Hassiotou, F. and D. Geddes, *Anatomy of the human mammary gland: Current status of knowledge.* Clin Anat, 2013. **26**(1): p. 29-48.
13. PANDYA, S. and R.G. MOORE, *Breast Development and Anatomy.* Clinical Obstetrics and Gynecology, 2011. **54**(1): p. 91-95.
14. Macias, H. and L. Hinck, *Mammary gland development.* Wiley Interdiscip Rev Dev Biol, 2012. **1**(4): p. 533-57.
15. Javed, A. and A. Lteif, *Development of the human breast.* Semin Plast Surg, 2013. **27**(1): p. 5-12.
16. Osmosis. *Anatomy of the Breast*. 2024; Available from: https://www.osmosis.org/learn/Anatomy_of_the_breast.
17. Javed, A. and A. Lteif, *Development of the Human Breast.* Seminars in plastic surgery, 2013. **27**(1): p. 005-012.
18. Sever, R. and J.S. Brugge, *Signal transduction in cancer.* Cold Spring Harb Perspect Med, 2015. **5**(4).
19. Veronesi, U., et al., *Breast cancer.* The Lancet, 2005. **365**(9472): p. 1727-1741.
20. Polyak, K., *Breast cancer: origins and evolution.* The Journal of Clinical Investigation, 2007. **117**(11): p. 3155-3163.

21. Beral, V., et al., *Familial breast cancer: collaborative reanalysis of individual data from 52 epidemiological studies including 58 209 women with breast cancer and 101 986 women without the disease.* The Lancet (British edition), 2001. **358**(9291): p. 1389-1399.
22. Allison, K.H., *Molecular pathology of breast cancer: what a pathologist needs to know.* Am J Clin Pathol, 2012. **138**(6): p. 770-80.
23. Brevard, C.C. *Types of Breast Cancer* 2012; Available from: https://www.cancercarebrevard.com/breast-cancer/types.
24. Bertos, N.R. and M. Park, *Breast cancer - one term, many entities?* J Clin Invest, 2011. **121**(10): p. 3789-96.
25. Tsang, J.Y.S. and G.M. Tse, *Molecular Classification of Breast Cancer.* Advances in anatomic pathology, 2020. **27**(1): p. 27-35.
26. Malhotra, G.K., et al., *Histological, molecular and functional subtypes of breast cancers.* Cancer Biol Ther, 2010. **10**(10): p. 955-60.
27. Subramaniam, S., et al., *Breast Cancer Outcomes as Defined by the Estrogen Receptor, Progesterone Receptor, and Human Growth Factor Receptor-2 in a Multi-ethnic Asian Country.* World J Surg, 2015. **39**(10): p. 2450-8.
28. Russnes, H.G., et al., *Breast Cancer Molecular Stratification: From Intrinsic Subtypes to Integrative Clusters.* Am J Pathol, 2017. **187**(10): p. 2152-2162.
29. Provenzano, E.M.P.F., G.A.M.D.P. Ulaner, and S.-F.B.P. Chin, *Molecular Classification of Breast Cancer.* PET clinics, 2018. **13**(3): p. 325-338.
30. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
31. Sorlie, T., et al., *Repeated observation of breast tumor subtypes in independent gene expression data sets.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8418-23.
32. Hu, Z., et al., *The molecular portraits of breast tumors are conserved across microarray platforms.* BMC Genomics, 2006. **7**(1): p. 96.
33. Sørlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10869-74.
34. Pace, L.E. and N.L. Keating, *A systematic assessment of benefits and risks to guide breast cancer screening decisions.* Jama, 2014. **311**(13): p. 1327-35.
35. Wang, L., *Early Diagnosis of Breast Cancer.* Sensors, 2017. **17**(7): p. 1572.
36. Chatterjee, S.K. and B.R. Zetter, *Cancer biomarkers: knowing the present and predicting the future.* Future Oncol, 2005. **1**(1): p. 37-50.
37. Morris, G.J., et al., *Differences in breast carcinoma characteristics in newly diagnosed African-American and Caucasian patients: a single-institution compilation compared with the National Cancer Institute's Surveillance, Epidemiology, and End Results database.* Cancer, 2007. **110**(4): p. 876-84.
