IDENTIFICATION AND INVESTIGATION OF METASTASIS SPECIFIC miRNAs IN BREAST CANCER CELLS FOR miRNA REPLACEMENT THERAPY

PhD Thesis

Emilia QOMI EKENEL

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PhD THESIS

Molecular Biology Program/ Department of Biology

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Anadolu University

Graduate School of Sciences
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This thesis titled "Identification and Investigation of Metastasis Specific miRNAs in Breast Cancer Cells for miRNA Replacement Therapy" has been prepared and submitted by Emilia QOMI EKENEL in partial fulfillment of the requirements in "Anadolu University Directive on Graduate Education and Examination" for the Degree of Doctor of Philosophy (PhD) in the Molecular Biology program/Department of Biology has been examined and approved on 09/05/2019.

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ABSTRACT

IDENTIFICATION AND INVESTIGATION OF METASTASIS SPECIFIC miRNAs IN BREAST CANCER CELLS FOR miRNA REPLACEMENT THERAPY

Emilia EKENEL

Molecular Biology Program, Department of Biology
Programme in Molecular Biology
Anadolu University, Graduate School of Science, May 2019

Supervisor: Assoc. Prof. Dr. Emel ERGENE
(Co-Supervisor: Assoc. Prof. Dr. Yeşim AYDIN SON)

Recent studies have shown that miRNAs can regulate cancer and metastatic processes. The aim of this study was to identify specific metastasis suppressor miRNA through meta-analysis method, which can be used as a therapeutic target in miRNA-based breast cancer therapy to prevent or delay breast cancer metastasis. This study presented the therapeutic ability of the three identified metastasis suppressor miRNAs through in-vitro miRNA replacement therapy. The mimics of "hsa-miR-145-5p", "hsa-miR-143-3p", and "hsa-miR-99A-5p" were transfected into "MDA-MB-231" cell lines. In-vitro cell migration assay was completed for functional analysis of the effect of the replacement therapy. Additionally, since a single miRNA can regulate many genes involved in different signaling pathways, we analyzed the effect of the transfected miRNAs at the transcription level through microarray analysis. Differential expression of selected genes was validated at the protein level by western blot and zymogram. Our results suggest that each of the three metastatic suppressor miRNAs is affecting different biological pathways in tumorigenesis. Also, the therapeutic potentials of "hsa-miR-145-5p", "hsa-miR-143-3p", and "hsa-miR-99a-5p" in breast cancer are observed, showing the tumor-suppressive functionalities.

Keywords: miRNA Mimic, Breast Cancer, miRNA Transfection, Western-Blot, miRNA Replacement Therapy, Meta-Analysis, Metastasis.

ÖZET

MEME KANSER HÜCRELERINDE miRNA REPLASMAN TEDAVİSİNDE KULLANILABİLECEK METASTAİK SPESİFİK miRNA'LARIN BELİRLENMESİ VE ARAŞTIRILMASI

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Son yıllardaki çalışmalar, miRNA'nın kanser ve metastatic süreçleri düzenleyebildiğini göstermiştir. Bu çalışmanın amacı, miRNA bazlı meme kanseri tedavisinde, terapötik hedef olarak kullanılabilecek özgül metastaz baskılayıcı miRNA'ların meta-analiz yöntemi ile belirlenmiştir. Ve bu Tespit edilen üç adet özgül miRNA'ların in-vitro miRNA replasman tedavi yöntemi ile, onların terapötik potansiyelleri sunulmuştur. "Hsa-miR-145-5p", "hsa-miR-143-3p" ve "hsa-miR-99A-5p" mimikleri, MDA-MB-231 hücrelerine transfekt edildi, bu transfeksiyon etkisinin fonksiyonel analizi için, in vitro hücre göçü deneyi uygulanmıştır. Tek bir miRNA farklı sinyal yollarında işe karışan çok sayıda genin ifadesini düzenleyebildiğinden, transfekt edilen özgül miRNA'ların metastaz yolağın üzerinde etkisi ve diğer sinyal yolaklarındaki genlerin ifadelereindeki değişimi, mikrodizi yöntemi ile araştırılmıştır. Western blot, zimogram, ve mikrodizi sonuclarındaki, seçilen bazı gen ifadelerindeki değişimi, protein düzeyinde doğrulanmıştır. Sonuçlarımız, tespit edilen üç metastatik baskılayıcı miRNA'nın her birinin, tümörgenezdeki farklı biyolojik yolları etkilediğini ortaya koymaktadır. Ayrıca, meme kanserinde "hsa-miR-145-5p", "hsa-miR-143-3p" ve "hsa-miR-99A-5p'nin" terapötik potansiyelleri gözlenmiştir ve tümör baskılayıcı işlevleri göstermektedir.

Anahtar Sözcükler: miRNA Mimik, Meme Kanseri, miRNA Transfeksiyonu, Western-Blot, miRNA Replasman Tedavi, Meta-Analiz, Metastaz.

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Emilia QOMI EKENEL

09/05/2019

STATEMENT OF COMPLIANCE WITH ETHICAL PRINCIPLES AND RULES

I hereby truthfully declare that this thesis is original work prepared by me; that I have behaved in accordance with the scientific ethical principles and rules throughout the stages of preparation, data collection, analysis and presentation of my work; that I have cited the sources of all data and information that could be obtained within the scope of this study, and included these sources in the references section; and that this study has been scanned for plagiarism with "scientific plagiarism detection program" used by Anadolu University, and that "it does not have any plagiarism" whatsoever. I also declare that, if a case contrary to my declaration is detected in my work at any time, I hereby express my consent to all the ethical and legal consequences that are involved.

(Signature)

Emilia QOMI EKENEL

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LIST OF SYMBOLS AND ABBREVIATIONS

ATCC	: American type culture collection
℃	: Degree Celsius
cDNA	: Complementary DNA
Da	: Dalton, a unit of mass; kDa: kilodaltons
DMSO	: Dimethyl sulfoxide
ECM	: Extracellular matrix
EMT	: Epithelial-mesenchymal transition
FBS	: Fetal bovine serum
G	: Gram; mg : milligram ; μg : micro gram
L	: Liter; ml: milliliter; μl: microliter
M	: Molar concentration, also called molarity; mM: millimolar
miRNA	: microRNA
mRNA	: Messenger RNA
RNA	: Ribonucleic acid
SDS-PAGE	: Sodium dodecylsulphate polyacrylamide gel
siRNA	: Small interfering RNA
TBS	: Tris-Buffered Saline
TNBC	: Triple negative breast cancer
UTR	: Untranslated region

1. INTRODUCTION

MicroRNAs (miRNAs) are a class of non-coding RNAs, they are short single strands of nearly 20-24 nucleotides in length that are processed from endogenous transcripts, they play an important role in gene expression regulation, by binding (complementary or partially complementary) to a sequence in the 3'UTR region of their specific target mRNA, resulting in gene silencing, which can occur via translational repression and/or mRNA degradation.

The discovery of miRNA changes the way in which we understand gene regulation. Bioinformatics analysis results suggest that miRNAs control up to one-third of all human genes (Tétreault and Guire, 2013). MicroRNAs are currently playing a vital role in biomedical research because of its role in a wide variety of cellular processes like; embryonic development, cell proliferation, and apoptosis. The abnormal expression of miRNAs is linked to various human diseases, such as Alzheimer's disease, cardiovascular disorders, schizophrenia, and cancer.

1.1. Statement of Problem

MicroRNA and its role in cancer are supported by numerous literatures, it suggests the inhibition of oncogenic miRNAs or substitution of tumor suppressive miRNAs, which could be used to develop novel treatment strategies. Since breast cancer has the highest morbidity rate in women, miRNA-based therapy like miRNA replacement therapy can be a promising method of treatment, further investigations are needed to identify the tumor suppressive miRNAs which can be substitute in miRNA replacement therapy.

1.2. Objectives of the Study

The first objective of this study is to identify specific metastasis suppressor miRNAs through meta-analysis method, which can be used as a therapeutic target in miRNA-based breast cancer therapy (miRNA replacement therapy), to prevent or delay breast cancer metastasis. Since one miRNA can regulate many genes involved in different signaling pathways, the second objective of this study is to analyze the effect of the substitution of the three identified metastasis suppressor miRNAs, on the gene expression profile and therefore functionally analyzes its effects on the cell signaling pathways, by microarray analysis technique.

2. REVIEW OF RELATED LITERATURE AND STUDIES

2.1. MicroRNAs

MicroRNAs (miRNAs) are a class of non-coding RNAs, they are short single-strands of nearly 20-24 nucleotides in length that are processed from endogenous transcripts, play key roles in the regulation of gene expression (RNA interference). RNA interference is an endogenous gene silencing process, where cells use short non-coding RNAs to bind partially or completely to the messenger RNAs and degrade it or prevent the translation process. In addition to miRNAs, the siRNAs which is also a class of non-coding RNAs, mostly exogenous source, as an example, it could enter the cells by viruses. Both miRNA and siRNA are currently important elements in gene silencing studies. The main difference in the gene silencing mechanism between them, that siRNAs is very specific because it completely binds to its mRNA target and results in its degradation, whereas miRNAs have multiple targets, as it partially binds to its mRNA target and inhibits the translation of it (Lam, et al., 2015). The differences between siRNA and miRNA are summarized in table 2.1.

Table 2.1. *Summary of the differences between siRNA and miRNA (Lam, et. al., 2015).*

The Difference	siRNA	miRNA
Prior to Dicer processing	Double-staranded RNA	Single-stranded precursor miRNA with hairpin structure
Complementary	Fully complementary to mRNA	Partially complementary to mRNA
mRNA target	One	Multiple (could be over 100 at the same time)
Mechanism of gene regulation	cleavage of mRNA	Translational repression or cleavage of mRNA
Clinical applications	Therapeutic agent	Drug target Therapeutic agent Diagnostic and biomarker tool

The first study suggests the ability of RNA to silence gene expression was in plants, but as the mechanism was not fully understood, so it was not reported. The first two miRNAs discovered, *lin-4* (1993) and *let-7* (2000), were characterized during a study

about developmental stage transition in *C.elegans* (Almeida, Reis, Calin, 2011). In 1993 cloning of the locus revealed that *lin-4* produces a 22- nucleotide non-coding RNA, rather than a protein-coding mRNA, and that *lin-4* could negatively regulate *lin-14* gene (Ahmadzada, Reid, and McKenzie, 2018). The second miRNA was dicoverd in 2000, it has been reported that let-7 repressing the expression of the *lin-41* and *hbl-1* mRNAs by binding to their 3′ UTRs.

miRNAs have been reported in humans, animals, plants, and the viruses that infect them. To date, a numerous number of miRNAs have been identified in different organisms, and the identification of the miRNAs target genes studies recently have a central interest. Recently, the identified miRNAs target genes through computational or experimental methods have been documented in miRNA-related public databases (Du and Zamore, 2005). Bacteria also have non-coding RNAs which are known as small RNA or sRNA.

The latest version of the miRBase Sequence Database, list (2654) number of mature human miRNAs (Figure 2.1.), and this list is expanding with time. The discovered miRNAs were designated according to the rules of nomenclature which is considered long names, for example, “hsa-miR-145-5p” is a mature miRNA name, the first three letters in the name refer to the species, which is in the example “hsa”, it refers to Homo sapiens. The “miR” refers to the mature miRNA, while “mir” for pre-miRNA. 5p it means that the mature sequence comes from the 5' arm of the precursor, and when it is 3p it's from the 3' arm of the precursor (http-1).

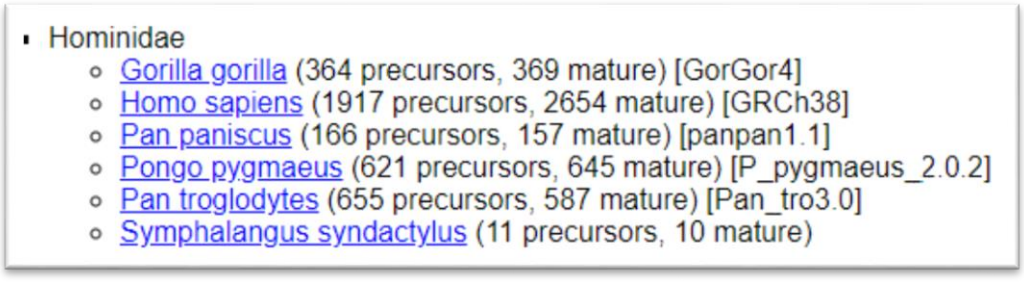


Figure 2.1. *miRBase database list (2654) number of mature human miRNAs (http-2).*

2.1.1. Biosynthesis of miRNA

About half of the known mammalian miRNAs have their own promoter, the other half are within the introns of protein-coding genes, or within either the introns or exons

of non-coding RNAs (Du and Zamore, 2005). Figure 2.2. shows the miRNAs biogenesis and their processing pathway. MicroRNA genes are transcribed by RNA polymerase II as large primary transcripts (pri-microRNA), they are processed by a protein complex containing the RNase III enzyme (Drosha) and its cofactor (Dgcr8), to form an approximately 70 nucleotides precursor microRNAs (pre-microRNA). These precursors are subsequently transported to the cytoplasm via the Exportin-5 complex, and again they are processed by a second RNase III enzyme (DICER), causes the miRNA duplex to unwound, forming the mature miRNAs of approximately 22 nucleotides. Then every mature miRNA transfers to an Argonaute (AGO) protein within the RNA-induced silencing complex (RISC), which is a ribonuclear particle, forms the RNA-induced silencing complex that mediates gene silencing. The mature single-stranded miRNA guides the RISC complex to its target mRNAs, and it hybridizes to the 3'-UTR of the target mRNAs completely or partially, resulting in translational repression and/or mRNA degradation (Zeinali, et al. 2019).

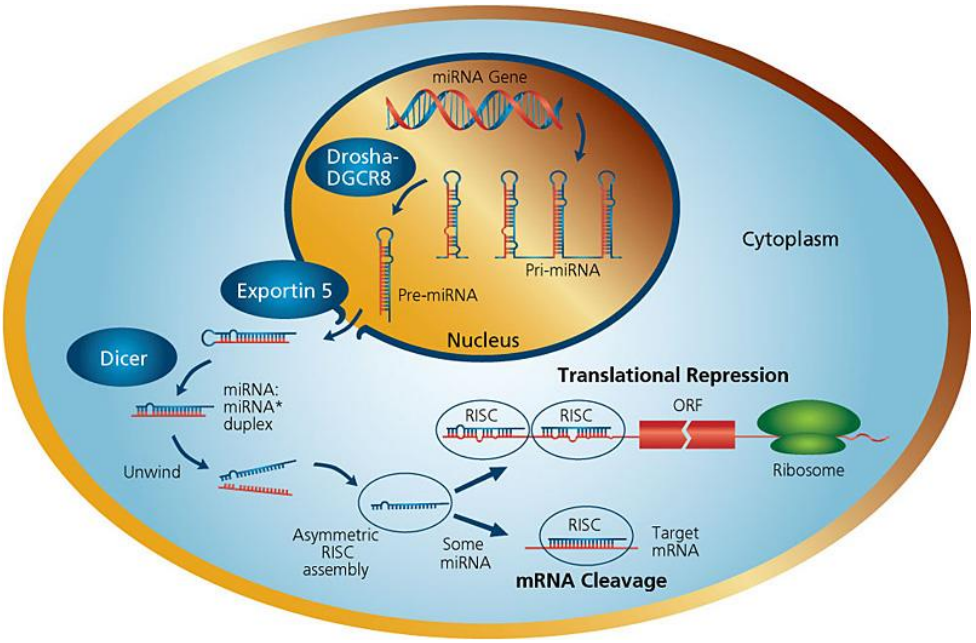


Figure 2.2. *MicroRNAs biogenesis and processing pathway (http-3).*

2.1.2. MicroRNAs and cancer

The first study suggests the role of miRNAs in cancer was in 2002, they found that miRNAs are associated with cancer by showing that the miR-15a/ 16-1 locus is

deleted or down-regulated in 68% of chronic lymphocytic leukemias (Ahmadzada, Reid, and McKenzie, 2018). Recently, miRNA microarrays and NGS next-generation sequencing techniques, have enabled researchers to analyze miRNA expression in tumors, and the role of miRNAs in cancer has been reported in almost all types of cancers. The abnormal miRNA expression can be due to deletion, amplification, mutation, or dysregulation of transcription factors that target them. Moreover, miRNAs can be controlled by epigenetic mechanisms (Almeida, Reis, Calin, 2011).

MicroRNA and its role in cancer are supported by the fact that about 50% of miRNA genes are located in a cancer-associated genomic region or in fragile sites (Abba, Patil, and Allgayet, 2014). The observed effects of the abnormal miRNA expression on tumor initiation, progression or metastasis can be explained by their mRNA targets, and the pathways they regulate.

MicroRNAs are generally classified as tumor suppressors miRNAs or oncomiRs. Tumor suppressors miRNAs are responsible for inhibiting oncogenes, and they are mostly down-regulated in cancers because of several mechanisms, such as genomic deletion, mutation, epigenetic silencing, and regulations in miRNA processing. An example of tumor suppressor miRNA is let-7, which targets many oncogenes like RAS, and myc. Another example of tumor suppressor miRNA is the miR-34 family, which targets many genes regulated cell cycle and apoptosis, such as *BCL2*, *CDK4*, and *CCND1* (Kim, et. Al., 2018), moreover, miR-15a and miR-16-1 are tumor suppressor miRNAs have been reported in chronic lymphocytic leukemia (CLL), they induced apoptosis in leukemia cells (Mollaei, Safaralizadeh, and Rostami, 2019).

OncomiRs, which have been reported to be overexpressed in several cancer studies, this overexpression could be because of gene deletion, duplication, or epigenetic regulations. Examples of oncomiRs; miR-21, which was overexpressed in most types of cancer, including lung cancer, breast cancer, ovarian cancer, glioblastoma, leukemia, and lymphoma. Also, miR-181a and miR-498 which they have been reported in breast cancer (Kim, et. Al., 2018).

Recent evidence has demonstrated that miRNA can regulate metastasis through the regulation of metastasis-associated genes. For example, miR-205 and the miR-200 family have been reported to inhibit EMT process in breast and lung cancers, also, miR-29b was inhibited EMT in breast cancer's cells. While miR-22 and miR-100 were reported to induce EMT in breast cancer (Kim, et. Al., 2018).

Cancer metastasis is a complex and multistep process this fact suggests that the only way for successful treatment might be in targeting multiple genes in different steps simultaneously, some miRNAs may have more than 100 target genes (Ibrahim, et al., 2011), this is emphasizing the therapeutic potential of the miRNAs as unique regulators of multiple target genes. In addition, miRNAs are smaller in size when compared to the protein-encoding genes. This is an advantage while developing gene therapy applications and for its systemic delivery via technologies that are also used for siRNAs. This provides a new opportunity for cancer therapy (Bader, Brown, and Winkler, 2010).

Various miRNAs were reported as promising therapeutic targets in different cancer types, for example, in breast cancer these reported therapeutic targets miRNAs where down-regulated; let-7, miR-31, miR-98, miR-205, and miR-9-3. While miR-27a, miR-96, miR182, and miR375 were up-regulated. Moreover, in prostate cancer miR-33, miR-200 and miR200b where down-regulated potential therapeutic targets. In hepatocellular carcinoma, miR-195, miR16, miR26a, miR101, and miR145 were down-regulated, while miR-18a reported as an up-regulated therapeutic target (Tan, et. Al., 2018). The therapeutic approach differs according to the expression pattern of the therapeutic targets miRNAs, it could be through the substitution of the suppressed miRNAs, like miRNA replacement therapy, or through the inhibition of the overexpressed miRNAs, by the “miRNA antagonists”; which are oligonucleotides containing the complementary sequences of the targeted miRNAs, it also called antagomir. In addition, there is another strategy which is the latest for inhibiting the miRNAs, it is called miRNA sponge therapy (Christopher, 2016). Generally, miRNAs are suppressed in tumor tissues compared to normal tissues, indicating that the probability for miRNAs to be tumor suppressors is greater than the ones of oncogenes (Bader, Brown, and Winkler, 2010). This supports the promising of miRNA replacement therapy strategy.

2.1.2.1. *MicroRNAs replacement therapy*

MicroRNA replacement therapy aims to restore the function of a miRNA in diseased cells, by inserting artificially synthesized RNA oligonucleotide that contains the same sequence as the mature endogenous miRNA, known as miRNA mimics. The substitution of the miRNA mimic in the diseased cells aims to achieve the same biological function as the naturally produced miRNA. Mimics should have the ability

to enter the RISC complex and affect the target mRNAs of the miRNA they are mimics (Zhang, Wang, and Gemeinhart, 2013).

Many studies prove the tumor suppressive effect after applying miRNA replacement therapy to cancerous cells or animals using viral vectors or non-viral delivery systems. For example, let-7 miRNAs family are one of the best-known miRNAs with tumor suppressor effect, which has been demonstrated in many types of cancers like breast, colorectal, gastric, melanoma and prostate cancers. Another example has been reported that the substitution of miR-34 using its mimic in lung cancer, triggers apoptosis by targeting different types of molecules including *p53, Bcl-2, KRAS*, and *MAPK* (Mollaei, Safaralizadeh, and Rostami, 2019). Moreover, miR-145-5p and miR-143-3p have been reported to down-regulate cancer growth and metastasis, after replacement of them in-vitro in prostate cancer cells (Pan, Jia, Li, and Dou, 2019). And also the transfection of miR-99a-5p into human urinary bladder urothelial carcinoma cells, it was demonstrated as a tumor suppressor gene, and it regulates cell growth and metastasis through inhibiting *mTOR* (Tsai, et. Al., 2018).