38. Haffty, B.G., et al., *Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer.* J Clin Oncol, 2006. **24**(36): p. 5652-7.
39. McDonald, E.S., et al., *Clinical Diagnosis and Management of Breast Cancer.* J Nucl Med, 2016. **57 Suppl 1**: p. 9s-16s.
40. Guo, L., et al., *Breast cancer heterogeneity and its implication in personalized precision therapy.* Experimental Hematology & Oncology, 2023. **12**(1): p. 3.

41. Mansoori, B., et al., *The Different Mechanisms of Cancer Drug Resistance: A Brief Review.* Adv Pharm Bull, 2017. **7**(3): p. 339-348.
42. Lehmann, B.D., J.A. Pietenpol, and A.R. Tan, *Triple-Negative Breast Cancer: Molecular Subtypes and New Targets for Therapy.* American Society of Clinical Oncology Educational Book, 2015(35): p. e31-e39.
43. Guedj, M., et al., *A refined molecular taxonomy of breast cancer.* Oncogene, 2012. **31**(9): p. 1196-1206.
44. Chamandi, G., et al., *ER Negative Breast Cancer and miRNA: There Is More to Decipher Than What the Pathologist Can See!* Biomedicines, 2023. **11**(8): p. 2300.
45. Lehmann, B.D., et al., *Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies.* J Clin Invest, 2011. **121**(7): p. 2750-67.
46. Lehmann, B.D., et al., *Refinement of Triple-Negative Breast Cancer Molecular Subtypes: Implications for Neoadjuvant Chemotherapy Selection.* PLOS ONE, 2016. **11**(6): p. e0157368.
47. Vranic, S., R. Feldman, and Z. Gatalica, *Apocrine carcinoma of the breast: A brief update on the molecular features and targetable biomarkers.* Bosn J Basic Med Sci, 2017. **17**(1): p. 9-11.
48. Lehmann-Che, J., et al., *de The H, Bertheau P (2013) Molecular apocrine breast cancers are aggressive estrogen receptor negative tumors overexpressing either HER2 or GCDFP15.* Breast Cancer Res. **15**(3): p. R37.
49. Farmer, P., et al., *Identification of molecular apocrine breast tumours by microarray analysis.* Oncogene, 2005. **24**(29): p. 4660-4671.
50. Doane, A.S., et al., *An estrogen receptor-negative breast cancer subset characterized by a hormonally regulated transcriptional program and response to androgen.* Oncogene, 2006. **25**(28): p. 3994-4008.
51. Guo, W., et al., *HER2 status in molecular apocrine breast cancer: associations with clinical, pathological, and molecular features.* Int J Clin Exp Pathol, 2015. **8**(7): p. 8008-17.
52. Wang, J., J. Chen, and S. Sen, *MicroRNA as Biomarkers and Diagnostics.* Journal of cellular physiology, 2016. **231**(1): p. 25-30.
53. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
54. Wang, J. and S. Sen, *MicroRNA functional network in pancreatic cancer: From biology to biomarkers of disease.* Journal of biosciences, 2011. **36**(3): p. 481-491.
55. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* Cell, 1993. **75**(5): p. 843-54.
56. Wightman, B., et al., *Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during Caenorhabditis elegans development.* Genes Dev, 1991. **5**(10): p. 1813-24.
57. Rodriguez, A., et al., *Identification of mammalian microRNA host genes and transcription units.* Genome Res, 2004. **14**(10a): p. 1902-10.
58. Ha, M. and V.N. Kim, *Regulation of microRNA biogenesis.* Nature Reviews Molecular Cell Biology, 2014. **15**(8): p. 509-524.

59. O'Brien, J., et al., *Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation.* Front Endocrinol (Lausanne), 2018. **9**: p. 402.
60. Denli, A.M., et al., *Processing of primary microRNAs by the Microprocessor complex.* Nature, 2004. **432**(7014): p. 231-235.
61. Okada, C., et al., *A high-resolution structure of the pre-microRNA nuclear export machinery.* Science, 2009. **326**(5957): p. 1275-1279.