Some miRNAs had an opposing effect in different types of cancer. For example, miR-9 a prometastatic and proangiogenic effects were reported in breast cancer while an antimetastatic and antiangiogenic effect in neuroblastoma and in gastric cancer (Bouyssou, et al., 2014). Therefore, these two factors can help in the application of the replacement therapy for clinical approaches; the appropriate miRNA mimic, and to overcome the limitations of the available delivery systems.

2.2. Cancer and Its Molecular Mechanism

Cancer is a genetic disease characterized by uncontrolled cell growth. There are many types of cancer, it is usually named according to the organs or tissues that cancer start in, mainly there are five groups of cancer (Chang, 2018):

- Carcinoma: It is the most common type of cancer, it starts in the epithelial tissues. It includes different subtypes according to the type of epithelial cells where it begins.
- Sarcoma: In the connective or supportive tissues like bone, cartilage, and muscle.
- Leukemia: Which is cancer of blood cells.
- Lymphoma and myeloma: Cancers that form in the immune system cells like lymphocytes and plasma cells.
- Brain and spinal cord cancers: Cancer which forms in the cells of the brain or spinal cord.

The transition from normal cells to cancerous cells is a result of genetic alterations, like point mutations, gene amplification, or chromosomal rearrangement, this can be due to inherited factors, or environmental factors like radiation, chemicals, and UV light, and also can be due to some biological factors like viruses (Malarkey, and Maronpot, 2013). A single mutation doesn't cause cancer, usually, mutations can accumulate over a person's lifetime until it becomes cancerous. The mutation number of a tumor can vary between tens to thousands (Nussbaum, et.al., 2007). Mutations in three types of genes lead to cancer, the oncogenes, tumor suppressor genes, and DNA repair genes. Oncogenes are a group of genes triggers cell growth and division, like growth factors, growth factor receptors, signal transducers, transcription factors, and inhibitors of programmed cell death, through activating growth related intracellular signaling pathways (Malarkey, and Maronpot, 2013). Tumor suppressor genes are responsible for suppresses cell growth or cell division, therefore, their inactivation or loss of their function leads to uncontrolled cell growth, and usually they are components of the intracellular signaling pathways that lead to growth inhibition. An example of common tumor suppressor genes observed in many cancers; *Rb* and *p53*, which are involved in cell cycle regulation mechanism (Malarkey, and Maronpot, 2013). The DNA repair genes have important role during the cell cycle after the DNA replication, any mutation leads to failure in DNA repair, allows subsequent mutations to be

accumulated, many mutated DNA repair genes, found to be related to cancer, for example, *BRCA1*, and *BRCA2* (Product, 2018). Cancer treatment studies are focusing on targeting these genes in molecular target therapy studies for the development of anticancer drugs.

2.2.1. Breast cancer and TNBC subtype

Breast cancer which is a type of adenocarcinoma starts when cells at any part of the breast begin to grow abnormally, it is one of the most common cancer-related mortality in women. Two histological subtypes of breast cancer are the most common, their origin is epithelial tissue cancers that begin in the ducts that carry milk to the nipple (ductal cancers), the second one that starts in the glands that make breast milk (lobular cancers), milk ducts and mammary glands are shown in Figure 2.3 of the breast anatomy (American Cancer Society, 2016).

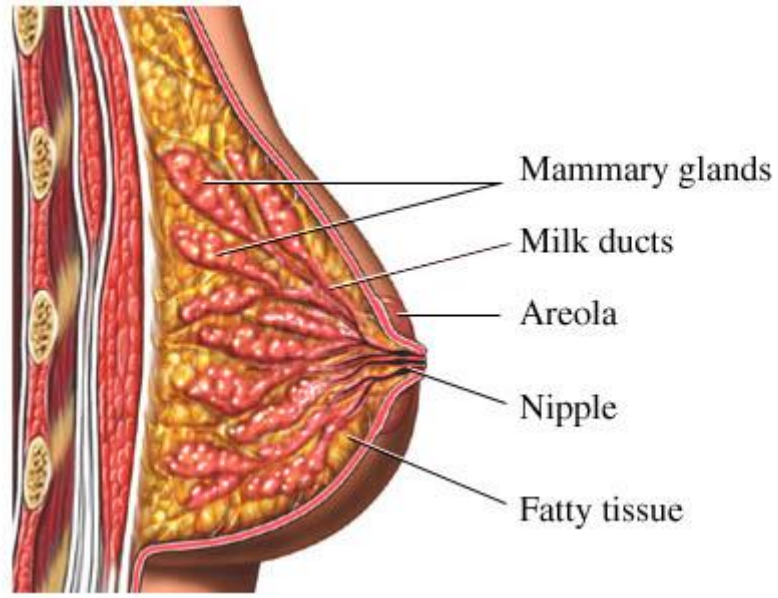


Figure 2.3. *Anatomy of the breast (http-4).*

The progress of breast cancer is incorporated with risk factors like family history, hormone, age, and the susceptibility of the person's genetic status. In terms of genetic susceptibility to breast cancer, the studies present several mutations of genes

that are related to breast cancer like, *BRCA1*, *BRCA2*, *ATM*, *PALB2*, and *CHEK2* (Tungsukruthai, Petpiroon, and Chanvorachote, 2018).

Breast cancers can also be classified according to the presence or absence of hormonal and growth receptors. Breast cancers which express estrogen receptor (ER), or progesterone receptor (PR), or human epidermal growth factor receptor 2 (HER2), are much more likely to be treated by targeting these receptors. The absence of these receptors known as triple negative breast cancers (TNBC); it spreads faster than most other types of breast cancer, and its treatment is harder because it can't be treated by targeting receptors and usually treated by chemotherapy (Ahmadzada, Reid, and McKenzie, 2018), TNBCs are classified into six different molecular subtypes, based on gene ontologies and gene expression characteristics, they are: Basal-like 1 (BL1), Basal-like 2 (BL2), immunomodulatory (IM), mesenchymal-like (M), mesenchymal stem-like (MSL), and luminal androgen receptors (LAR) subtypes (Varghese, et.al., 2018). Table 2.2 shows the molecular subtyping of TNBC based on gene ontology and gene expression characteristics.

Table 2.2. *The molecular subtyping of TNBC based on distinct gene ontology and differential gene expression (Varghese, et.al., 2018).*

Subtypes	**Characteristics (Based on Gene Ontology and Differential Gene Expression)**
Basal-like 1 (BL1)	• Enriched cell cycle components. • DNA replication reactome, G2 cell-cycle pathway, RNA polymerase and G1 to S cell cycle. • Elevated DNA damage response (ATR/BRCA) • High proliferation rate (Ki-67 mRNA). • Respond to cisplatin/ taxanes.
Basal-like 2 (BL2)	• Activated EGF pathway, NGF pathway, MET pathway. • Wnt/ β-catenin pathway, and IGF-1R. • Activated glycolysis and gluconeogenesis.
immunomodulatory (IM),	• Activated immune cell signaling (TH1/TH2 pathway, NK cell pathway, • B cell receptor [BCR] signaling pathway, DC pathway, and T cell receptor

	• signaling), • Activated cytokine signaling (IL-12 pathway, and IL-7 pathway) • Activated antigen processing and presentation • Activated immune signaling pathway (NFKB, TNF, and JAK/STAT signaling)
mesenchymal-like (M),	• Enhanced expression of genes involved in motility • Heightened ECM receptor interaction • Activated cell differentiation pathways (Wnt, TGF- β) and genes associated • with epithelial-to-mesenchymal transition (EMT).
mesenchymal stem-like (MSL)	• Low levels of proliferation genes and enriched expression of stem • cell-associated genes • Activated cell motility (Rho pathway), • Cellular differentiation, and growth pathways (ALK pathway, TGF- β) • signaling and Wnt/ β-catenin pathway • Altered genes linked to EGFR, PDGF, Calcium signaling, G-protein coupled • receptor, ERK1/2 signaling, ABC transporters and adipocytokine signaling • Enriched in genes involved in angiogenesis
luminal androgen receptors (LAR)	• Heavily enriched in hormonally regulated pathways including steroid • synthesis, porphyrin metabolism, and androgen/estrogen metabolism. • AR mRNA is highly expressed.

2.2.2. Metastasis

Metastasis is the spread of cancer to a different part of the body from a primary tumor, it may invade the nearby tissue or travel through the blood or lymph system to form a new tumor in other organs or tissues of the body and it is the main leading cause

of cancer-related deaths (Tungsukruthai, Petpiroon, and Chanvorachote, 2018). The major steps of the metastasis are shown in Figure 2.4.

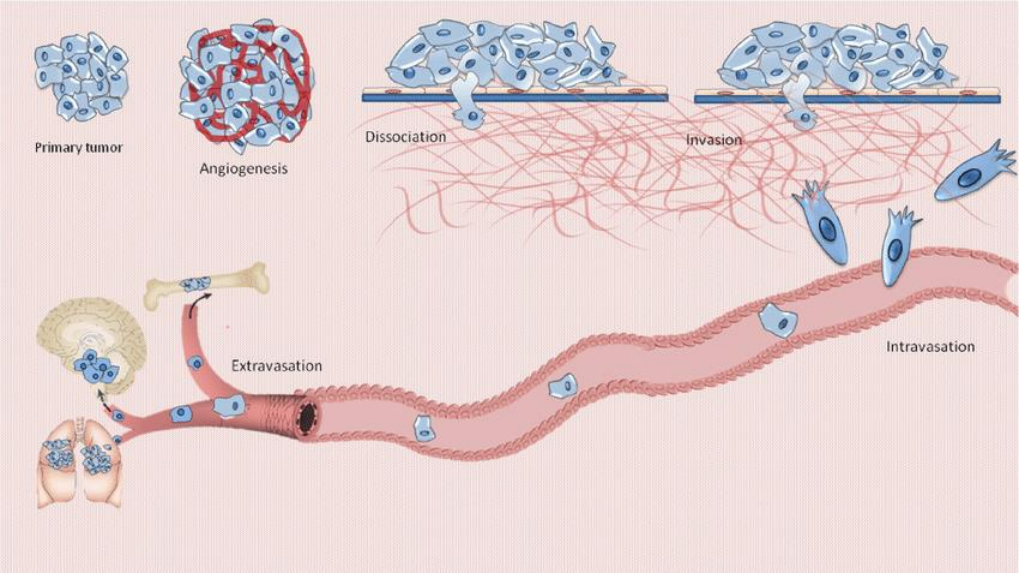


Figure 2.4. *The major steps of metastasis (Alizadeh, Shiri, and Farsinejad, 2014).*

Metastasis is a complex process that occurs through a series of steps, two major steps are needed to form a new tumor in another distant place from the primary one, they are angiogenesis and epithelial-mesenchymal transition (EMT) (Ibrahim, et al., 2011).

Angiogenesis provides the cancer cells with the nutrients and oxygen required for growth, through the formation of new blood vessels from pre-existing vessels, many molecules which are component in many signalling pathways responsible for the angiogenesis process such as, the angiogenic factors and their receptors for example, *VEGF* and *VEGFR*, *Angiopoietin-Tie*, *Ephrin-EphRs*, and *Delta-Notch*, also there are many other molecules associated with the angiogenesis directly or indirectly like, Fibroblast Growth Factor (*FGF*) and Thrombin receptors (Marson, Miller, and Dixon, 1998).

In epithelial-mesenchymal transition (EMT) step, cells go through a morphological change, acquiring mesenchymal characteristics to detach from the primary tumor, through the suppression of "E-cadherin" expression, and the loss of their tight membrane junctions, therefore, they acquire a mobile phenotype and invade

through ECM, and intravasate into the blood system, to establish a new tumor at a distant site (Xia, and Hu, 2009). When cells exiting the blood system at the target site, they go through the reverse process of mesenchymal-to-epithelial transition (MET), to retrieve their original epithelial like characteristics (Harrington, 2016). Therefore, tumor cells go through alterations in cell-cell and cell-ECM adhesion, via regulation in the adhesion receptors such as, cadherins, integrins, cell surface proteoglycans, and tetraspanins, these adhesion proteins with the extracellular ligands they transduce many signals from the ECM, to enhance cancer cell migration, Invasion, and proliferation (Thiery, 2003). Moreover, the fragmentation of the ECM, which provides paths for cancer cells to migrate, and releases the ECM bound growth factors, mainly induced by the matrix metalloproteinases (MMPs) (Oskarsson, 2013). Embryonic signaling pathways, such as Hedgehog (Hh), Notch, Wnt, and transforming growth factor (TGF)-β signaling, also other signaling pathways like, PI3K/AKT/mammalian target of rapamycin (mTOR), and MAPK signaling pathway has been reported to induce the (EMT) process in many cancers, through the regulation of many transcription factors such as; *Snail, Slug, KLF8, Twist, Foxc1, Foxc2, Zeb1,* and *Zeb2* (Takebe, Warren, and Ivy, 2011).

2.2.4. MDA-MB-231 cell line

The MDA-MB-231 cell line is a human breast cancer cell line, of epithelial cell type, that was derived from a metastatic site: pleural effusion of a 51-year-old Caucasian female with adenocarcinoma, and it is commonly used in most of the in-vitro breast cancer medical researches (http:5).

MDA-MB-321 is triple negative breast cancers (TNBC) and it is a highly aggressive cell line, its molecular subtype is mesenchymal stem cell-like (MSL), figure 2.5 shows the shape of the cultured MDA-MB-231cell line, at high and low densities. The Gene expression characteristic of this subtype is: (Varghese, et.al., 2018)

- Low levels of proliferation genes and enriched expression of stem cell-associated genes.
- Activated cell motility (Rho pathway).
- Cellular differentiation, and growth pathways (ALK pathway, TGF-β signaling and Wnt/ β-catenin pathway).
- Altered genes linked to *EGFR, PDGF*, Calcium signaling, G-protein coupled receptor, ERK1/2 signaling, ABC transporters and adipocytokine signaling.

- Enriched in genes involved in angiogenesis.

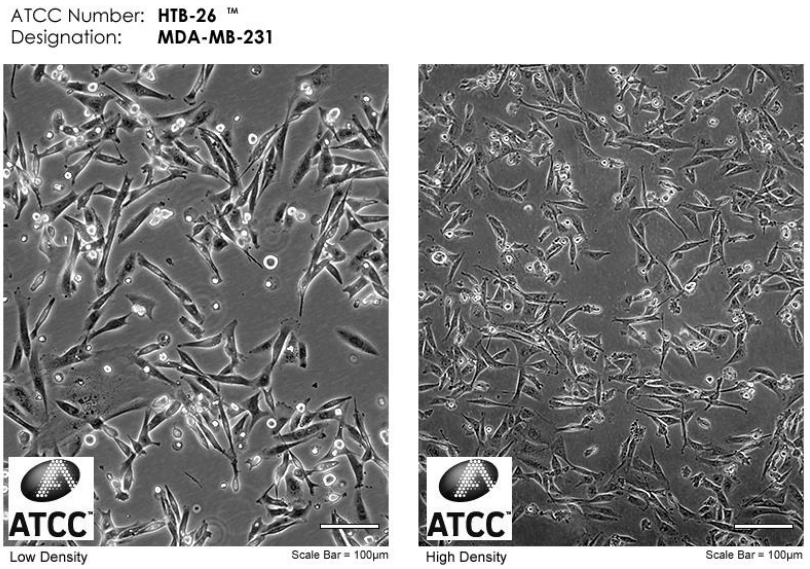


Figure 2.5. *MDA-MB-231 cell line images (http:6).*

2.3. Meta-Analysis

Meta-analysis is a statistical calculation aims to combine all available similar studies done in different times, by different researchers, different centers, and different platforms, the more sample number is, the more the statistical power will be (Oztemur, et al., 2015). Its benefit especially when studies have conflicting conclusions, because any new hypothesis cannot be considered according to a single study. Meta-analysis is widely used in many research field, it is widely used in the medical researches, mainly to assess the performance of the diagnostic tests (Ab, 2010), also it is used in many other research types, such as the pharmaceutical researches, education, economy, ecology, and genetics.

There are some issues related to the meta-analysis of gene expression studies, some are common with the traditional meta-analysis problems such as the different aims, the design and the populations between the studies. In addition, there are some concerns specific to gene expression data includes the differences between probes and probe sets of the compared studies (Anna, and Yee, 2010). The ranking-based meta-analysis method calculates the weighted mean of the interested value across all studies, which is a successful approach in eliminating the differences that originate from platform variability (Anna, and Yee, 2010). An example for a ranking-based meta-

analysis study, where breast cancer miRNA and mRNA microarray studies were collected, and then combined by a newly developed ranking-based meta-analysis approach to identify miRNA biomarkers according to their grades (Oztemur, et al., 2015).

2.4. Gene Expression Microarray

Gene expression microarray is a technique used to detect the expression of thousands of genes simultaneously. DNA microarrays are microscope solid supports that are printed with thousands of spots that contain a known gene probe. The DNA probes attached act as probes to measure gene expression levels of a known sample. Different microarray platforms are existing today, the differences between them are related to the difference in manufacturing them (spotting or in-situ synthesis), the detection type (single or dual channel microarrays), or probe types (cDNA or oligonucleotides).

The principle of gene expression microarray involves the hybridization of the mRNA of a known sample with the DNA on the microarray chip. The amount of mRNA hybridize to each probe on the array indicates the expression level of its related gene. One microarray chip determines the expression levels of thousands of genes simultaneously, aims to study the effects of certain treatments, disease, or developmental stages studies. In single channel platforms, one array per sample are used, and the gene expression level is determined by comparison of the expression regarded to a control sample. While for dual channel microarray, one array compares two samples by labeling them with two different fluorophores and the gene expression levels are determined by analyzing the different intensities between them (Qomi Ekenel, 2012). The major steps of the gene expression single and dual channel microarray experiments are shown in figure 2.6, the major steps are target preparation and labeling, target hybridization, array scanning, and data analysis.

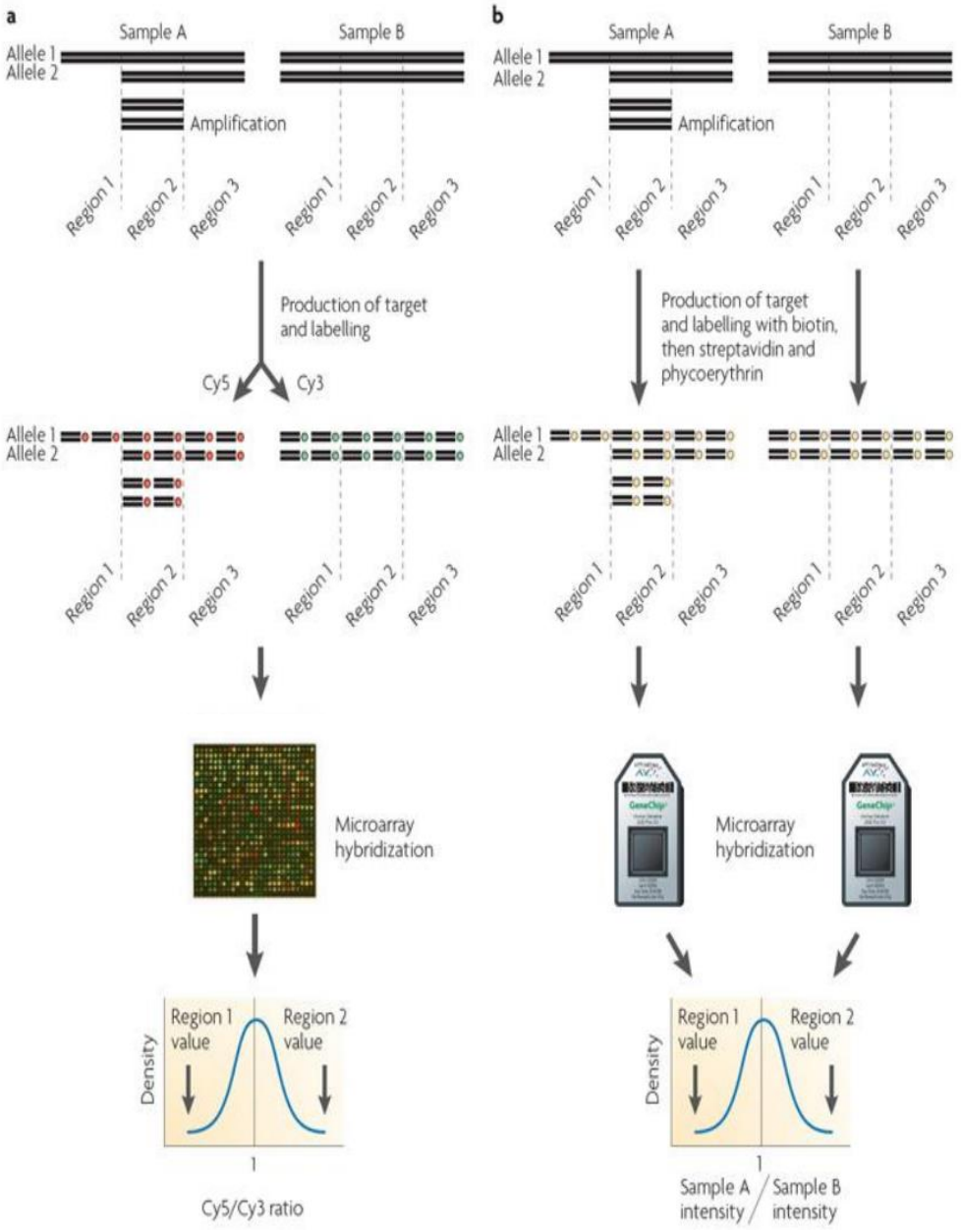


Figure 2.6. *The major steps of the gene expression single and dual channel microarray experiments (Qomi Ekenel, 2012). In the figure a: shows the major steps for the single-channel microarray experiment, and b: shows the major steps for the dual-channel microarray experiment*

Affymetrix GeneChips platform, are single channel, consist of short oligonucleotides, synthesized by the photolithography technology on the chip. Generally, GeneChips are designed with 16-20 oligonucleotides representing each gene on the array. Each gene is represented with a set of probes with a perfect match and, single base mismatch probes, this serves as a control to determine the degree of

nonspecific binding by comparison of target binding intensity between perfect match vs mismatch probes (Macgregor, and Squire, 2002).