62. Zhang, H., et al., *Single processing center models for human Dicer and bacterial RNase III.* Cell, 2004. **118**(1): p. 57-68.
63. Yoda, M., et al., *ATP-dependent human RISC assembly pathways.* Nature structural & molecular biology, 2010. **17**(1): p. 17-23.
64. Ruby, J.G., C.H. Jan, and D.P. Bartel, *Intronic microRNA precursors that bypass Drosha processing.* Nature, 2007. **448**(7149): p. 83-86.
65. Babiarz, J.E., et al., *Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs.* Genes & development, 2008. **22**(20): p. 2773-2785.
66. Yang, J.-S., et al., *Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis.* Proceedings of the National Academy of Sciences, 2010. **107**(34): p. 15163-15168.
67. Park, S., I. Kang, and C. Shin, *MicroRNA clustering on the biogenesis of suboptimal microRNAs.* Applied Biological Chemistry, 2021. **64**(1): p. 51.
68. Macfarlane, L.A. and P.R. Murphy, *MicroRNA: Biogenesis, Function and Role in Cancer.* Curr Genomics, 2010. **11**(7): p. 537-61.
69. Kozomara, A. and S. Griffiths-Jones, *miRBase: annotating high confidence microRNAs using deep sequencing data.* Nucleic Acids Res, 2014. **42**(Database issue): p. D68-73.
70. Mitra, R., et al., *Concordant dysregulation of miR-5p and miR-3p arms of the same precursor microRNA may be a mechanism in inducing cell proliferation and tumorigenesis: a lung cancer study.* Rna, 2015. **21**(6): p. 1055-65.
71. Carthew, R.W. and E.J. Sontheimer, *Origins and Mechanisms of miRNAs and siRNAs.* Cell, 2009. **136**(4): p. 642-55.
72. Brodersen, P., et al., *Widespread translational inhibition by plant miRNAs and siRNAs.* Science, 2008. **320**(5880): p. 1185-90.
73. Giraldez, A.J., et al., *Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs.* Science, 2006. **312**(5770): p. 75-9.
74. Bagga, S., et al., *Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation.* Cell, 2005. **122**(4): p. 553-63.
75. Wu, L., J. Fan, and J.G. Belasco, *MicroRNAs direct rapid deadenylation of mRNA.* Proc Natl Acad Sci U S A, 2006. **103**(11): p. 4034-9.
76. Lim, L.P., et al., *Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs.* Nature, 2005. **433**(7027): p. 769-73.
77. Behm-Ansmant, I., et al., *mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes.* Genes Dev, 2006. **20**(14): p. 1885-98.
78. Tomankova, T., M. Petrek, and E. Kriegova, *Involvement of microRNAs in physiological and pathological processes in the lung.* Respiratory Research, 2010. **11**(1): p. 159.

79. Vidigal, J.A. and A. Ventura, *The biological functions of miRNAs: lessons from in vivo studies.* Trends Cell Biol, 2015. **25**(3): p. 137-47.
80. Mencía, A., et al., *Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss.* Nat Genet, 2009. **41**(5): p. 609-13.
81. Tüfekci, K.U., R.L.J. Meuwissen, and Ş. Genç, *The Role of MicroRNAs in Biological Processes*, in *miRNomics: MicroRNA Biology and Computational Analysis*, M. Yousef and J. Allmer, Editors. 2014, Humana Press: Totowa, NJ. p. 15-31.
82. Sokol, N.S., *The role of microRNAs in muscle development.* Curr Top Dev Biol, 2012. **99**: p. 59-78.
83. Peng, Y. and C.M. Croce, *The role of MicroRNAs in human cancer.* Signal Transduction and Targeted Therapy, 2016. **1**(1): p. 15004.
84. Cheson, B.D., *Chronic lymphoid leukemias*. 2001: CRC Press.
85. Cimmino, A., et al., *miR-15 and miR-16 induce apoptosis by targeting BCL2.* Proc Natl Acad Sci U S A, 2005. **102**(39): p. 13944-9.