Bioinformatics analysis of the microarray data results are needed, because of the huge volume of the data generated from a microarray experiment, the results are analyzed using statistical and mathematical methods. The major steps of the microarray Data analysis are preprocessing, Analysis, visualization of results and biological interpretation. The preprocessing step includes filtering signals, background correction, and data normalization. The analysis step is done to select the differentially expressed gene list and could be visualized by many plots and diagrams such as volcano plot, and scatter plot. In order to understand the biological functions of the selected gene set, several bioinformatics tools could be done like gene annotation, pathway enrichment analysis and network analysis.

3. RESEARCH METHODOLOGY

3.1. Meta-Analysis

In order to obtain statistically significant suppressed miRNAs list in breast cancer, meta-analysis was done. Ranking-based meta-analysis were calculated according to two features (the t-test probability (p-value), and the logFC value), additionally, two types of comparison were applied (normal breast tissue vs breast cancer) and (breast cancer vs metastatic breast cancer).

Meta-analysis major steps were: Datasets collection from GEO database and literature search, comparison between the samples of each dataset, extracting meta-list and selection of three metastasis suppressor therapeutic targets. The major steps of the meta-analysis work shown in figure 3.1.

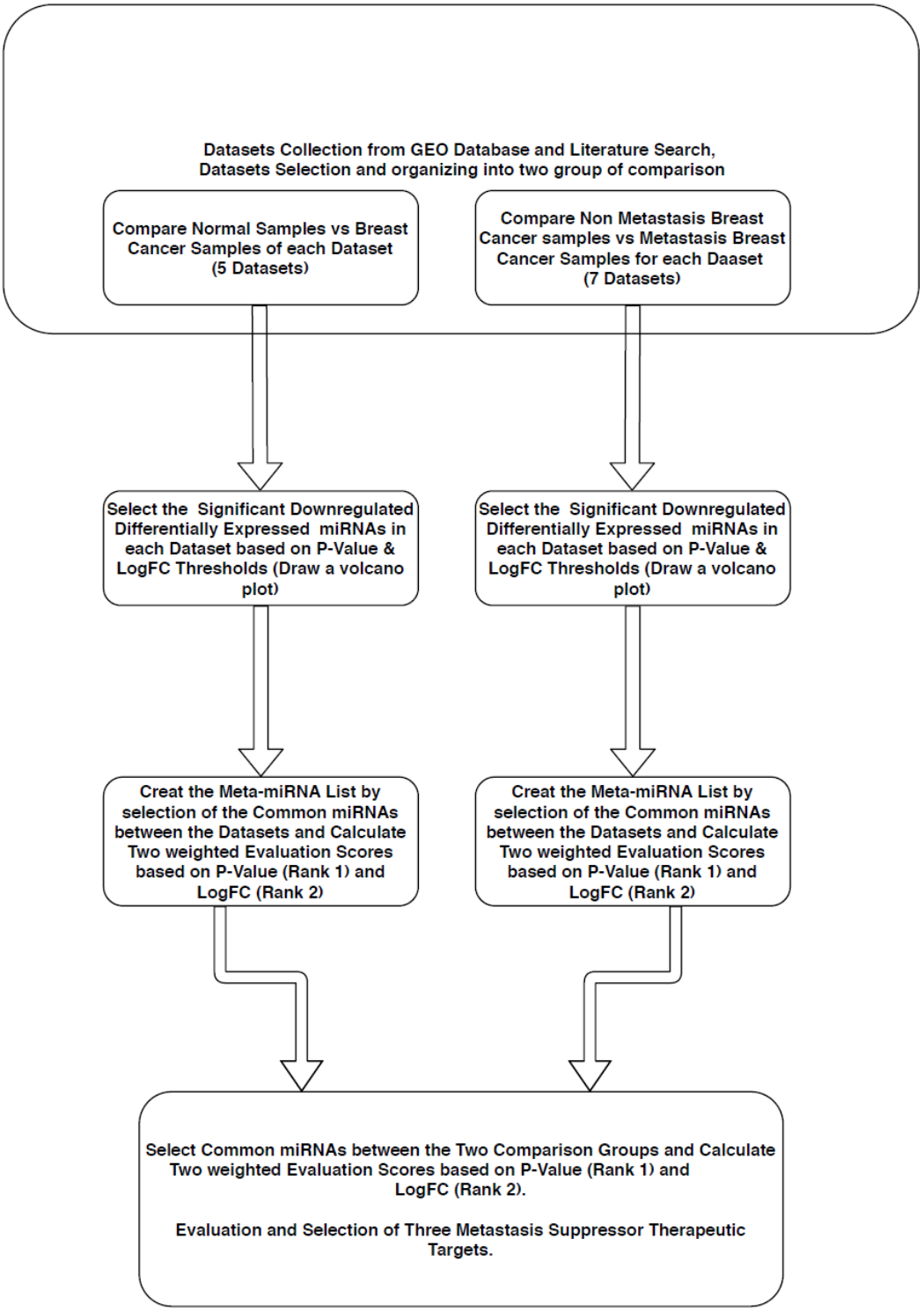


Figure 3.1. *The workflow of the meta-analysis steps.*

3.1.1. Datasets collection from GEO database and literature search

Three miRNA microarray datasets (GSE7842, GSE15885 and GSE22216) related to breast cancer and miRNA microarray were found in the literature (Oztemur Y, et al., 2015). The other datasets were searched and extracted from "Gene Expression Omnibus" (GEO, last visited: 20/11/2016), (http-7). Geo dataset was searched using the keywords ("miRNA microarray" and "breast cancer"). We found 92 studies, then filtered (homo sapiens) to 87. The 87 study samples were scanned for data which can be analyzed in any of two ways: normal breast tissue vs breast cancer or breast cancer vs metastatic breast cancer. FFPE and blood samples studies were excluded. Totally, 8-miRNA microarray datasets were used for the meta-analysis. Table 3.1 shows the informations of the collected datasets, and which comparison was done for their samples in the meta-analysis work.

Table 3.1. *The informations of the collected breast cancer datasets, and the type of comparison done for their samples in the meta-analysis work.*

GEO Accession	Microarray Platform	Study Published Date	Comparing Non-Met. vs Met.	Comparing Normal vs Cancer	Cancer Type
GSE7842	Bead-based microRNA profiling platform version 3	2007	NO comparison	5 Normal breast samples vs 93 Primary human breast tumor.	Breast
GSE26659	Agilent-019118 Human miRNA Microarray 2.0 G4470B (miRNA ID version)	2012	4 Tumor 1st grade vs 34 tumor 3rd grade.	17 Normal breast tissue vs 77 breast cancer tissue.	Breast
GSE44124	Agilent-021827 Human miRNA Microarray G4470C (Feature Number version)	2013	NO comparison	3 Normal breast vs 50 breast tumor.	Breast
GSE45666	Agilent-021827 Human miRNA Microarray	2013	10 Patient 1st grade vs 45 patient 3rd grade.	15 Normal breast tissue vs 101 breast tumor.	Breast

	G4470C (Feature Number version)				
GSE40525	Agilent-019118 Human miRNA Microarray 2.0 G4470B (miRNA ID version)	2012	3 Tumor 1st grade vs 27 tumor 3rd grade.	59 Pre-tumor tissue vs 61 tumor tissue.	Breast
GSE22216	Illumina Human v1 MicroRNA expression beadchip	2011	42 Patient 1st grade vs 63 Patient 3rd grade.	NO comparison	Breast
GSE15885	Homo sapiens 0.4K miChip v8	2009	5 Patient 1st grade vs 17 patient 3rd grade.	NO comparison	Breast
GSE75685	Agilent-019118 Human miRNA Microarray 2.0 G4470B (Feature Number version)	2015	4 Breast tumor metastas to (1 liver, 3 lung) vs no metastasis.	NO Comparison	Breast

3.1.2. Comparison between the samples of each dataset

The next step was analyzing the selected datasets using the web tool "Geo2R" (http-8). This tool allows the user to compare two or more groups of samples in the GEO database datasets, to identify the differentially expressed genes. Firstly, the samples were defined in two groups, five datasets could be compared between normal and breast cancer samples (normal vs cancer), and seven datasets could be compared between non-metastasis breast cancer and metastatic breast cancer samples (non-met. vs met.). Then, a box plot of the value distribution for the Samples was done to make sure that the value data are median-centered across Samples, this ensures that the samples are comparable. Then all the results of the “differentially expressed miRNA list” saved in excel files for each study alone.

3.1.3. Extracting meta-list

From the “differentially expressed miRNAs list” of each dataset, “the down-regulated differentially expressed miRNAs lists” were extracted based on (p-value) versus (logFC) thresholds, it was done through drawing a volcano plot using online tool (http:9). On the y-axis: the -log10 (p-values), the values range between [(-2.5) -(2.5)], the x-axis: log2 (fold-change) values between (0-4). The cut-off selection was for -log10 (p-values) = (1), while for log2 (FC) range was between = [(-0.5) -(0.5)]. Then all the selected “significant down-regulated gene lists” of each dataset were collected together in one excel sheet, for the two comparisons separately. The miRNA names updated according to miRBase database (http-10), by checking the previous name field. Additionally, it was confirmed by checking the datasets, which their platform contains information page for the mature sequence of the miRNA, it was controlled if it's on the 5' or 3' prime of the precursor.

Meta-miRNA list created by the selection of the common “significant suppressed miRNA’s” between the datasets in each comparison, this was done manually using Microsoft Office Excel’s options. Two weighted evaluation scores (Ranks) calculated for every common miRNA, based on p-value (rank1), and logFC (Rank2), through calculating the total pooled value, divided to the number of the studies, that the miRNA was significant in its result.

3.1.3.1. *The volcano-plots of the differentially expressed miRNA lists of each dataset*

The “downregulated differentially expressed miRNAs lists” were extracted based on (p-value) versus (logFC) thresholds, it was done through drawing a volcano plot for every dataset. The cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figures, while for log2(FC) range, it was between = [(-0.5) - (0.5)], which is represented by the horizontal blue lines.

3.1.3.1.1. *Volcano-plots of the datasets from the comparison between non-met. vs met.*

To extract the “downregulated differentially expressed miRNAs lists” from the datasets of the comparison between (non-metastasic breast cancer) vs (metastatic breast cancer), a volcano plot was drawn for every dataset.

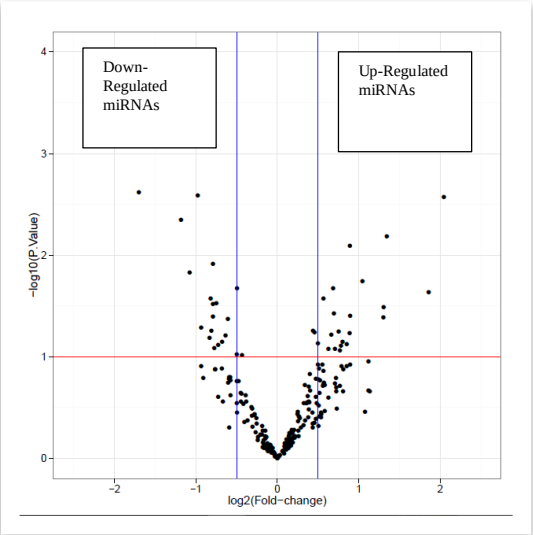


Figure 3.2. *The volcano-plots of the differentially expressed genes from GSE26659 Ddtaset. To extract the "down-regulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

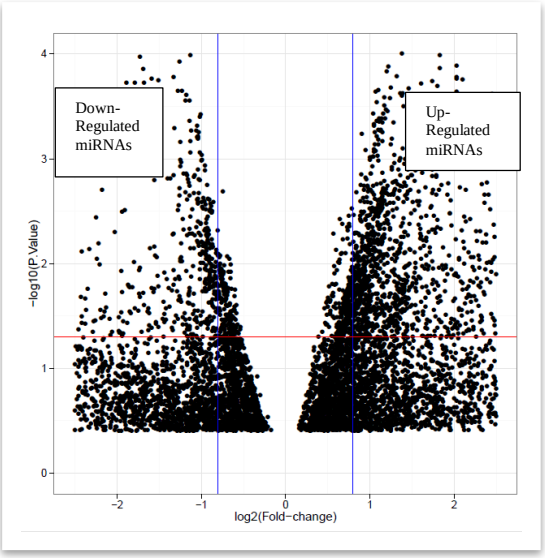


Figure 3.3. *The volcano-plots of the differentially expressed genes from GSE45666 dataset. To extract the "downregulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

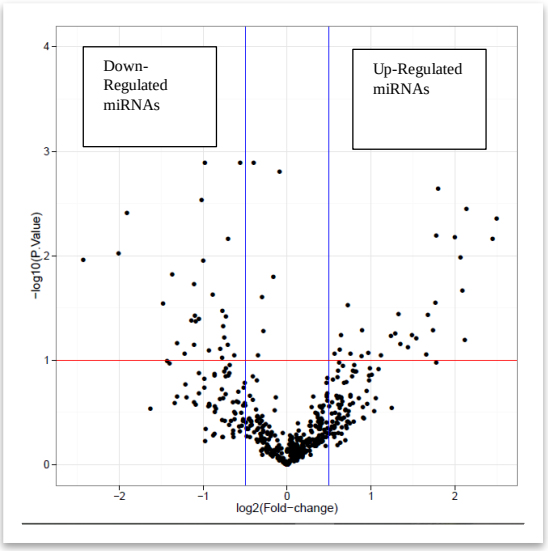


Figure 3.4. *The volcano-plots of the differentially expressed genes from GSE40525 dataset. To extract the "downregulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

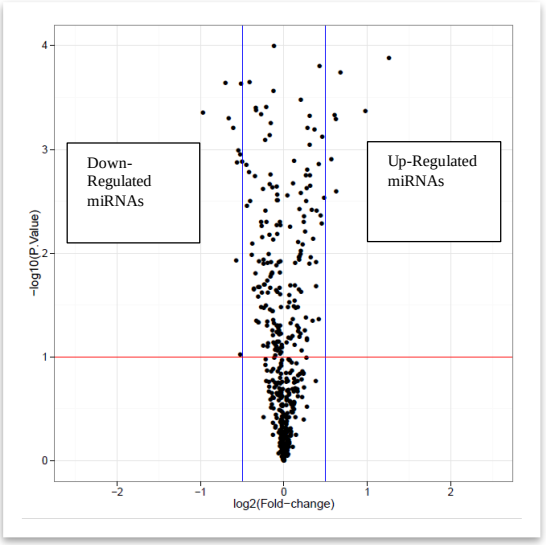


Figure 3.5. *The volcano-plots of the differentially expressed genes from GSE22216 dataset. To extract the "downregulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

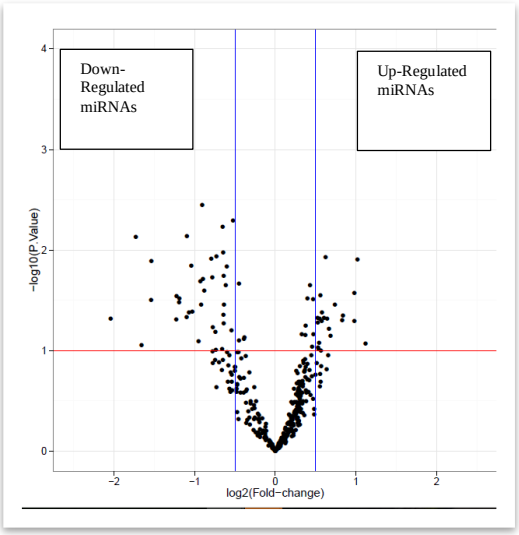


Figure 3.6. *The volcano-plots of the differentially expressed genes from GSE15885 dataset. To extract the "downregulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

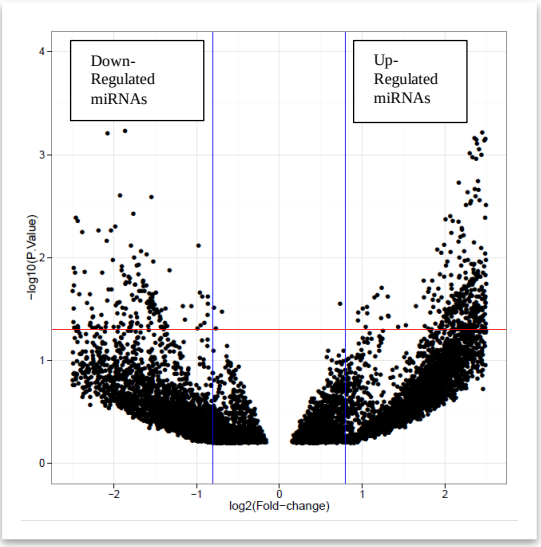


Figure 3.7. *The volcano-plots of the differentially expressed genes from GSE75685 dataset. To extract the "downregulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

3.13.1.2. *Volcano-plots of the datasets from the comparison between normal vs breast cancer*

To extract the "down-regulated differentially expressed miRNAs lists" from the datasets of the comparison between normal vs breast cancer, a volcano plot was drawn for every dataset.

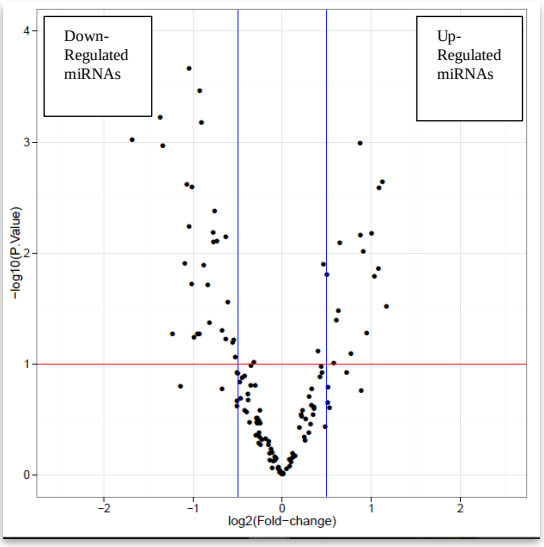


Figure 3.8. *The volcano-plots of the differentially expressed genes from GSE7842 dataset. To extract the "downregulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

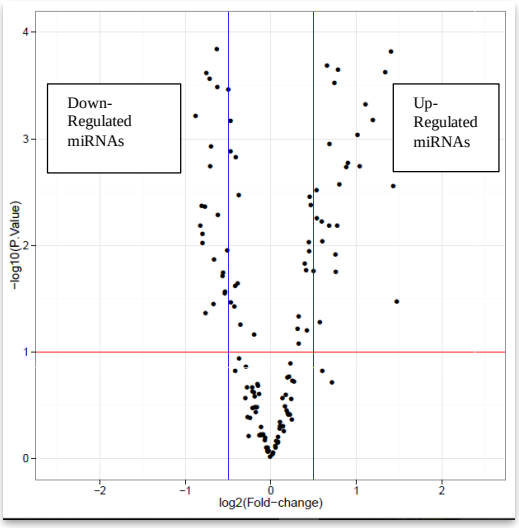


Figure 3.9. *The volcano-plots of the differentially expressed genes from GSE26659 dataset. To extract the "downregulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

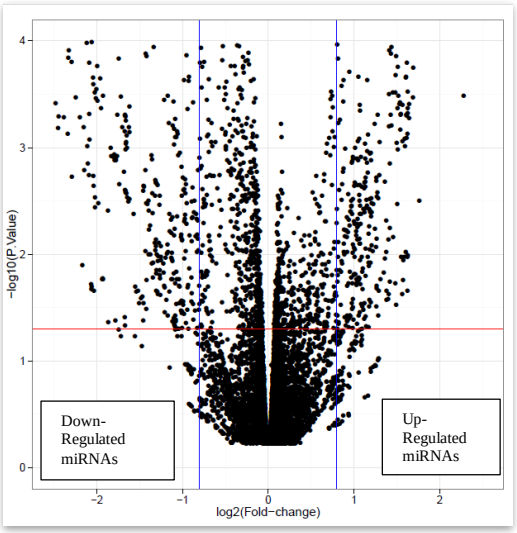


Figure 3.10. *The volcano-plots of the differentially expressed genes from GSE44124 dataset. To extract the "downregulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