86. Calin, G.A., et al., *MiR-15a and miR-16-1 cluster functions in human leukemia.* Proceedings of the National Academy of Sciences, 2008. **105**(13): p. 5166-5171.
87. Hayashita, Y., et al., *A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation.* Cancer Res, 2005. **65**(21): p. 9628-32.
88. Tagawa, H. and M. Seto, *A microRNA cluster as a target of genomic amplification in malignant lymphoma.* Leukemia, 2005. **19**(11): p. 2013-2016.
89. Zhang, B., et al., *microRNAs as oncogenes and tumor suppressors.* Developmental Biology, 2007. **302**(1): p. 1-12.
90. Takamizawa, J., et al., *Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival.* Cancer Res, 2004. **64**(11): p. 3753-6.
91. Johnson, S.M., S.-Y. Lin, and F.J. Slack, *The time of appearance of the C. elegans let-7 microRNA is transcriptionally controlled utilizing a temporal regulatory element in its promoter.* Developmental Biology, 2003. **259**(2): p. 364-379.
92. Lewis, B.P., et al., *Prediction of mammalian microRNA targets.* Cell, 2003. **115**(7): p. 787-98.
93. He, H., et al., *The role of microRNA genes in papillary thyroid carcinoma.* Proc Natl Acad Sci U S A, 2005. **102**(52): p. 19075-80.
94. Svoronos, A.A., D.M. Engelman, and F.J. Slack, *OncomiR or Tumor Suppressor? The Duplicity of MicroRNAs in Cancer.* Cancer Res, 2016. **76**(13): p. 3666-70.
95. Shaham, L., et al., *MiR-125 in normal and malignant hematopoiesis.* Leukemia, 2012. **26**(9): p. 2011-8.
96. Sun, Y.-M., K.-Y. Lin, and Y.-Q. Chen, *Diverse functions of miR-125 family in different cell contexts.* Journal of Hematology & Oncology, 2013. **6**(1): p. 6.
97. Roberts, A.W., et al., *Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease.* J Clin Oncol, 2012. **30**(5): p. 488-96.

98. Tili, E., et al., *The down-regulation of miR-125b in chronic lymphocytic leukemias leads to metabolic adaptation of cells to a transformed state.* Blood, 2012. **120**(13): p. 2631-8.
99. Kong, W., et al., *Upregulation of miRNA-155 promotes tumour angiogenesis by targeting VHL and is associated with poor prognosis and triple-negative breast cancer.* Oncogene, 2014. **33**(6): p. 679-89.
100. Babar, I.A., et al., *Nanoparticle-based therapy in an in vivo microRNA-155 (miR-155)-dependent mouse model of lymphoma.* Proc Natl Acad Sci U S A, 2012. **109**(26): p. E1695-704.
101. Levati, L., et al., *MicroRNA-155 targets the SKI gene in human melanoma cell lines.* Pigment Cell Melanoma Res, 2011. **24**(3): p. 538-50.
102. Li, C.L., et al., *microRNA-155 is downregulated in gastric cancer cells and involved in cell metastasis.* Oncol Rep, 2012. **27**(6): p. 1960-6.
103. Qin, W., et al., *MicroRNA-155 is a novel suppressor of ovarian cancer-initiating cells that targets CLDN1.* FEBS Lett, 2013. **587**(9): p. 1434-9.
104. Calin, G.A., et al., *Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers.* Proceedings of the National Academy of Sciences, 2004. **101**(9): p. 2999-3004.
105. Lehmann, U., et al., *Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer.* The Journal of pathology, 2008. **214**(1): p. 17-24.
106. Selcuklu, S.D., et al., *MicroRNA-9 inhibition of cell proliferation and identification of novel miR-9 targets by transcriptome profiling in breast cancer cells.* J Biol Chem, 2012. **287**(35): p. 29516-28.
107. Wang, Y., et al., *MicroRNA-224 is up-regulated in hepatocellular carcinoma through epigenetic mechanisms.* Faseb j, 2012. **26**(7): p. 3032-41.
108. Feng, Z., et al., *Tumor suppressor p53 meets microRNAs.* J Mol Cell Biol, 2011. **3**(1): p. 44-50.
109. Sachdeva, M., et al., *p53 represses c-Myc through induction of the tumor suppressor miR-145.* Proc Natl Acad Sci U S A, 2009. **106**(9): p. 3207-12.
110. Suzuki, H.I., et al., *Modulation of microRNA processing by p53.* Nature, 2009. **460**(7254): p. 529-533.
111. Merritt, W.M., et al., *Dicer, Drosha, and outcomes in patients with ovarian cancer.* N Engl J Med, 2008. **359**(25): p. 2641-50.
112. Karube, Y., et al., *Reduced expression of Dicer associated with poor prognosis in lung cancer patients.* Cancer Sci, 2005. **96**(2): p. 111-5.
113. Iorio, M.V., et al., *MicroRNA signatures in human ovarian cancer.* Cancer Res, 2007. **67**(18): p. 8699-707.
114. Lebanony, D., et al., *Diagnostic assay based on hsa-miR-205 expression distinguishes squamous from nonsquamous non-small-cell lung carcinoma.* J Clin Oncol, 2009. **27**(12): p. 2030-7.
115. Iorio, M.V. and C.M. Croce, *MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review.* EMBO Mol Med, 2012. **4**(3): p. 143-59.
116. du Rieu, M.C., et al., *MicroRNA-21 is induced early in pancreatic ductal adenocarcinoma precursor lesions.* Clin Chem, 2010. **56**(4): p. 603-12.

117. Caramuta, S., et al., *MicroRNA expression profiles associated with mutational status and survival in malignant melanoma.* J Invest Dermatol, 2010. **130**(8): p. 2062-70.
118. Ji, J., et al., *MicroRNA expression, survival, and response to interferon in liver cancer.* N Engl J Med, 2009. **361**(15): p. 1437-47.
119. Lu, J., et al., *MicroRNA expression profiles classify human cancers.* Nature, 2005. **435**(7043): p. 834-8.
120. Volinia, S., et al., *A microRNA expression signature of human solid tumors defines cancer gene targets.* Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2257-61.
121. Iorio, M.V., et al., *MicroRNA gene expression deregulation in human breast cancer.* Cancer Res, 2005. **65**(16): p. 7065-70.
122. Andorfer, C.A., et al., *MicroRNA signatures: clinical biomarkers for the diagnosis and treatment of breast cancer.* Trends in molecular medicine, 2011. **17**(6): p. 313-319.
123. Blenkiron, C., et al., *MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype.* Genome Biology, 2007. **8**(10): p. R214.
124. Søkilde, R., et al., *Refinement of breast cancer molecular classification by miRNA expression profiles.* BMC Genomics, 2019. **20**(1): p. 503.
125. Baffa, R., et al., *MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets.* The Journal of pathology, 2009. **219**(2): p. 214-221.
126. Denkert, C., et al., *Expression of the ELAV-Like Protein HuR Is Associated with Higher Tumor Grade and Increased Cyclooxygenase-2 Expression in Human Breast Carcinoma.* Clinical Cancer Research, 2004. **10**(16): p. 5580-5586.
127. Guo, X., Y. Wu, and R.S. Hartley, *MicroRNA-125a represses cell growth by targeting HuR in breast cancer.* RNA Biol, 2009. **6**(5): p. 575-83.
128. Qi, L., et al., *Expression of miR-21 and its targets (PTEN, PDCD4, TM1) in flat epithelial atypia of the breast in relation to ductal carcinoma in situ and invasive carcinoma.* BMC Cancer, 2009. **9**: p. 163.
129. Frankel, L.B., et al., *Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells.* J Biol Chem, 2008. **283**(2): p. 1026-33.
130. Wu, H., S. Zhu, and Y.Y. Mo, *Suppression of cell growth and invasion by miR-205 in breast cancer.* Cell Res, 2009. **19**(4): p. 439-48.
131. Tavazoie, S.F., et al., *Endogenous human microRNAs that suppress breast cancer metastasis.* Nature, 2008. **451**(7175): p. 147-152.
132. Fassan, M., et al., *MicroRNA expression profiling of male breast cancer.* Breast Cancer Research, 2009. **11**(4): p. R58.