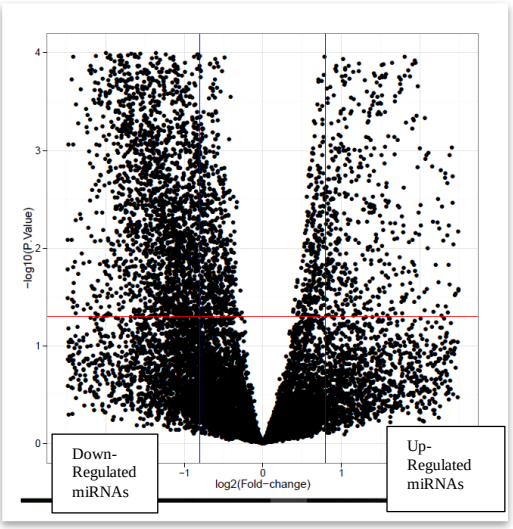


Figure 3.11. *The volcano-plots of the differentially expressed genes from GSE45666 dataset. To extract the "downregulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

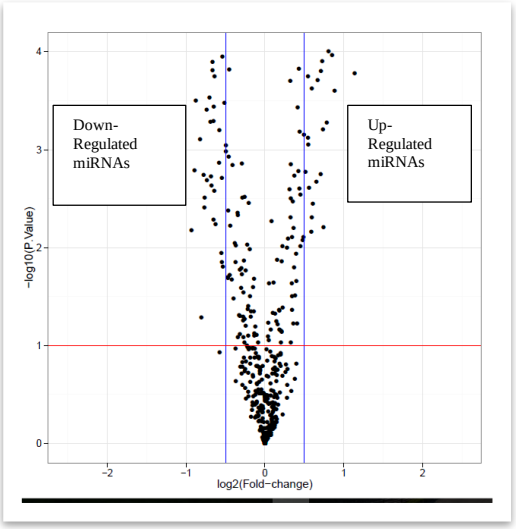


Figure 3.12. *The volcano-plots of the differentially expressed genes from GSE40525 dataset. To extract the "downregulated differentially expressed miRNAs lists" from this dataset, the cut-off selection was for -log10 (p-values) = (1) and it is represented by the vertical red line in the figure, while for log2 (FC) range, it was between = [(-0.5) -(0.5)], which is represented by the horizontal blue lines.*

3.1.4. Selection of three metastasis suppressor therapeutic targets

The common miRNAs in the meta-list of the two comparisons groups: normal vs cancer and non-met. vs met. were selected, based on p-value (rank1), and logFC (Rank2). Both ranking scores were evaluated to select the top three significant down-regulated miRNAs. In addition to the meta-analysis work result, other miRNAs list available from the literature was considered, a miRNA list from the Review (Bouyssou, et.al., 2014), it is including a table of Metastasis regulating miRNAs, which has been reported to directly regulates the metastatic process both in vitro and in vivo. Another consideration was the experimentally validated miRNA targets lists from miRTarBase Database (http-11), the target gene was accepted if it has been validated by the three techniques, qPCR, western blot and reporter assay), this was done for the top 4 "significant miRNAs" in the meta-analysis result, "hsa-miR-139-5p", "hsa-miR-145-5p", "hsa-miR-143-3p", and "hsa-miR-99A-5p". In order to understand the biological functions of their targets lists, a Pathway enrichment analysis was done using WebGestalt 2017 web-based gene set analysis toolkit (http-12). The selected enrichment method was ORA "Overrepresentation Enrichment Analysis", the database result was requested from "Wikipathway-Cancer", the other parameters were the default settings.

Considering the meta-analysis result, the review contains metastasis regulating miRNAs table, and the "pathway enrichment analysis" result of the top 4 significant miRNAs targets, three metastasis suppressor miRNAs in breast cancer was selected as therapeutic targets for miRNA replacement therapy.

3.2. In-Vitro MicroRNA Replacement Therapy

3.2.1. Cell culture

"MDA-MB-231" cells from ATCC (American type culture collection) were cultured in "RPMI-1640" plus 2.05mM (L-Glutamine) medium, supplemented with 10% (fetal bovine serum) and 1% (10,000 units/ml penicillin plus 10 mg/ml streptomycin), under conditions of 5% CO2 and 37°C. Cells were subcultured when reaching 70-80% confluency. A stock of 500,000 cell/ml in freezing medium were stored in liquid nitrogen (vapor phaset), in the presence of a cryoprotective agent: 5% DMSO. The freezing medium was "RPMI-1640" with L-Glutamine medium,

supplemented with 20% “fetal bovine serum” and 1% (10,000 unit/ml penicillin plus 10 mg/ml streptomycin).

3.2.2. In-vitro transfection of the identified microRNA mimics into MDA-MB-231 cells

The used miRNA mimics were “miScript miRNA Mimics” from QIAGEN: “hsa-miR-145-5p” (Cat. No. MS00003528), “hsa-miR 143-3p” (Cat. No. MS00003528), “hsa-miR 99A-5p” (Cat. NoMS00032158). The mimics were provided at lyophilized state, and resuspended as the recommendations of the manufacturer’s manual. “HiPerFect” transfection reagent, from QIAGEN was used (Cat No./ID: 301705).

The transfection (miRNA replacement therapy) of the identified miRNAs mimics was done in-vitro, using a commercial lipid-based transfection reagent. The miRNA mimic transfection in 24-well plates protocol was considered, according to the guidelines manual of the manufacturer company. In order to achieve the best results in miRNA’s mimic transfection to the cells, an optimization step was done to determine the following parameters: The ratio of HiPerFect Transfection Reagent to the miRNA’s mimic, the cell density, and the transfection method.

The transfection experiment for our cell line “MDA-MB-231” was done after the optimization as the following:

- Fast-Forward Protocol considered as our transfection protocol, which means that the cell seeding and the transfection were carried out on the same day. Figure 3.2, shows the difference between the traditional transfection protocol vs fast-forward protocol.

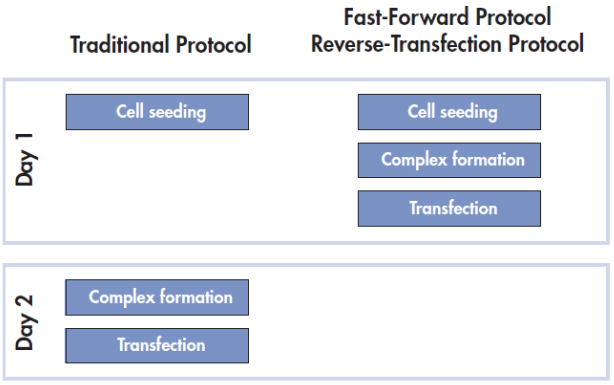


Figure 3.13. *Traditional transfection protocol vs fast-forwarded protocol (http-13).*

- Cells seeding number was (30000 cells) in 24 well plate. Cell Passage number was (4).
- The HiPerFect transfection reagent and the miRNA mimic amount used: 10 nM miRNA and 4.5 µl HiPerFect Transfection Reagent.

Every miRNA type was transfected in one 24 well plate with suitable transfection experiment controls, the controls were performed in every plate, the design of the transfection experiment are shown in figure 3.3. The transfection positive control mimic was used to confirm the transfection efficiency, a commercial positive control from QIAGEN was used: AllStars Hs Cell Death Control siRNA (Cat. No. SI04381048), which is a siRNA blend that targets essential human genes, the transfection of this control caused a high degree of cell death that observed by the light microscopy. The transfection negative control, was a commercial siRNA mimic has no homology to any known mammalian gene (Cat No. 1022076). The other controls were: Mock-transfection control, which means that cells were treated with transfection reagent only, it aims to determine whether the transfection reagent causes nonspecific or cytotoxic effects, and the control of the untransfected cells. Images of the controls wells were captured every 24 hours by an inverted microscope (10X).

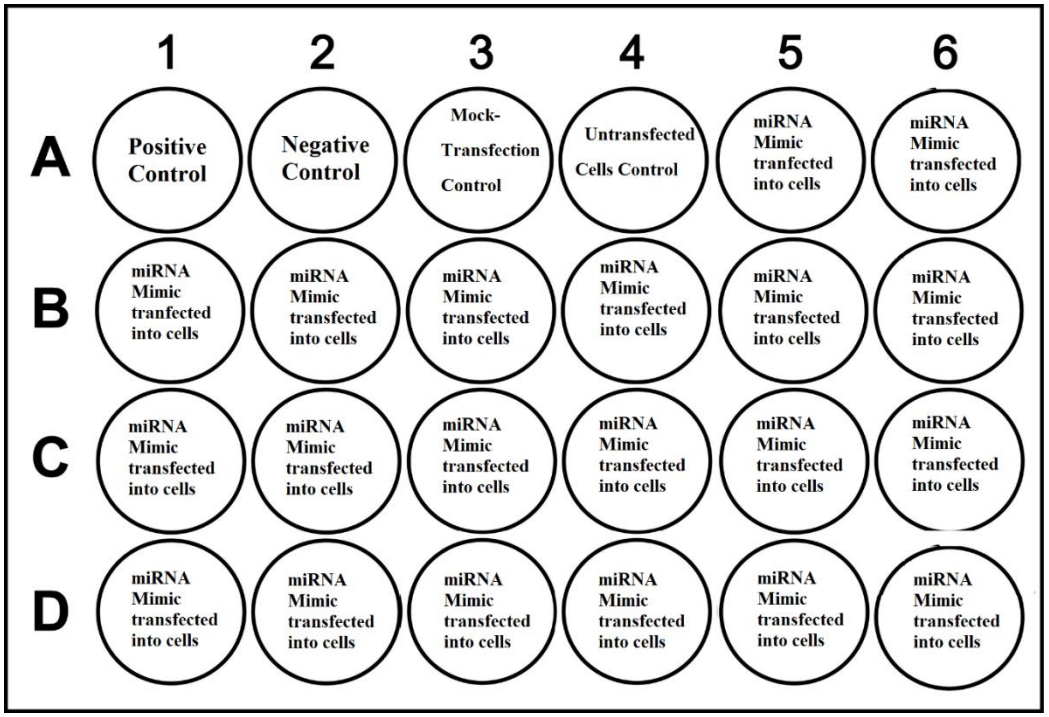


Figure 3.14. *The design of the transfection experiment on 24 well plate.*

3.2.3. RNA extraction

The total RNA extraction was on the third day after transfection, and performed using miRNeasy Mini kit, from Qiagen (Cat No./ID: 217004). The protocol was from the manufacturer manual: Purification of Total RNA, Including Small RNAs, from animal cells.

The RNAs concentration was measured using the NanoDrop 1000 spectrophotometer machine from ThermoFisher, after following the ND-1000 software instruction, 1.5 µl of elution water (DNase and RNase free water) was loaded to the machine to blank the system, then RNA was selected on the software setting, and the RNA concentration was measured by loading1.5 µl of the sample to the machine.

3.2.4. Protein extraction

Cell lysate proteins were extracted at the fourth day after the transfection. The protein extraction was according to the standard laboratory method. For cell lysis, RIPA (Radioimmunoprecipitation) Lysis Buffer system from SantaCruz Biotechnology (Cat. No. sc-24948A) was used, the system composed of a lysis buffer, PMSF in DMSO, protease inhibitor cocktail in DMSO, and (100mM) sodium orthovanadate in water.

Immediately prior to lysing cells, we combined 10 µl PMSF solution, 10 µl sodium orthovanadate solution and 15 µl protease inhibitor cocktail solution per ml of 1X RIPA Lysis buffer to prepare complete RIPA.

The protein concentration was determined by Qubit® 3.0 fluorometer's protein assay kit (life technologies Cat. No. Q33211), according to the manufacturer protocol.

3.3. In-Vitro Cell Migration Assay

The cells were seeded (30000 cells) in 24 well plate, each well in the plate were partially covered by a small piece of sterile glass slide, then the miRNA mimic were transfected to the cells, and when cells reached 70-80% confluence, the glass pieces were taken off to leave an empty space (gap) in the middle surrounding by the transfected cells. The control was a well that contains non-transfected cells. The degree of the cells observed by light microscopy. Images of cells migrating into the empty space were captured every 24 h by an inverted microscope (4X).

3.4. Microarray Gene Expression Experiment

The isolated total RNAs was sent to a commercial company (AYKA LTD.ŞTI-Ankara/Turkey), for the microarray experiment service. Affymetrix GeneChip Microarray technology was worked using the GeneChip® Human Genome U133 plus 2.0 Array.

3.4.1. Microarray Data analysis

In order to study the effect of the three transfected miRNAs, microarray samples were: The three transfected cell samples, one control (untransfected cells), and their biological replica. Thus, in total 8 arrays were analyzed.

Analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team: R version 3.5.1, BRB-ArrayTools Version: 4.6.0 – Stable, project annotated by bioconductor (www.bioconductor.org) annotation package hgu133plus2.db (Version: 3.2.3).

3.4.1.1. *Preprocessing*

MAS 5.0 Affymetrix algorithm was used for background correction, calculation of the probe summary, and normalization. Filtering parameters:

- Normalization: normalize (center) each array using quantile normalization.
- Filtering: genes under the following thresholds are exclude from the study; less than 20 % of expression data have at least a 1.5-fold change in either direction from gene's median value, and with 50% percent of missing data.

3.4.1.2. *Comparison analysis (gene set selection analysis)*

The differentially expressed genes between the classes were extracted by the class comparison analysis option in the BRB-array tool, for the genes that passed the filtering criteria. Three comparison analysis were done:

1. Between the transfection of "has-miR-145-5p" and the untransfected control.
2. Between the transfection of "has-miR-143-3p" and the untransfected control.
3. Between the transfection of "has-miR-99A-5p" and the untransfected control.

The class comparison parameters:

- The number of classes: 2.
- The number of genes that passed the filtering criteria: 50873.
- The type of univariate test used: Two-sample t-test.
- Class variable: 145 v cont.
- The nominal significance level of each univariate test: 0.01.

3.4.1.3. *Visualization of results and biological interpretation*

3.4.1.3.1. *Network visual analysis*

A network visual analysis was done for the top 10 (up and down) regulated genes separately, to extract the genes that have the strongest relationships to genes in the uploaded list (seed genes). It was done using Network Analyst online web application (http:15). "Gene regulatory network/TF-gene interaction" analysis was applied, the requested database: ENCODE, the other parameters were the default settings. The Network tool gives the opportunity to perform gene set overrepresentation analysis for the extracted genes (genes related to the uploaded list), the cancer-related pathways were considered.

3.4.1.3.2. *Pathway enrichment analysis*

In order to understand the biological functions of the class comparison's gene lists, a Pathway enrichment analysis was done using Web Gestalt 2019 web-based gene set analysis toolkit (http:12). The selected Enrich method was ORA (Overrepresentation Enrichment Analysis), the database result was requested: (Wikipathway-Cancer), the other parameters were the default settings.

3.5. Western Blot

3.5.1. Electrophoresis of proteins using SDS-PAGE

SDS-PAGE was carried out using Invitrogen Bolt Mini Gel Tank electrophoresis chamber system from Thermo Fisher, NuPAGE 3-8% Tris-Acetate ready to use gels (Cat. No. EA0375PK2), and NuPAGE Tris-Acetate SDS Running Buffer (Cat. No. LA0041). Protein samples preparation performed according to the instructions of using bolt mini gels electrophoresis, reduced sample was prepared using: LDS (Lithium dodecyl sulfate) Sample Buffer 4x (life technologies Cat. No. B0007), Reducing Agent 10X (life technologies Cat. No. B0009), the samples heated at ,70°C for 10 minutes. Proteins were resolved in NuPAGE 3-8% Tris-Acetate Pre-Cast Gels (Invitrogen Life Technologies Cat. No EA0375BOX), MagicMark XP Western Protein Standard was used from Invitrogen Life Technologies (Cat. No. LC5602) and SeeBlue pre-stained Protein standards 1 x (life technologies Cat. No. LC5625). Gels were run at 165 V for approximately 40 minutes.

3.5.2. Proteins transfer

Proteins were transferred to a nitrocellulose membrane using the iBlot2 Gel transfer device (dry, buffer-free system), the Gel was transferred with iBlot2 Transfer Stacks, PVDF (life technologies Cat. No. IB24001) using the P0 program (8 min, at 25V).

3.5.3. Detection of proteins

Once proteins were transferred onto the PVDF membrane, the membrane was blocked in blocking buffer (10% w/v Milk Powder, 0.1% v/v Tween 20 in 1x TBS buffer) for 75 minutes. The blot was then incubated with p-mTOR mouse monoclonal

antibody (stock at 1 mg/ml) (SantaCruz biotechnology sc-293132) at a dilution of 1/200 in blocking buffer for 16 hours at 4°C. The blot was washed 3 x for 5 minutes in 1x TBS-T (1x TBS buffer, 0.1v/v Tween 20, pH adjusted to 7.6). The secondary antibody, a goat anti-mouse IgG-HRP (stock at 0.32 mg/ml) (SantaCruz biotechnology sc-2005) was used at a dilution of 1/500 in blocking buffer. Again, the blot was washed for 3 x for 5 minutes in TBS-T. Detection of the Protein on the western blot membrane were done using Santa Cruz Biotechnology Luminol reagent (sc-2048), according to the manufacturer's instructions. The protein detection image was visualized using LI-COR C-DiGiT Blot scanner device.

3.6. Gelatin Zymography

3.6.1. Protein isolation

Conditioned media (FBS free media) was collected and concentrated by precipitation with cold acetone (1 ml condition media: 4 ml acetone), the mix was incubated for 2 hours at -20°C, then centrifuged at 3000-4000 rpm for 15 minutes at 4°C, and suspended in 4x LDS (Lithium dodecyl sulfate) Sample Buffer (life technologies Cat. No. B0007). The protein concentration was determined by Qubit® 3.0 fluorometer's protein assay kit (life technologies Cat. No. Q33211)

3.6.2. SDS-PAGE

Zymogram gels were prepared as following, 10% separating, 0.1% gelatin gel (10%SDS, 1.5M Tris PH: 8.8), and 4% stacking gel (10%SDS, 1M Tris PH: 6.8). The electrophoresis apparatus was filled with 1xTris/Glycine/SDS buffer, then protein samples and SeeBlue pre-stained Protein standards 1 x (life technologies Cat. No. LC5625) were electrophoresed at 100 volts for 75 min. at 4°C. The marker lane was cut off before the washing and staining steps, and then considered to know the bands molecular weight of the result.

3.6.3. Gel washing and staining

After electrophoresis, gels were rinsed (2× for 30 minutes) in washing buffer (2.5% Triton X-100, 1M Tris (pH 7.8), 0.15M NaCl, 10mM CaCl2) to remove SDS, followed by incubation at 37 °C overnight in incubation buffer (1% Triton X-100, 1M

Tris (pH 7.8), 0.15M NaCl, 10mM CaCl2). After incubation, the gels were stained in staining solution (0.5% Coomassie Brilliant Blue) and destained with 7% methanol and 5% acetic acid. Areas of enzymatic activity appeared as clear bands over dark background.

4. RESULTS AND FINDINGS

4.1. Meta-Analysis

As meta-analysis integrates results from different studies and from different centers, time, and platforms, increases the statistical power of the results, so a statistically significant Meta-miRNA list (significant suppressed in cancerous list) was obtained. It is based on ranking according to two features (the t-test probability (p-value), and the logFC value), additionally, two types of comparison were applied (normal Breast tissue vs breast cancer) and (Breast cancer vs metastatic breast cancer).

4.1.2. The common significant down-regulated miRNA's (meta-miRNA) list

The meta-miRNA list was created based on two ranks, the t-test probability (p-value), and the logFC. To identify the most significant suppressed miRNA, the two comparisons applied were considered, table 4.1 shows their ranks separately, and then the overall meta-list ranks was extracted by calculating the average of the ranks in both comparisons (Table 4.2). In addition to the meta-analysis work results, miRNA list available from the literature was considered to identify the therapeutic targets.

Table 4.1. *The meta-miRNA list shows the two ranks in each comparison (p-value & logFC) and the availability of it in the "metastatic miRNA list" from the considered review.*

Updted miRNA ID	Comparison Between Non-Met. Vs Met.		Comparison Between Normal Vs Breast Cancer		In the Metastatic miRNA List from the Review: (Bouyssou, et al., 2014)
	P-Value Rank	logFC Rank	P-Value Rank	logFC Rank	
hsa-let-7c-5p	0.004464286	-0.323495571	6.50001E-05	-0.50708664	yes
hsa-miR-10a-5p	0.008042714	-0.322312671	0.003126	-0.44892722	yes
hsa-miR-10b-5p	0.020552743	-0.592411186	0.004056	-0.93728752	yes
hsa-miR-26a-5p	0.020541929	-0.4403089	9.24001E-09	-0.62615918	yes
hsa-miR-30a-3p	6.49531E-08	-0.560901286	0.007100001	-0.63217388	No
hsa-miR-30a-5p	0.002792303	-0.432541571	0.001894027	-0.59402202	yes
hsa-miR-99a-5p	0.008388571	-0.311295486	8.91594E-07	-1.56889608	No
hsa-miR-100-5p	0.027642857	-0.302643929	4.44008E-05	-1.44055022	No
hsa-miR-125b-5p	0.014522857	-0.167237229	2.08201E-05	-1.58810022	yes
hsa-miR-126-3p	0.004255714	-0.327012214	0.002075753	-0.8439726	No
hsa-miR-126-5p	0.027642857	-0.302643929	4.24E-07	-0.63207792	No
hsa-miR-139-5p	0.001100957	-0.366714743	8.7762E-11	-1.95558228	yes
hsa-miR-143-3p	0.001108571	-0.278157314	0.000260788	-1.13509736	yes
hsa-miR-145-5p	0.002421429	-0.352888243	3.10011E-06	-1.75547986	yes
hsa-miR-152-5p	0.010661429	-0.416652286	0.00238914	-0.32497436	No
hsa-miR-205-5p	0.010794286	-0.387881257	0.012940251	-1.77062618	yes
hsa-miR-328-5p	0.00016696	-0.210566671	4.30132E-09	-0.52073926	No
hsa-miR-497-5p	0.00496	-0.242904286	0.0001282	-1.2548973	No

Table 4.2. *The meta-miRNA list shows the two ranks from the average of the both comparison (p-value & logFC).*

Updted miRNA ID	Average P-Value in the Both comparison	Average logFC in the Both Comparison
hsa-let-7c-5p	0.002264643	-0.41529
hsa-miR-10a-5p	0.005584357	-0.38562
hsa-miR-10b-5p	0.012304371	-0.76485
hsa-miR-26a-5p	0.010270969	-0.53323
hsa-miR-30a-3p	0.003550033	-0.59654
hsa-miR-30a-5p	0.002343165	-0.51328
hsa-miR-99a-5p	0.004194732	-0.9401
hsa-miR-100-5p	0.002626486	-0.84975
hsa-miR-125b-5p	0.007271839	-0.87767
hsa-miR-126-3p	0.003165733	-0.58549
hsa-miR-126-5p	0.013821641	-0.46736
hsa-miR-139-5p	0.000550479	-1.16115
hsa-miR-143-3p	0.00068468	-0.70663
hsa-miR-145-5p	0.001212264	-1.05418
hsa-miR-152-5p	0.006525284	-0.37081
hsa-miR-205-5p	0.011867268	-1.07925
hsa-miR-328-5p	8.34822E-05	-0.36565
hsa-miR-497-5p	0.0025441	-0.7489

In order to select the three metastasis suppressor miRNA, the both ranking scores were evaluated to select the top four significant downregulated miRNAs from the meta list, which they are ("hsa-miR-139-5p", "hsa-miR-145-5p", "hsa-miR-143-3p", and "hsa-miR-99A-5p"), of these four, three miRNAs will be selected. In addition to the meta-analysis work results, other miRNAs list available from the literature were considered (Bouyssou, et. al., 2014). The other consideration was the pathway enrichment result of the experimentally validated miRNA targets lists of the top 4 significant miRNAs.

4.1.3. Pathway enrichment analysis results for the target gene list of the selected "metastasis suppressor miRNA"

According to meta-analysis result, the top four "metastasis suppressor miRNA" was: "hsa-miR-139-5p", "hsa-miR145-5p", "hsa-miR-143-3p", and "hsa-miR-99a-5p". In order to understand the biological functions of these four miRNAs, a Pathway enrichment analysis was done for their experimentally validated mRNA targets lists collected from miRTarBase Database. Table 4.3 shows the pathway enrichment analysis results for the target gene list of "hsa-miR-145-5p". Moreover, table 4.4, table 4.5, and table 4.6 respectly show shows the pathway enrichment analysis results for the target gene list of "hsa-miR-143-3p", "hsa-miR-99a-5p" and "hsa-miR-139-5p".

Table 4.3. *Pathway enrichment analysis results for the target gene list of "hsa-miR-145-5p"*

Wikipathway-Cancer	
Pathway Name	#Gene from 61 gene list
Pancreatic adenocarcinoma pathway	9
Chromosomal and microsatellite instability in colorectal cancer	6
Bladder Cancer	4
MET in type 1 papillary renal cell carcinoma	5
Breast cancer pathway	8
Endometrial cancer	5
Non-small cell lung cancer	5
TGF-beta Signaling Pathway	7
TGF-beta Receptor Signaling	4
Type 2 papillary renal cell carcinoma	3

Table 4.4. *Pathway enrichment analysis results for the target gene list of "hsa-miR-143-3p".*

Wikipathway-Cancer	
Pathway Name	#Gene from 31 gene list
Chromosomal and microsatellite instability in colorectal cancer	5
MET in type 1 papillary renal cell carcinoma	4
Endometrial cancer	4
Non-small cell lung cancer	4
Breast cancer pathway	5
EMT in colorectal cancer	5
Integrin-mediated Cell Adhesion	4
ErbB Signaling Pathway	3
DNA Damage Response (only ATM dependent)	4
MAPK Signaling Pathway	6

Table 4.5. *Pathway enrichment analysis results for the target gene list of "hsa-miR-99a-5p".*

Wikipathway-Cancer	
Pathway Name	#Gene from 10 gene list
Focal Adhesion-PI3K-Akt-mTOR-signaling pathway	3
Breast cancer pathway	2
4-hydroxytamoxifen, Dexamethasone, and Retinoic Acids Regulation of p27 Expression	1
Ras Signaling	2
Bladder Cancer	1
PI3K-AKT-mTOR signaling pathway and therapeutic opportunities	1
Target Of Rapamycin (TOR) Signaling	1
ATM Signaling Network in Development and Disease	1
Wnt Signaling Pathway	1
Interferon type I signaling pathways	1

Table 4.6. *Pathway enrichment analysis results for the target gene list of "hsa-miR-139-5p".*

Wikipathway-Cancer	
Pathway Name	#Gene from 13 gene list
Apoptosis	4
Breast cancer pathway	3
MET in type 1 papillary renal cell carcinoma	2
Chromosomal and microsatellite instability in colorectal cancer	2
MFAP5-mediated ovarian cancer cell motility and invasiveness	1
DNA Damage Response (only ATM dependent)	2
Wnt Signaling Pathway	2
TP53 Network	1
TGF-beta Signaling Pathway	2
PDGFR-beta pathway	1

4.1.4. Selection of three metastasis suppressor therapeutic targets

In order to select the three metastasis suppressor miRNA, the both ranking scores were evaluated to select the top four significant downregulated miRNAs from the meta list, which they are ("hsa-miR-139-5p", "hsa-miR-145-5p", "hsa-miR-143-3p", and "hsa-miR-99A-5p"), of these four, three miRNAs will be selected. In addition to the meta-analysis work results, other miRNAs list available from the literature were considered (Bouyssou, 2014). The other consideration was the pathway enrichment result of the experimentally validated miRNA targets lists of the top 4 significant miRNAs. A similar result was observed in the pathway enrichment result, the top four miRNAs have pathways involved in cancer and cancer metastasis. "has-miR-99A-p" was selected because it is not on the list of the considered review, so it will be a significant finding to prove its therapeutic ability, as it has a significant rank in the meta-list. "hsa-miR-145-5p" and "hsa-miR-143-3p" were selected because, they both have different targets genes but they are involved in the same metastasis processes and were validated in adenocarcinoma (Table 4.7).

Table 4.7. *Shows a part from the list which is from a review includes a table of metastasis regulating miRNAs (Bouyssou, 2014), it contains three of the identified miRNAs.*

MicroRNA	Properties	Known targets	Type of cancer
miR-139	Metastasis suppressor, invasion suppressor	IGF-IR	Colorectal cancer, Laryngeal squamous carcinoma, Liver cancer
miR-143	Metastasis suppressor, EMT inhibitor, Invasion suppressor	CD44v3, GEF1, GEF2, KRAS	Prostate cancer, Non-small cell lung cancer, Pancreatic cancer
miR-145	Metastasis suppressor, EMT inhibitor, Antiangiogenic, Invasion suppressor	HIF-2α, mucin 1, N-cadherin, Ets1	Prostate cancer, Neuroblastoma, Breast cancer, Gastric cancer

4.2. MicroRNA Replacement Therapy

4.2.1. In-Vitro transfection of the three identified metastasis suppressor miRNAs mimics to MDA-MB-231 Cells

The in-vitro transfection (miRNA replacement therapy) of the identified miRNAs mimics, was done using a commercial lipid-based transfection reagent. A suitable transfection experiment controls were performed. In order to confirm the transfection efficiency, a commercial positive control was used, which is a blend of siRNA that targets essential human genes, the transfection of this control causes a high degree of cell death that could be observed by light microscopy (Figure 4.7), shows that at the third day after the transfection, cells died and this is confirming the high efficiency of the transfection experiments. The transfection's negative control is a commercial siRNA mimic that has no homology to any known mammalian gene, the negative control (Figure 4.6) shows no differences with the untransfected cell control. About the mock-transfection control (Figure 4.5), cells were treated with transfection reagent only to determine whether the transfection reagent causes nonspecific or cytotoxic effects, and also in this control was no differences considered compared with the untransfected cells control (Figure 4.4), this validates that the lipid-based commercial transfection reagent used has no toxicity effects on the cells.

The captured cells images during the in-vitro transfection of "hsa-miR-145-5p", "hsa-miR-143-3p", and "hsa-miR-99A-5p" (Figures 4.1, 4.2, and 4.3) respectively, clearly show the transfected cells at the third day after the transfection with lower cell number, cells at the death stage, and some morphological changes are observed in comparison with the control cells. This verifies the antitumor effect of "hsa-miR-145-5p"," hsa-miR-143-3p", and "hsa-miR-99A-5p" miRNAs. Images of the transfected cells and their controls were captured every 24 hours by inverted microscope (10X), the images are represented below (Figure 4.1, Figure 4.2, Figure 4.3, Figure 4.4, Figure 4.5, Figure 4.6, and Figure 4.7), they were captured after three days of the transfection.

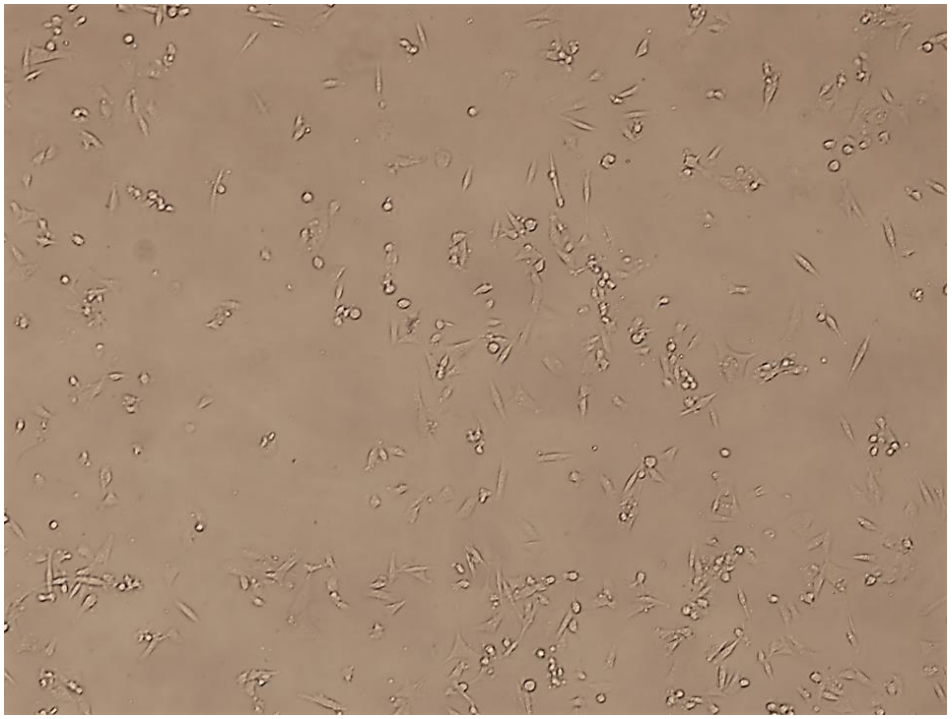


Figure 4.1. *Image of cells at the third day after in-vitro transfection of "hsa-miR-145-5p" Mimics to MDA-MB-231 cells (10X).*

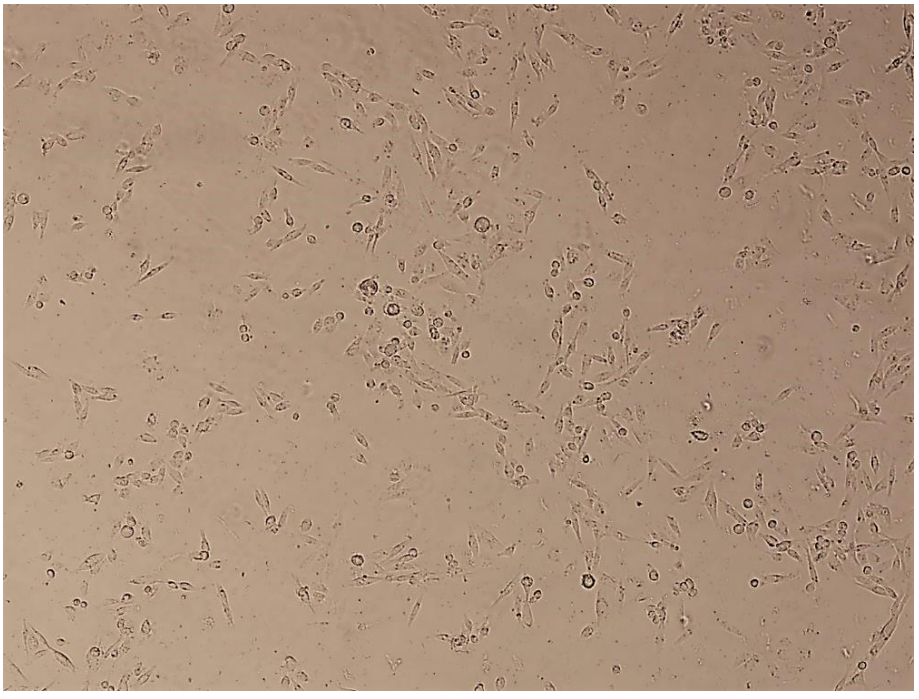


Figure 4.2. *Image of cells at the third day after in-vitro transfection of "hsa-miR-143-3p" mimics to MDA-MB-231 cells (10X).*

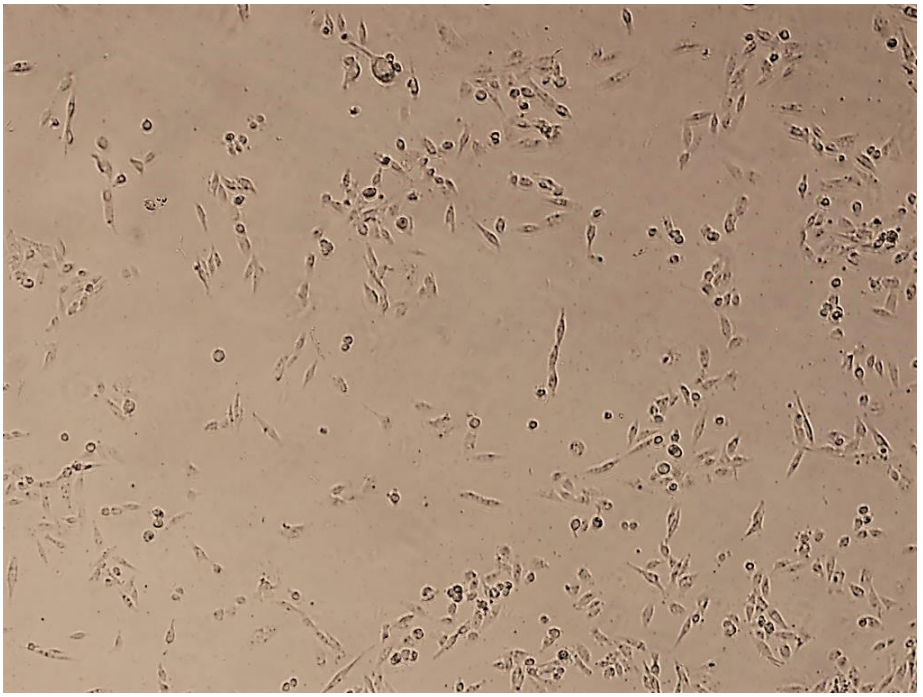


Figure 4.3. *Image of cells at the third day after in-vitro transfection of "hsa-miR-99A-5p" mimics to MDA-MB-231 cells (10X).*

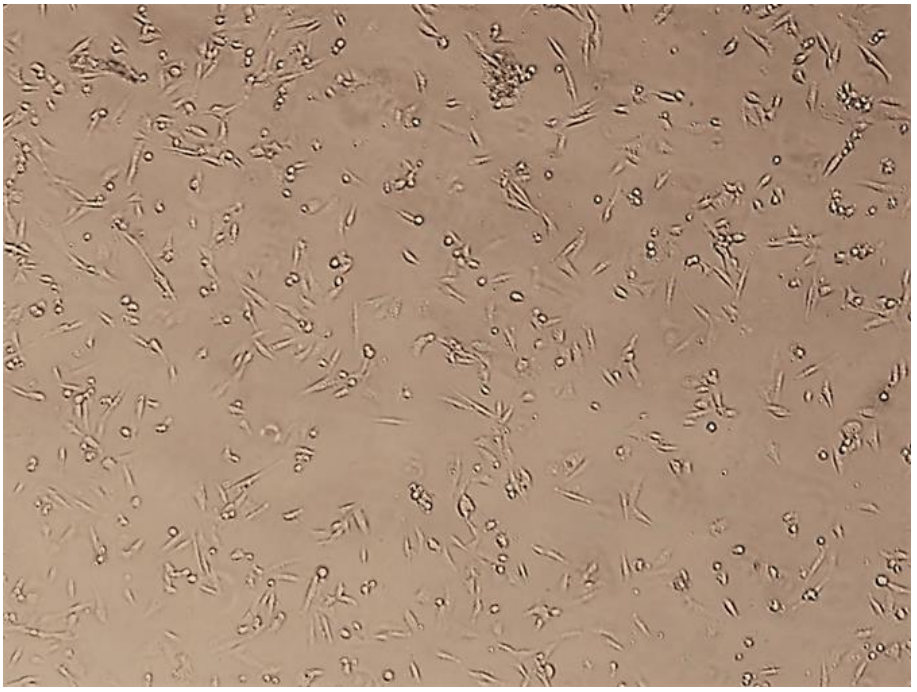


Figure 4.4. *Image of cells at the third day after in-vitro transfection of an untransfected control (MDA-MB-231) cells (10X).*

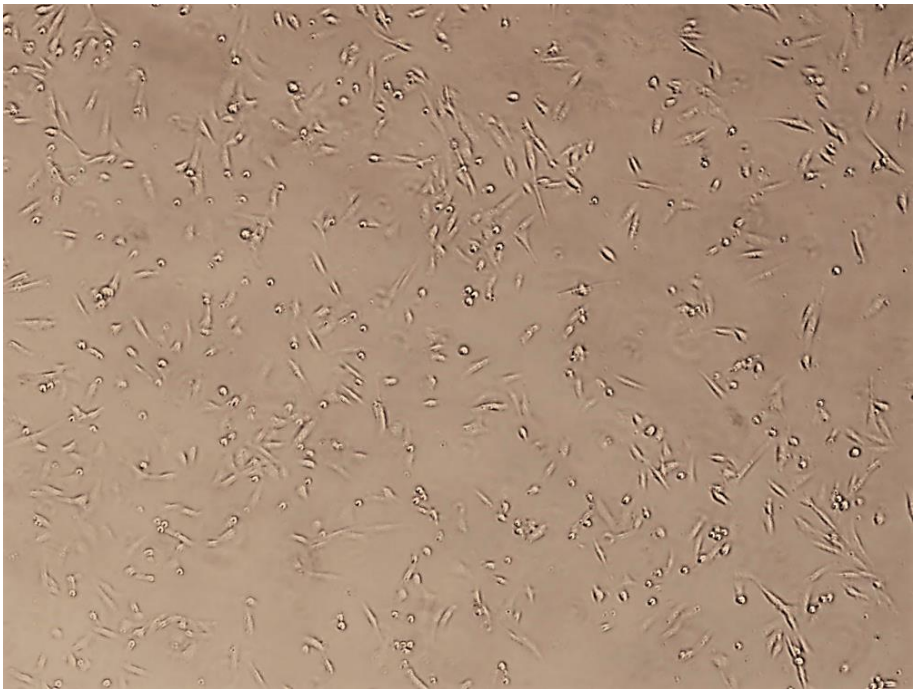


Figure 4.5. *Image of cells at the Third Day after In-Vitro Transfection of mock transfection control to MDA-MB-231 cells (10X).*