133. Thiery, J.P., et al., *Epithelial-Mesenchymal Transitions in Development and Disease.* Cell, 2009. **139**(5): p. 871-890.
134. Boyer, B., et al., *Rearrangements of desmosomal and cytoskeletal proteins during the transition from epithelial to fibroblastoid organization in cultured rat bladder carcinoma cells.* J Cell Biol, 1989. **109**(4 Pt 1): p. 1495-509.

135. Comijn, J., et al., *The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion.* Mol Cell, 2001. **7**(6): p. 1267-78.
136. Eger, A., et al., *DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells.* Oncogene, 2005. **24**(14): p. 2375-85.
137. Gregory, P.A., et al., *The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1.* Nature Cell Biology, 2008. **10**(5): p. 593-601.
138. Huber, M.A., et al., *NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression.* J Clin Invest, 2004. **114**(4): p. 569-81.
139. Fletcher, C.E., et al., *Androgen-regulated processing of the oncomir MiR-27a, which targets Prohibitin in prostate cancer.* Human Molecular Genetics, 2012. **21**(14): p. 3112-3127.
140. Shi, Y., et al., *Differential microRNA expression is associated with androgen receptor expression in breast cancer.* Mol Med Rep, 2017. **15**(1): p. 29-36.
141. Lyu, S., et al., *Androgen receptor decreases CMYC and KRAS expression by upregulating let-7a expression in ER-, PR-, AR+ breast cancer.* Int J Oncol, 2014. **44**(1): p. 229-237.
142. Peng, W., et al., *Role and mechanism of miR-187 in human cancer.* Am J Transl Res, 2020. **12**(9): p. 4873-4884.
143. Ambrozkiewicz, F., et al., *Challenges in Cancer Biomarker Discovery Exemplified by the Identification of Diagnostic MicroRNAs in Prostate Tissues.* Biomed Res Int, 2020. **2020**: p. 9086829.
144. Dou, C., et al., *miR-187-3p inhibits the metastasis and epithelial-mesenchymal transition of hepatocellular carcinoma by targeting S100A4.* Cancer Lett, 2016. **381**(2): p. 380-90.
145. Ng, L., et al., *High Levels of Tumor miR-187-3p-A Potential Tumor-Suppressor microRNA-Are Correlated with Poor Prognosis in Colorectal Cancer.* Cells, 2022. **11**(15).
146. Xiao, Y., et al., *MicroRNA-187 Inhibits Growth and Metastasis of Osteosarcoma by Downregulating S100A4.* Cancer Invest, 2018. **36**(1): p. 1-9.
147. Dou, C., et al., *miR-187-3p inhibits the metastasis and epithelial–mesenchymal transition of hepatocellular carcinoma by targeting S100A4.* Cancer Letters, 2016. **381**(2): p. 380-390.
148. Xu, W., et al., *Deciphering the role of miR-187-3p/LRFN1 axis in modulating progression, aerobic glycolysis and immune microenvironment of clear cell renal cell carcinoma.* Discover Oncology, 2022. **13**(1): p. 59.
149. Wu, Y., et al., *miR-187-3p increases gemcitabine sensitivity in breast cancer cells by targeting FGF9 expression.* Exp Ther Med, 2020. **20**(2): p. 952-960.
150. Mandujano-Tinoco, E.A., et al., *miRNA expression profile in multicellular breast cancer spheroids.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2017. **1864**(10): p. 1642-1655.
151. De Luca, A., et al., *The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches.* Expert Opin Ther Targets, 2012. **16 Suppl 2**: p. S17-27.

152. Martinez-Gutierrez, A.D., et al., *miRNA profile obtained by next-generation sequencing in metastatic breast cancer patients is able to predict the response to systemic treatments.* Int J Mol Med, 2019. **44**(4): p. 1267-1280.
153. Generali, D., et al., *Phosphorylated ERalpha, HIF-1alpha, and MAPK signaling as predictors of primary endocrine treatment response and resistance in patients with breast cancer.* J Clin Oncol, 2009. **27**(2): p. 227-34.