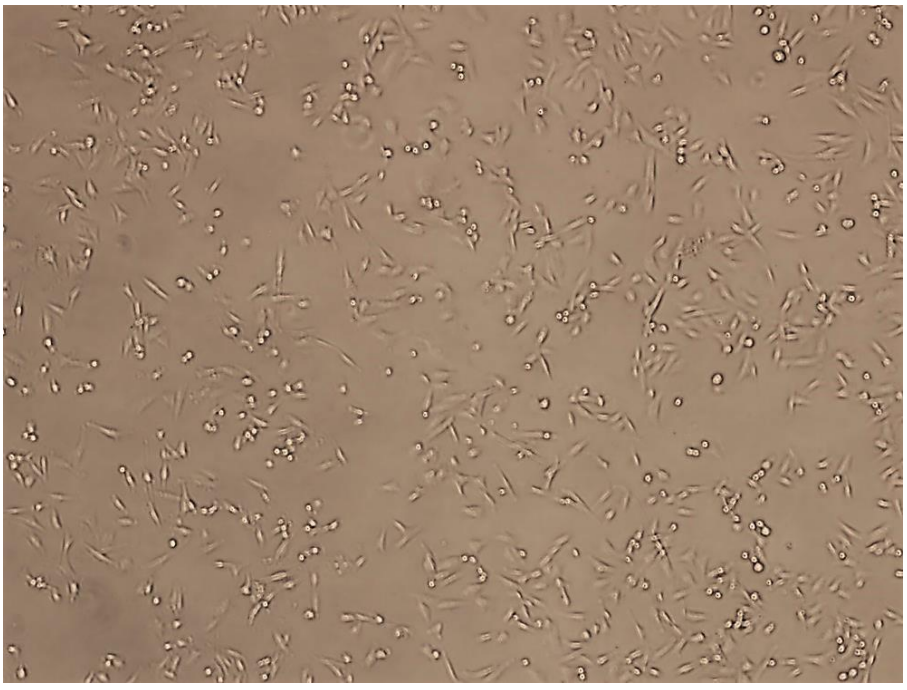


Figure 4.6. *Image of cells at the third day after in-vitro transfection of the negative control (a commercial siRNA mimic has no homology to any known mammalian gene) to MDA-MB-231 cells(10X).*

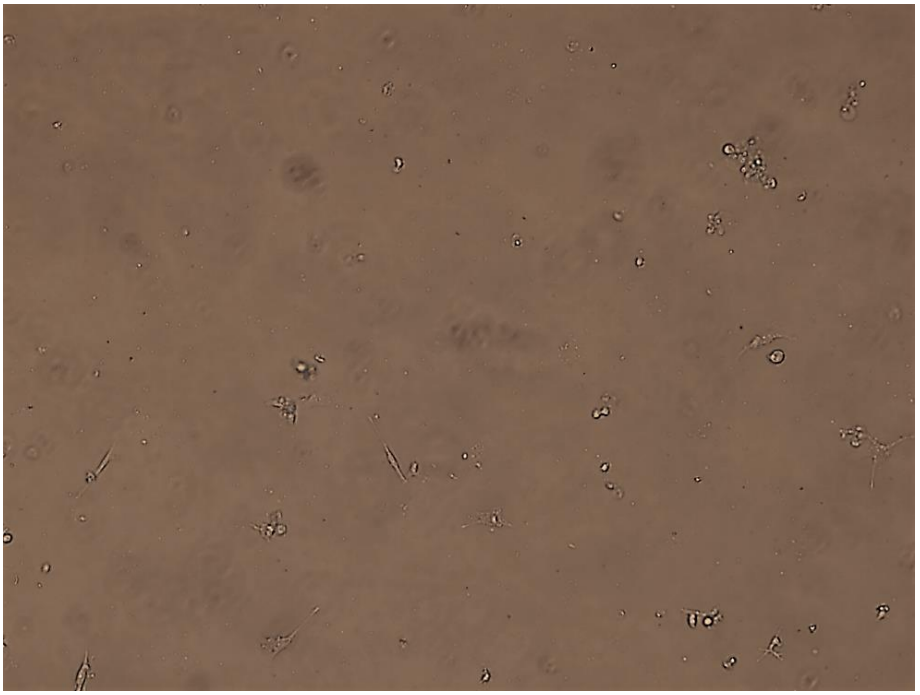
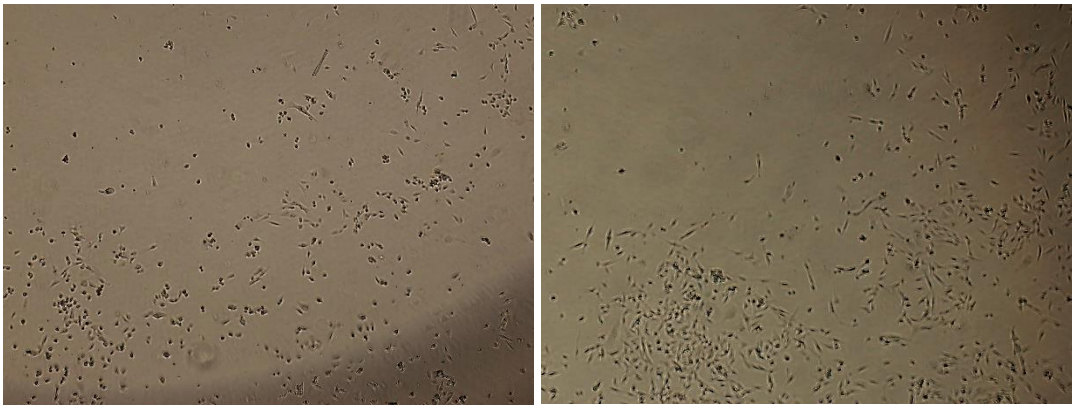


Figure 4.7. *Image of cells at the third day after in-vitro transfection of the postive control (AllStars Hs Cell Death Control siRNA) to MDA-MB-231 Cells (10X).*

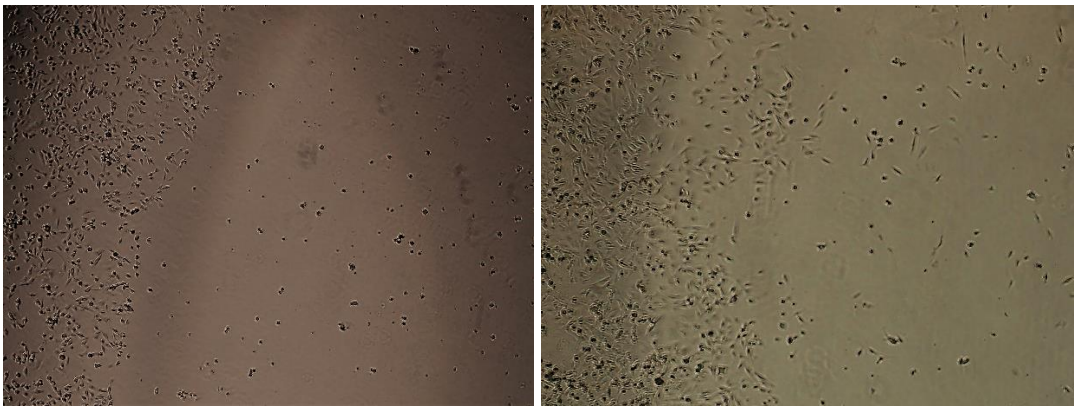
4.2.2. In-vitro cell migration assay

To confirm the functions of the transfected metastasis suppressor miRNAs, specifically to monitor its effect on cell migration, in-vitro cell migration assay was done. Control of non-transfected cells was performed. The figures below show the migration degree of the cells in 72 hours observed by light microscopy (4X), the photos were taken at (4X) magnification to observe the cell migration on a large space from the well. The figures (4.8, 4.9, 4.10, and 4.11) respectively, show the migration degree of the transfected cells in comparison with the control of the transfected cells, in 72 hours observed by light microscopy. Obviously, the migration level was inhibited after the transfection of the three miRNAs separately (“hsa-miR-145-5p”, “hsa-miR-143-3p”, and “hsa-miR-99A-5p”). This illustrates that every one of the three miRNAs consider anti-metastatic miRNA.



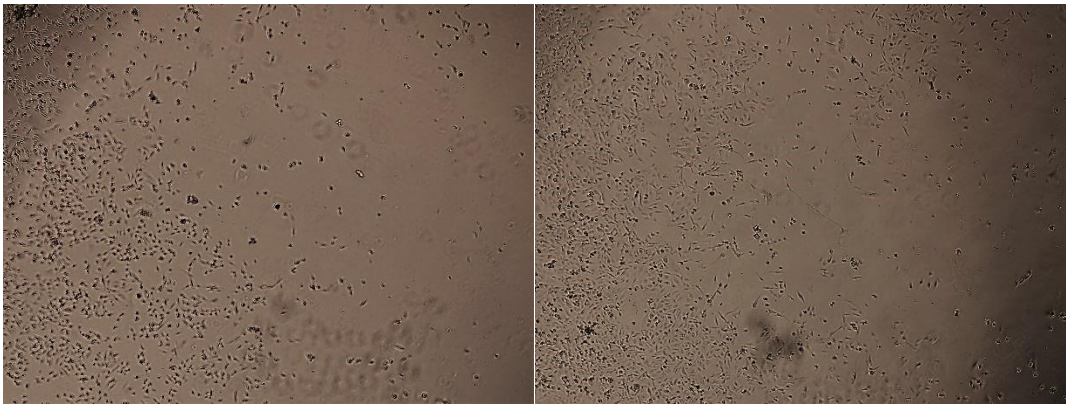
A **B**

Figure 4.8. *Shows the migration degree in 72 hours, for the transfected cells of "hsa-miR-145-5p" mimics into MDA-MB-231 cells, observed by light microscopy (4X). The image (A), shows the cell on the first day when the glass pieces were taken off to leave an empty space (gap) in the middle surrounded by the cells. The image (B), shows the cell on the third day after removing the glass slide. The migration degree is observed through comparing between image (A) and (B).*



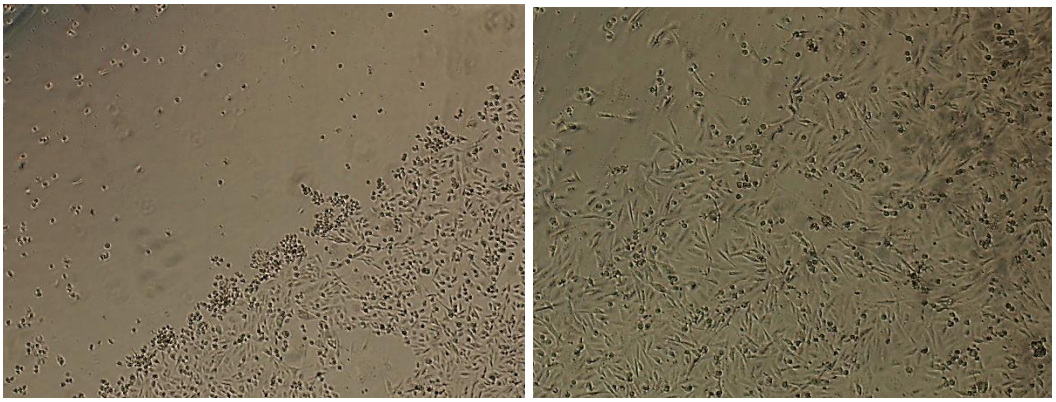
A **B**

Figure 4.9. *Shows the migration degree in 72 hours, for the transfected cells of "hsa-miR-143-3p" mimics into MDA-MB-231 cells, observed by light microscopy (4X). The image (A), shows the cell on the first day when the glass pieces were taken off to leave an empty space (gap) in the middle surrounded by the cells. The image (B), shows the cell on the third day after removing the glass slide. The migration degree is observed through comparing between image (A) and (B).*



A **B**

Figure 4.10. *Shows the migration degree in 72 hours, for the transfected cells of "hsa-miR-99A-5p" mimics into MDA-MB-231 cells, observed by light microscopy (4X). The image (A), shows the cell on the first day when the glass pieces were taken off to leave an empty space (gap) in the middle surrounded by the cells. The image (B), shows the cell on the third day after removing the glass slide. The migration degree is observed through comparing between image (A) and (B).*



A **B**

Figure 4.11. *Shows the migration degree in 72 hours, for the Control (non-transfected) MDA-MB-231 cells, observed by light microscopy (4X). The image (A), shows the cell on the first day when the glass pieces were taken off to leave an empty space (gap) in the middle surrounded by the cells. The image (B), shows the cell on the third day after removing the glass slide. The migration degree is observed through comparing between image (A) and (B).*

4.3. Microarray Data Analysis

This technique was done to analyze the effect of the transfected miRNAs at the transcription level, aiming to identify the whole genome gene expression change happened to the cells, after the substitution of the metastasis suppressor.

"GeneChip® Human Genome U133Plus 2.0 Array", from Affymetrix microarray technology was used, it monitors the expression level of 54675 genes simultaneously, in order to remove the non-biological variances a preprocessing step was done after normalization and filtering steps.

4.3.1. Preprocessing Evaluation

Boxplot diagrams of intensity data for each array was performed, before and after the normalization step. The quantile normalization method aims to make the intensity distributions in the samples identical, therefore this plot allows us to evaluate the preprocessing step. Figure 4.12, and figure 4.13 shows boxplot diagrams of gene expression on each array before and after the normalization step. Obviously, all the intensity distribution in the arrays were identical after normalization, which means all the non-biological differences between the arrays were removed after the normalization step.

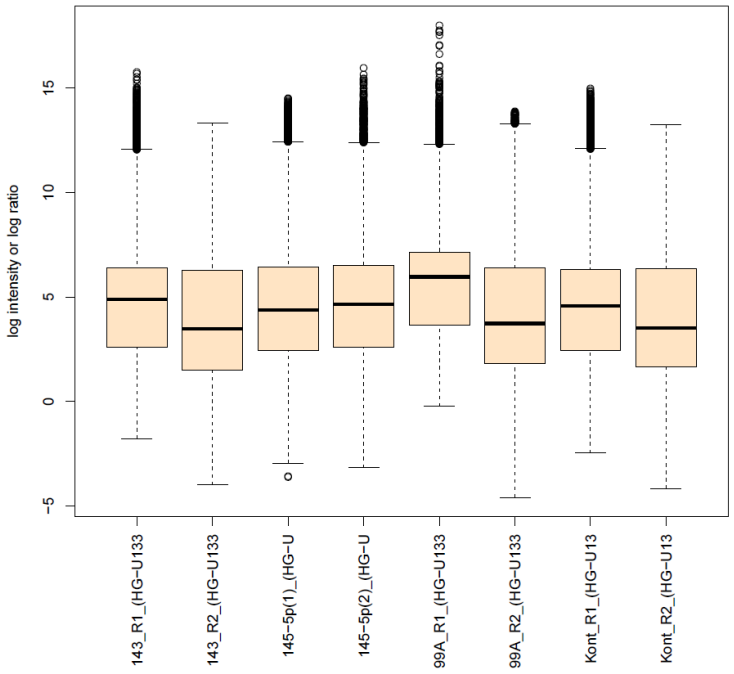


Figure 4.12. *Boxplot diagrams of gene expression on each array before the normalization step.*

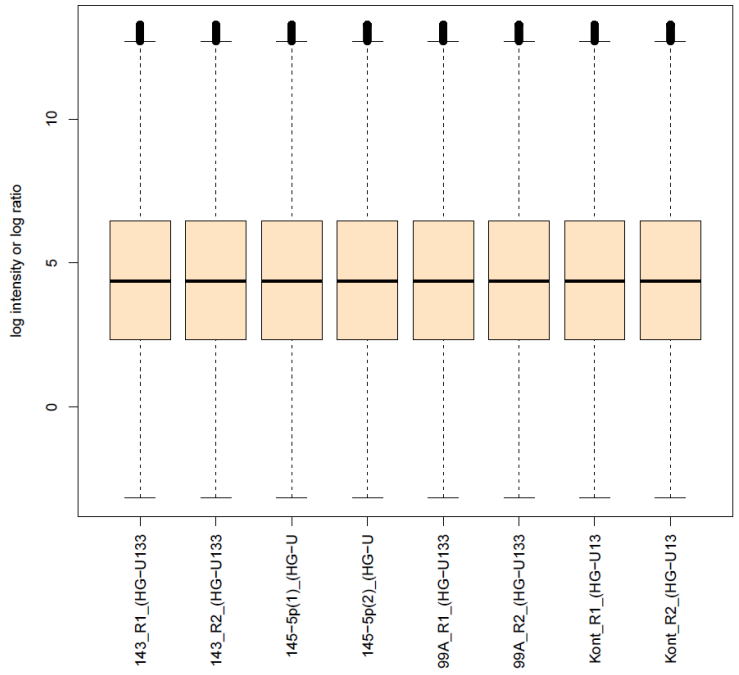


Figure 4.13. *Boxplot diagrams of gene expression on each array after the normalization step.*

4.3.2. Visualization and biological interpretation results

4.3.2.1. *Scatter plot*

Scatter plot was applied on all the genes that passing the filtering criteria, it visualizes the differences in gene expression between the arrays. Figure 4.14, Figure 4.15, and Figure 4.16 respectively shows scatter plot of the phenotype average between two classes, control and cells transfected with “hsa-miR-145-5p”, “hsa-miR-143-3p”, and “hsa-miR-99A-5p” miRNA mimics respectively. They were done to compare the general expression patterns between the Control and the transfected cells. The genes above the diagonal represent the upregulated genes, while the genes below the diagonal represent the downregulated genes.

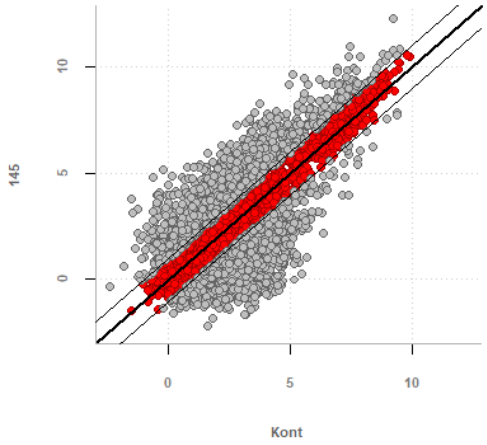


Figure 4.14. *Scatter plot of the phenotype average between two classes, control and cells transfected with “hsa-miR-145-5p” miRNa mimic.*

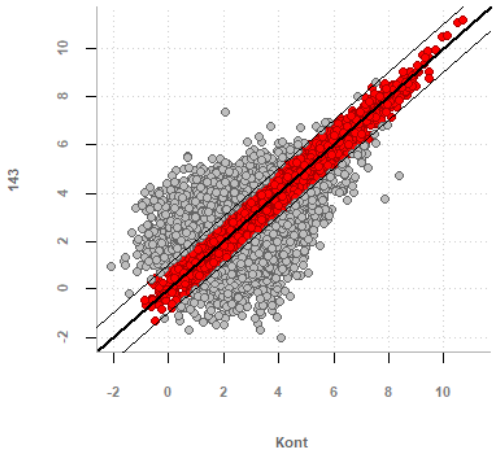


Figure 4.15. *Scatter plot of the phenotype average between two classes, control and cells transfected with "hsa-miR-143-3p" miRNa mimic.*

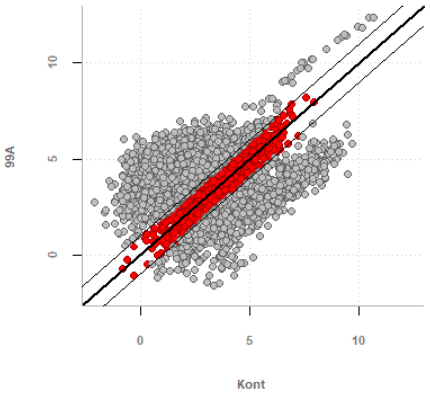


Figure 4.16. *Scatter plot of the phenotype average between two classes, control and cells transfected with "hsa-miR-99A-5p" miRNa mimic.*

4.3.2.2. *Analysis of the comparison between control cells and the cells transfected with "hsa-miR-145-5p"*

From The class comparison analysis, the differentially expressed gene set was extracted based on univariate test's (p-value), the cut-off value was 0.01, the gene number was: 277gene. Figure 4.17 shows the volcano plot diagram of the differentially expressed gene, and Figure 4.18 illustrate the clustered heatmap of them. In addition, GO annotation analysis were done for the gene list of 0.01 cut-off p-value of the differentially expressed gene set, the analysis of biological process, cellular component and molecular function category is represented in figure 4.19.

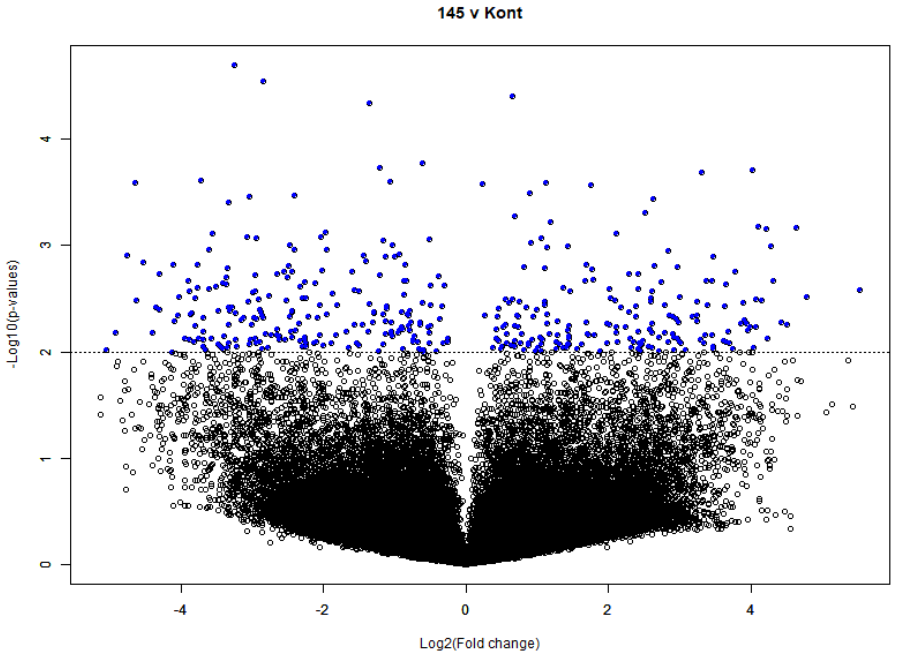


Figure 4.17. *The volcano plot diagram of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-145-5p" miRNA mimic. The p-value cut off was at 0.01.*

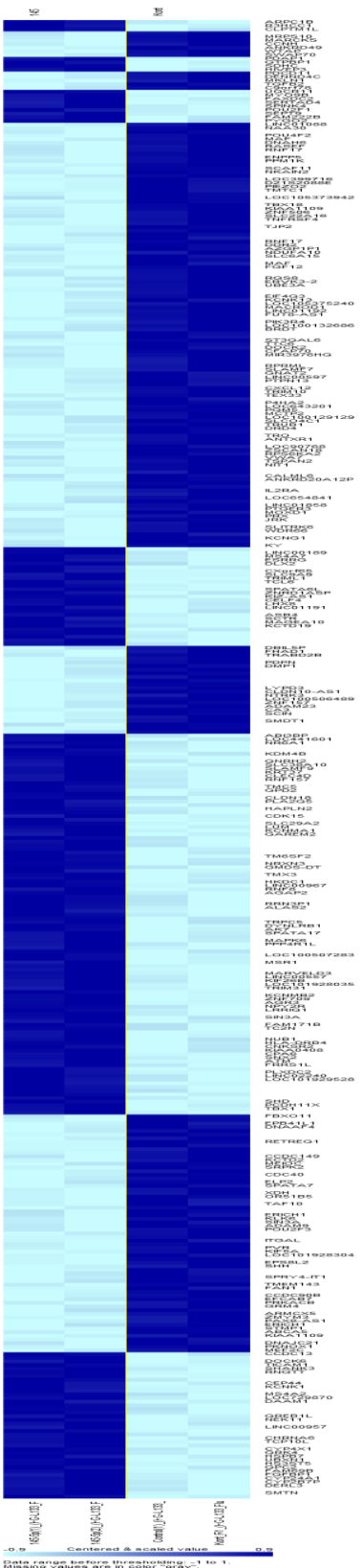


Figure 4.18. *Clustered heatmap of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-145-5p" miRNA mimic.*

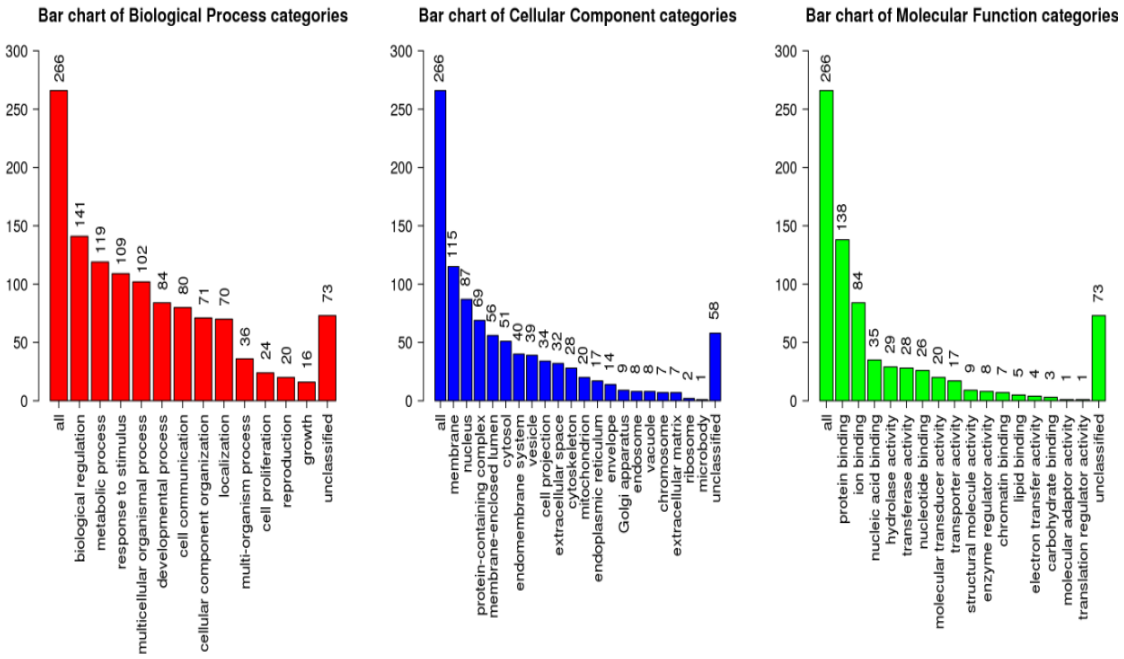


Figure 4.19. *GO annotation result of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-mir-145-5" mirna mimic. biological process, cellular component and molecular function category is represented respectively.*

The lists of the top 10 down-regulated genes, and top 10 up-regulated genes are shown in Table 4.8 and Table 4.9 respectively. The lists based on fold change values, from the class comparison analysis between control and cells transfected with "hsa-miR-145-5p" mimic. Furthermore, visual Network Analysis were done for each of the two tables, to extract the genes that have the strongest relationships to genes in the uploaded list (top 10 up or down-regulated list), and in order to understand the network result a "gene set overrepresentation analysis" for the extracted genes (the strongly related genes) was done, the cancer-related pathways were considered and all the result have pathways involved in cancer and cancer metastasis.

Table 4.8. *The list of the top 10 down-regulated genes based on fold change values from the class comparison analysis between control and cells transfected with "hsa-miR-145-5p" mimic.*

NO	Gene Symbol	Gene Name	Fold Change	p-value	Pathways
1	SLC22A16	solute carrier family 22 member 16	-33.42	0.0095421	
2	ZNF506	zinc finger protein 506	-30.433	0.006656	
3	TJP2	tight junction protein 2	-24.915	0.000253	Kegg Pathways: Tight junction
4	TNFRSF4	TNF receptor superfamily member 4	-24.718	0.003297	Kegg Pathways: Cytokine-cytokine receptor interaction
5	TRO	Trophinin	-20.391	0.003821	
6	EIF4G3	eukaryotic translation initiation factor 4 gamma 3	-19.711	0.0040456	BioCarta Pathways: mTOR Signaling Pathway
7	POU4F2	POU class 4 homeobox 2	-17.194	0.0015214	
8	KCNK13	potassium two pore domain channel subfamily K member 13	-17.067	0.0051048	
9	PIEZO2	piezo type mechanosensitive ion channel component 2	-16.5	0.0045766	
10	P4HA2	prolyl 4-hydroxylase subunit alpha 2	-16.47	0.0030449	KEGG Pathways: 1: Arginine and proline metabolism 2: Metabolic pathways

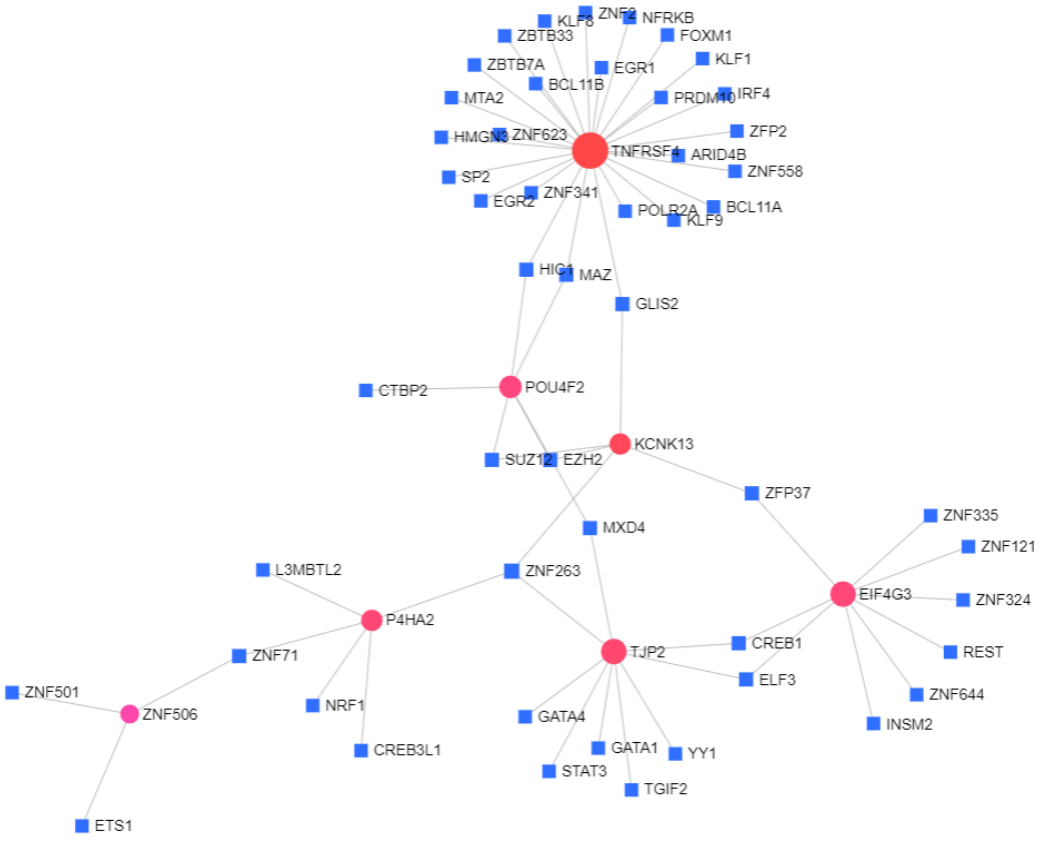


Figure 4.20. *The visual network analysis of top 10 down-regulated genes, from the class comparison analysis between control and cells transfected with "hsa-miR-145-5p" mimic.*

The cancer-related pathways result, from the gene set overrepresentation analysis of the extracted genes that have the strongest relationships to the top 10 up-regulated genes was:

- Melanogenesis
- Dorso-ventral axis formation
- Pathways in cancer
- Prostate cancer

Table 4.9. *The list of the top 10 up-regulated genes based on fold change values from the class comparison analysis between control and cells transfected with "hsa-miR-145-5p" mimic.*

NO	Gene Symbol	Gene Name	Fold Change	p-value	Pathways
1	TBX1	T-box 1	27.459	0.003077	
2	AGR3	anterior gradient 3, protein disulphide isomerase family member	24.855	0.0006755	
3	NPY2R	neuropeptide Y receptor Y2	21.324	0.0052904	KEGG Pathways: 1: Neuroactive ligand-receptor interaction
4	LRRIQ1	eucine rich repeats and IQ motif containing 1	19.679	0.0021352	
5	ZNF709	zinc finger protein 709	19.528	0.0010072	
6	SIN3A	SIN3 transcription regulator family member A	18.701	0.0074564	
7	KCNMB2	potassium calcium-activated channel subfamily M regulatory beta subunit 2	18.478	0.0006973	KEGG Pathways: Vascular smooth muscle contraction
8	SHD	Src homology 2 domain containing transforming protein D	17.184	0.0006606	
9	DERL3	derlin 3	16.796	0.0058866	KEGG Pathways: Protein processing in endoplasmic reticulum
10	PCDH11X	protocadherin 11 X-linked	16.729	0.0032079	

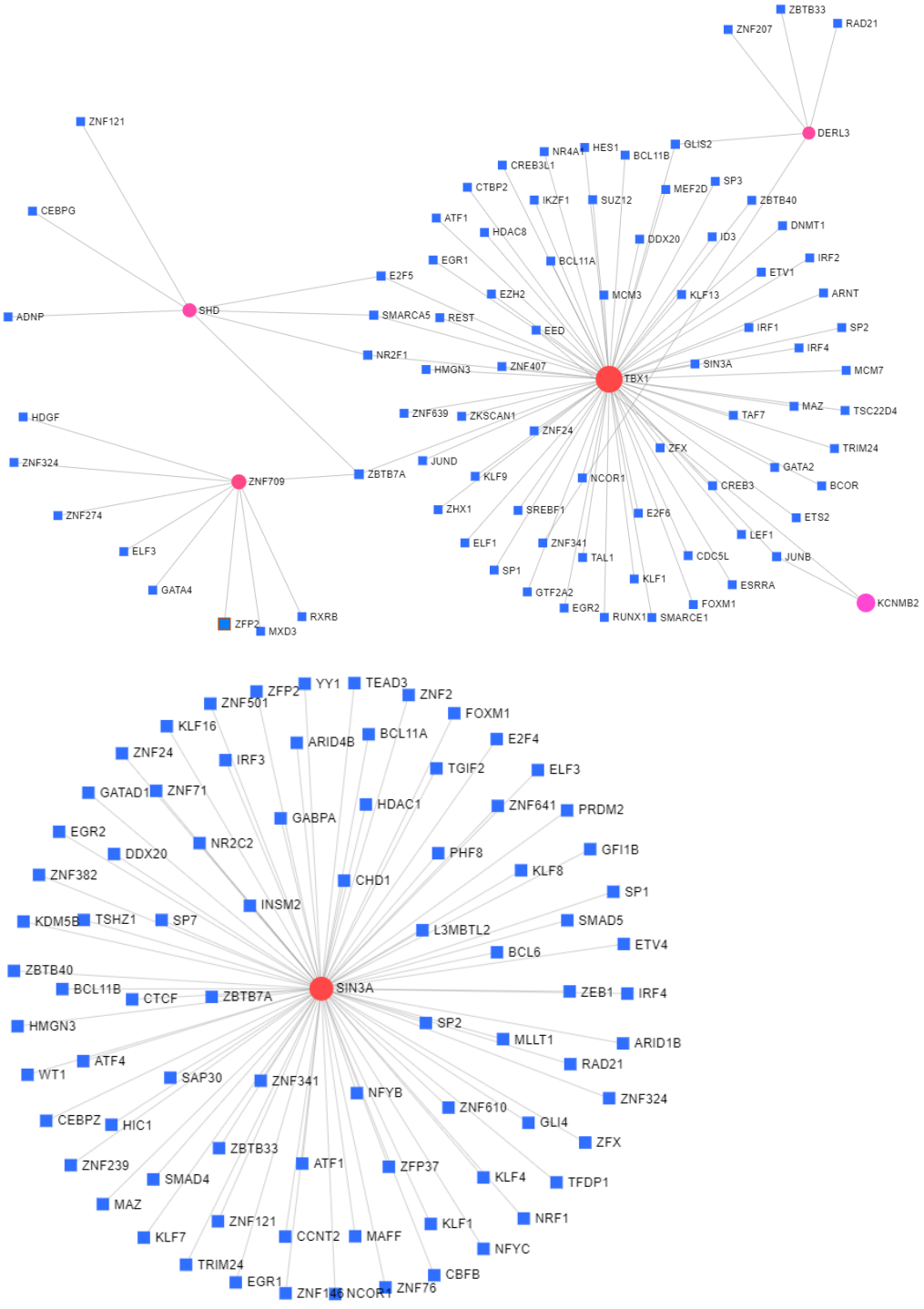


Figure 4.21. *The visual network analysis of top 10 up-regulated genes, from the class comparison analysis between control and cells transfected with "hsa-miR-145-5p" mimic.*

The cancer-related pathways result, from the gene set overrepresentation analysis of the extracted genes that have the strongest relationships to the top 10 up-regulated genes was:

- TGF-beta signaling pathway
- Cell cycle
- Transcriptional misregulation in cancer
- Prostate cancer
- Notch signaling pathway
- Pathways in cancer
- Thyroid cancer

Specifically, to analyze the effect of the transfection of “hsa-miR-145-5p” into MDA-MB-231 cell lines at the molecular mechanisms level and specially to understand the anti-metastatic mechanism of the transfected miRNA, through analyzing its effect on the whole genome expression profile, and obtain the regulated signaling pathway, gene set overrepresentation analysis was done for the differentially expressed gene set that extracted from the “class comparison analysis” based on univariate test’s (p-value), the cut off value was (0.01).

Table 4.10. *The pathway enrichment result of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-145-5" miRNA mimic.*

Enrichment Results	Significant regulated Genes in the enrichment Results	Fold change
Hedgehog Signaling Pathway	protein kinase cAMP-activated catalytic subunit beta (PRKACB)	-3.539
	sonic hedgehog signaling molecule (SHH)	-4.017
Chemokine signaling pathway	C-X-C motif chemokine ligand 12 (CXCL12)	-7.801
	dedicator of cytokinesis 2 (DOCK2)	-10.545
	G protein-coupled receptor kinase 4 (GRK4)	2.151
	protein kinase cAMP-activated catalytic subunit beta (PRKACB)	-3.539
Cell Cycle	ATM serine/threonine kinase (ATM)	12.462
	cyclin H (CCNH)	-1.198
	transforming growth factor beta 2 (TGFB2)	-1.877

4.3.2.3. *Analysis of the comparison between control cells and the cells transfected with "hsa-miR-143-3p"*

From The class comparison analysis, the differentially expressed gene set was extracted based on univariate test's (p-value), the cut off value was 0.01, the gene number was: 254 genes. Figure 4.22 shows the volcano plot diagram of the differentially expressed gene, and figure 4.23 illustrate the clustered heatmap of them. In addition, GO annotation analysis were done for the gene list of 0.01 cutt-off p-value of the differentially expressed gene set, the analysis of biological process, cellular component and molecular function category is represented in figure 4.24.

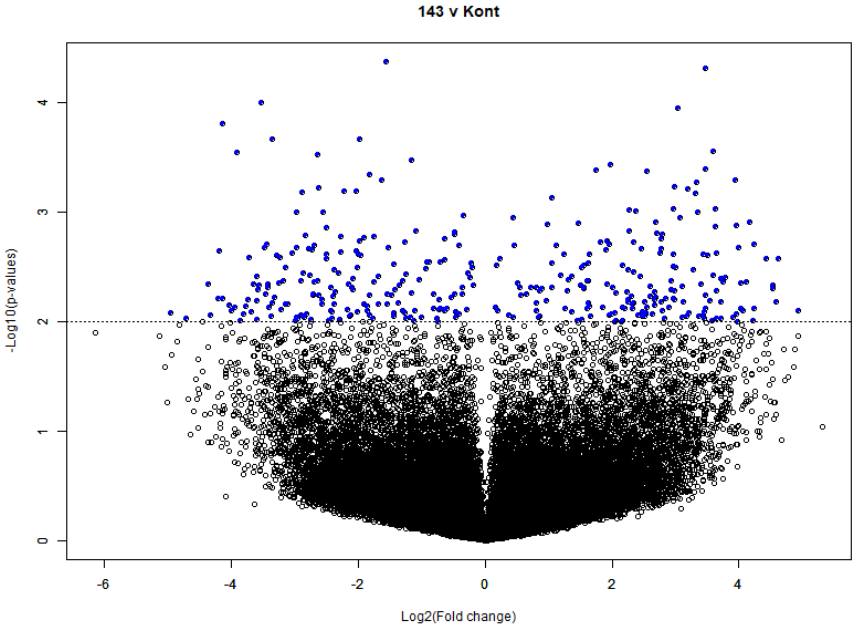


Figure 4.22. *The volcano plot diagram of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-143-3p" miRNA mimic. The p-value cut off was at 0.01.*

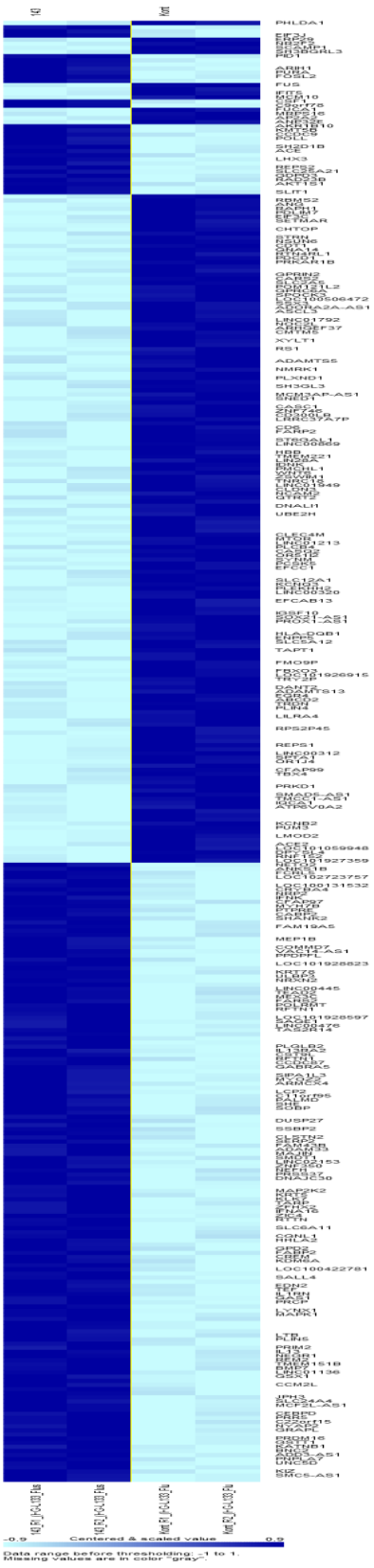


Figure 4.23. *Clustered heatmap of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-143-3p" miRNA mimic.*

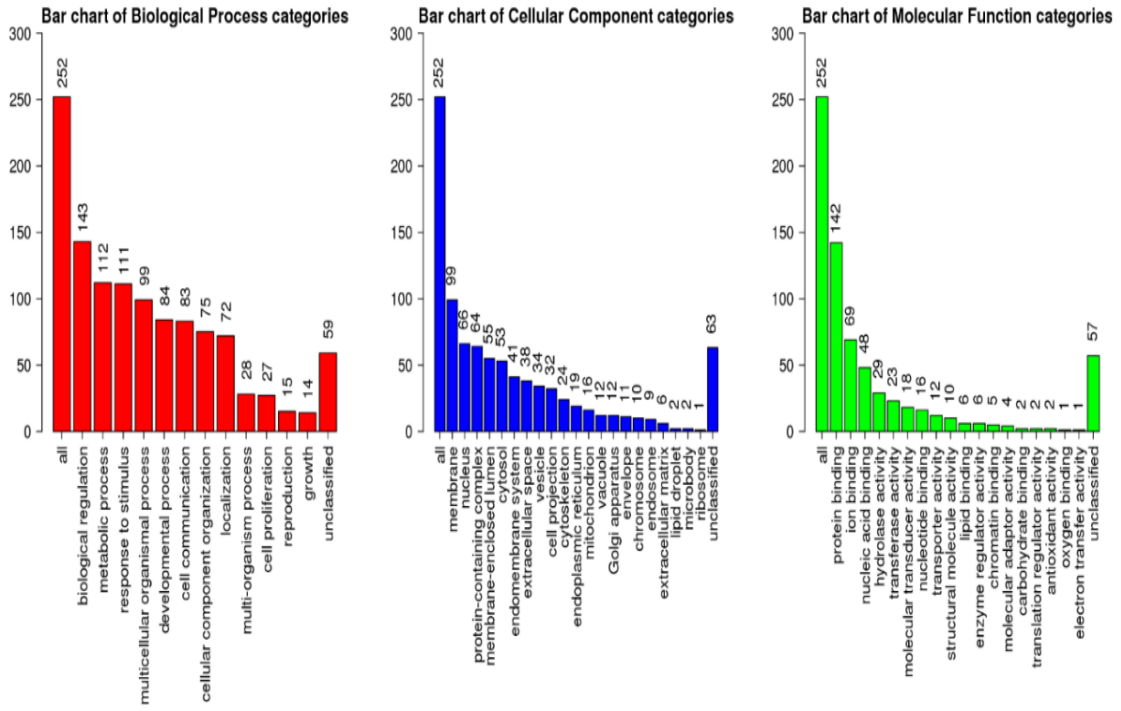


Figure 4.24. *GO annotation result of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-143-3p" miRNA mimic. biological process, cellular component and molecular function category is represented respectively.*

The lists of the top 10 down-regulated genes, and top 10 up-regulated genes are shown in Table 4.11 and Table 4.12 repectively. The lists based on fold change values, from the class comparison analysis between Control and cells transfected with "hsa-miR-145-5p" mimic. Furthermore, visual Network Analysis were done for each of the two tables, to extract the genes that have the strongest relationships to genes in the uploaded list (top 10 up or down-regulated list), and in order to understand the network result a "gene set overrepresentation analysis" for the extracted genes (the strongly related genes) was done, the cancer-related pathways were considered and all the result have pathways involved in cancer and cancer metastasis.