154. Luo, R., et al., *POU2F2 promotes the proliferation and motility of lung cancer cells by activating AGO1.* BMC Pulm Med, 2021. **21**(1): p. 117.
155. Daugan, M.V., et al., *Complement C1s and C4d as Prognostic Biomarkers in Renal Cancer: Emergence of Noncanonical Functions of C1s.* Cancer Immunol Res, 2021. **9**(8): p. 891-908.
156. Zhang, Q., et al., *The Regulatory Role of Both MBNL1 and MBNL1-AS1 in Several Common Cancers.* Curr Pharm Des, 2022. **28**(7): p. 581-585.
157. Deng, J., et al., *Decreased INPP5B expression predicts poor prognosis in lung adenocarcinoma.* Cancer Cell Int, 2022. **22**(1): p. 189.
158. Chen, J., et al., *Inositol 1,4,5-trisphosphate receptor gene variants are related to the risk of breast cancer in a Chinese population.* J Gene Med, 2023. **25**(2): p. e3463.
159. Ding, Y., et al., *LncRNA MBNL1-AS1 Represses Proliferation and Cancer Stem-Like Properties of Breast Cancer through MBNL1-AS1/ZFP36/CENPA Axis.* J Oncol, 2022. **2022**: p. 9999343.
160. Zhang, J., et al., *SOX4 promotes the growth and metastasis of breast cancer.* Cancer Cell Int, 2020. **20**: p. 468.
161. Zhang, F., et al., *MicroRNA-187, a downstream effector of TGFβ pathway, suppresses Smad-mediated epithelial-mesenchymal transition in colorectal cancer.* Cancer Lett, 2016. **373**(2): p. 203-13.
162. He, C.Y. and J. Yang, *miR-187 induces apoptosis of SiHa cervical carcinoma cells by downregulating Bcl-2.* Genet Mol Res, 2017. **16**(1).
163. ATCC. *Cell Products, Human Cells*. 2024; Available from: https://www.atcc.org/cell-products/human-cells#t=productTab&numberOfResults=24.
164. Itskovich, S.S., et al., *MBNL1 regulates essential alternative RNA splicing patterns in MLL-rearranged leukemia.* Nature Communications, 2020. **11**(1): p. 2369.
165. Konieczny, P., E. Stepniak-Konieczna, and K. Sobczak, *MBNL proteins and their target RNAs, interaction and splicing regulation.* Nucleic Acids Research, 2014. **42**(17): p. 10873-10887.
166. Liang, C., et al., *MBNL1 and MRTF-A form a positive feedback loop in regulating the migration of esophageal cancer cells.* J Cancer Res Ther, 2022. **18**(5): p. 1312-1319.
167. Fish, L., et al., *Muscleblind-like 1 suppresses breast cancer metastatic colonization and stabilizes metastasis suppressor transcripts.* Genes Dev, 2016. **30**(4): p. 386-98.
168. Huang, V., et al., *Ago1 Interacts with RNA Polymerase II and Binds to the Promoters of Actively Transcribed Genes in Human Cancer Cells.* PLOS Genetics, 2013. **9**(9): p. e1003821.

169. Luo, R., et al., *POU2F2 promotes the proliferation and motility of lung cancer cells by activating AGO1*. BMC Pulmonary Medicine, 2021. **21**(1): p. 117.
170. Wu, Y., et al., *LncRNA PVT1 promotes the progression of ovarian cancer by activating TGF-β pathway via miR-148a-3p/AGO1 axis.* J Cell Mol Med, 2021. **25**(17): p. 8229-8243.
171. Wang, M., et al., *AGO1 may influence the prognosis of hepatocellular carcinoma through TGF-β pathway.* Cell Death Dis, 2018. **9**(3): p. 324.
172. Parisi, C., et al., *Ago1 and Ago2 differentially affect cell proliferation, motility and apoptosis when overexpressed in SH-SY5Y neuroblastoma cells.* FEBS Lett, 2011. **585**(19): p. 2965-71.
173. Fawzy, M.S., et al., *The prognostic value of microRNA-biogenesis genes Argonaute 1 and 2 variants in breast cancer patients.* Am J Transl Res, 2020. **12**(5): p. 1994-2006.