Table 4.11. *The list of the top 10 down-regulated genes based on fold change values from the class comparison analysis between control and cells transfected with "hsa-miR-143-3p" mimic*.

NO	Gene Symbol	Gene Name	Fold Change	p-value	Pathways
1	PLCB4	phospholipase C beta 4	-31.319	0.0082111	KEGG Pathways: 1: Calcium signaling pathway 2: Chemokine signaling pathway 3: Gap junction 4: Inositol phosphate metabolism
2	LILRA4	leukocyte immunoglobulin like receptor A4	-20.732	0.0045443	
3	RPS2P45	ribosomal protein S2 pseudogene 45	-18.537	0.0061192	
4	RNF152	ring finger protein 152	-18.325	0.0022224	
5	DPYSL4	dihydropyrimidinase like 4	-17.581	0.0001525	
6	RTN4RL1	reticulon 4 receptor like 1	-16.432	0.0071149	
	SLC5A12	solute carrier family 5 member 12	-15.914	0.0079671	
8	SOX21-AS1	SOX21 antisense divergent transcript 1	-14.955	0.0002828	
9	TAPT1	transmembrane anterior posterior transformation 1	-14.622	0.0098019	
10	ACE2	angiotensin I converting enzyme 2	-12.263	0.0095399	KEGG Pathways: Protein digestion and absorption

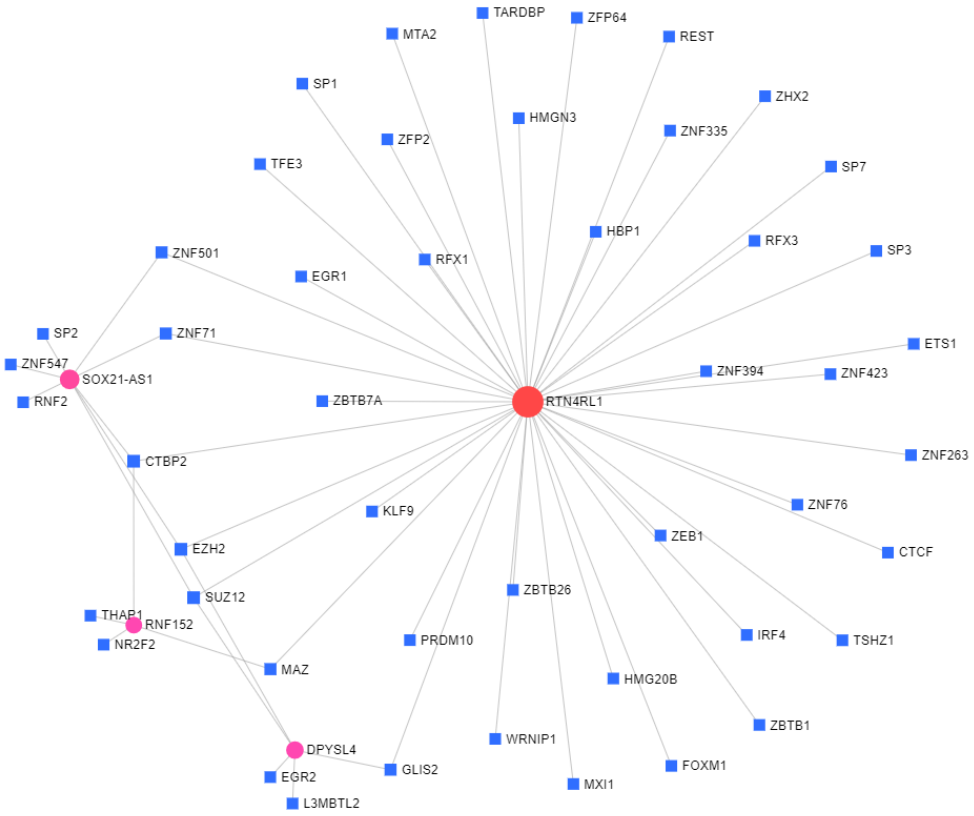


Figure 4.25. *The visual network analysis of top 10 down-regulated genes, from the class comparison analysis between control and cells transfected with "hsa-miR-143-3p" mimic.*

The cancer-related pathways result, from the gene set overrepresentation analysis of the extracted genes that have the strongest relationships to the top 10 up-regulated genes was:

- Dorso-ventral axis formation
- Pathways in cancer
- Notch signaling pathway
- Renal cell carcinoma
- TGF-beta signaling pathway
- Wnt signaling pathway

Table 4.12. *The list of the top 10 up-regulated genes based on fold change values from the class comparison analysis between control and cells transfected with "hsa-miR-143-3p" mimic.*

NO	Gene Symbol	Gene Name	Fold Change	p-value	Pathways
1	MEX3C	mex-3 RNA binding family member C	24.633	0.0026722	
2	FARS2	phenylalanyl-tRNA synthetase 2, mitochondrial	23.927	0.0065591	KEGG Pathways: Aminoacyl-tRNA biosynthesis
3	FAM19A5	family with sequence similarity 19 member A5, C-C motif chemokine like	22.935	0.0049956	
4	SHANK2	SH3 and multiple ankyrin repeat domains 2	22.919	0.0045984	
5	CABP2	calcium binding protein 2	21.494	0.0026198	
6	TEAD2	TEA domain transcription factor 2	18.568	0.009658	
7	POLRMT	RNA polymerase mitochondrial	17.951	0.0012204	
8	LINC00445	long intergenic non-protein coding RNA 445	16.744	0.0079617	
9	PTPRE	protein tyrosine phosphatase, receptor type E	16.262	0.0075978	
10	MYH7B	myosin heavy chain 7B	16.039	0.0021129	

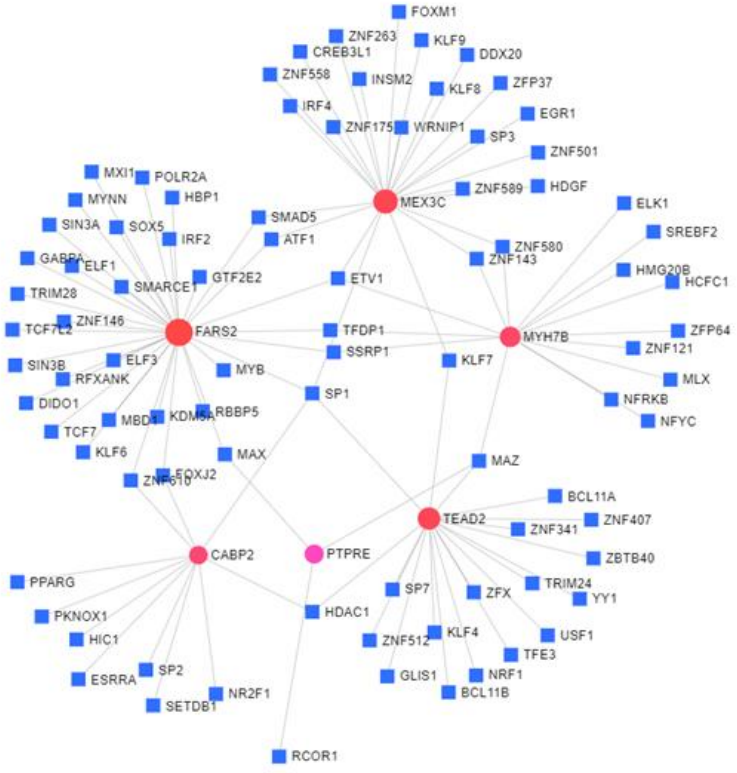


Figure 4.26. *The visual Network Analysis of top 10 up-regulated genes, from the class comparison analysis between control and cells transfected with "hsa-miR-143-3p" mimic.*

The cancer-related pathways result, from the gene set overrepresentation analysis of the extracted genes that have the strongest relationships to the top 10 up-regulated genes was:

- Transcriptional misregulation in cancer
- Thyroid cancer
- Endometrial cancer
- TGF-beta signaling pathway
- Prostate cancer
- Pathways in cancer
- Basal cell carcinoma
- Colorectal cancer
- Adherens junction

Specifically, to analyze the effect of the transfection of "hsa-miR-143-3p" into MDA-MB-231 cell lines at the molecular mechanisms level and specially to understand the anti-metastatic mechanism of the transfected miRNA, through analyzing its effect on the whole genome expression profile, and obtain the regulated signaling pathway, gene set overrepresentation analysis was done for the differentially expressed gene set that extracted from the "class comparison analysis" based on univariate test's (p-value), the cut off value was (0.01).

Table 4.13. *The pathway enrichment result of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-143-3p" miRNA mimic.*

Enrichment Results	Significant regulated Genes in the enrichment Results	Fold change
Target Of Rapamycin (TOR) Signaling	AKT1 substrate 1(AKT1S1) mechanistic target of rapamycin kinase (MTOR) proline rich 5 (PRR5)	2.576 -3.91 6.52
Epithelial to mesenchymal transition in colorectal cance	claudin 3 (CLDN3) mitogen-activated protein kinase kinase 2 (MAP2K2) mitogen-activated protein kinase 1 (MAPK1) neuropilin 2 (NRP2) Wnt family member 6 (WNT6)	-2.36 2.93 12.418 3.021 -1.764
Cytokines and Inflammatory Response	colony stimulating factor 1 (CSF1) interleukin 13 (IL13)	2.08 4.756

4.3.2.4. *Analysis of the comparison between control cells and the cells transfected with "hsa-miR-99A-5p"*

From The class comparison analysis, the differentially expressed gene set was extracted based on univariate test's (p-value), the cut off value was 0.01, the gene number was: 188 genes. Figure 4.27 shows the volcano plot diagram of the differentially expressed gene, and figure 4.28 illustrate the clustered heatmap of them. In addition, GO annotation analysis were done for the gene list of 0.01 cutt-off p-value of the

differentially expressed gene set, the analysis of biological process, cellular component and molecular function category is represented in figure 4.29.

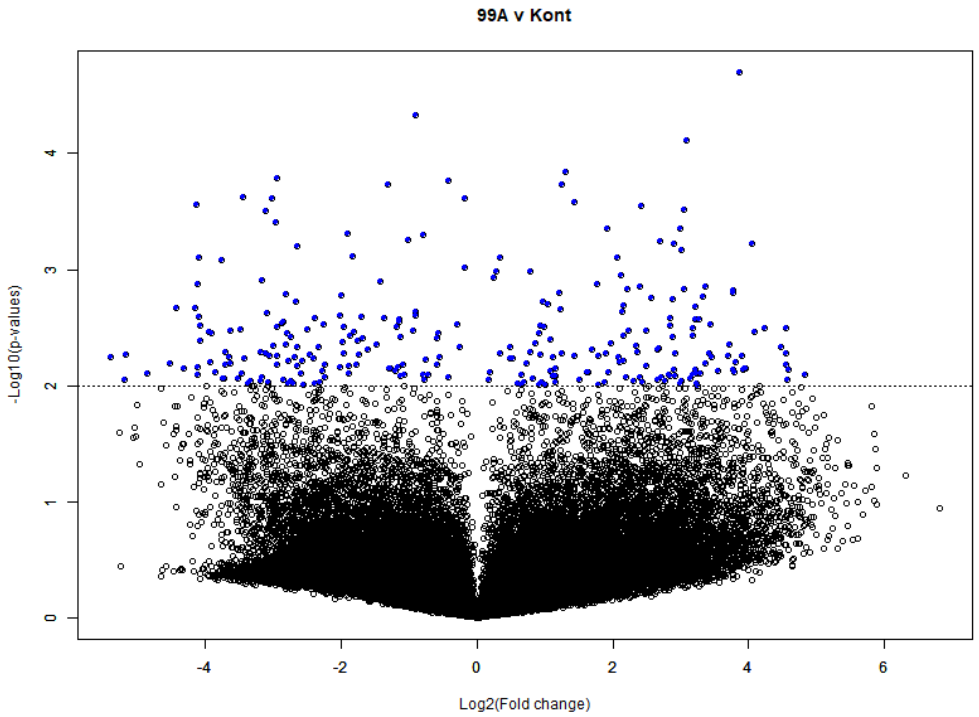


Figure 4.27. *The volcano plot diagram of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-99A-5p" miRNA mimic. The p-value cut off was at 0.01.*

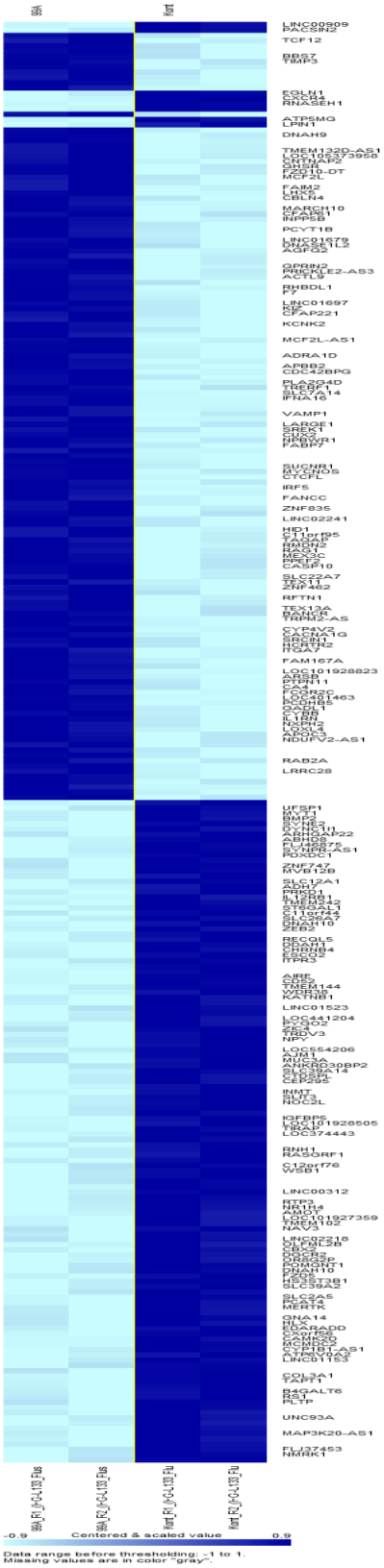


Figure 4.28. *Clustered heatmap of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-99A-5p" miRNA mimic.*

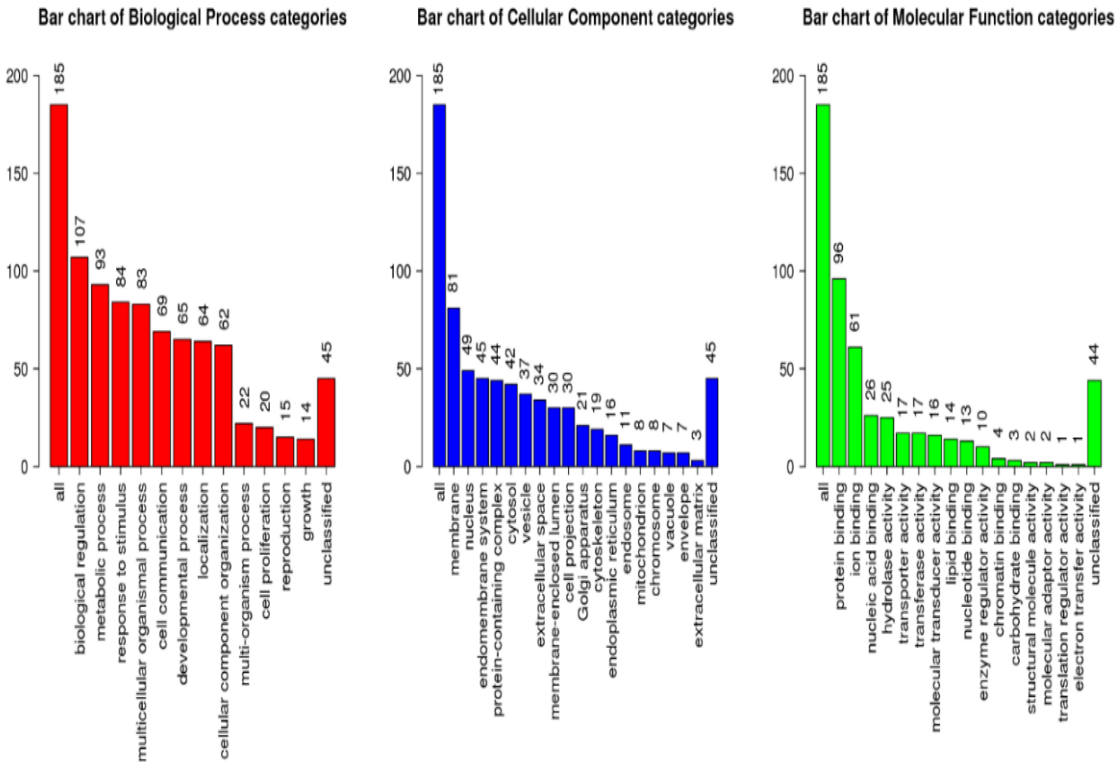


Figure 4.29. *GO annotation result of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-99A-5" miRNA mimic. biological process, cellular component and molecular function category is represented respectively.*

The lists of the top 10 down-regulated genes, and top 10 up-regulated genes are shown in Table 4.14 and Table 4.15 repectively. The lists based on fold change values, from the class comparison analysis between Control and cells transfected with "hsa-miR-145-5p" mimic. Furthermore, visual Network Analysis were done for each of the two tables, to extract the genes that have the strongest relationships to genes in the uploaded list (top 10 up or down-regulated list), and in order to understand the network result a "gene set overrepresentation analysis" for the extracted genes (the strongly related genes) was done, the cancer-related pathways were considered and all the result have pathways involved in cancer and cancer metastasis.

Table 4.14. *The list of the top 10 down-regulated genes based on fold change values from the class comparison analysis between control and cells transfected with "hsa-miR-99A-5p" mimic.*

NO	Gene Symbol	Gene Name	Fold Change	p-value	Pathways
1	NMRK1	Nicotinamide riboside kinase 1	-36.26	0.0054153	**KEGG Pathways:** Nicotinate and nicotinamide metabolism
2	RS1	Retinoschisin 1	-23.026	0.0064635	
3	NOC2L	NOC2 like nucleolar associated transcriptional repressor	-21.717	0.0021682	
4	SLIT3	Slit guidance ligand 3	-17.736	0.0021526	**KEGG Pathways:** Axon guidance
5	COL3A1	Collagen type III alpha 1 chain	-17.548	0.0002745	KEGG Pathways: 1: ECM-receptor interaction 2: Focal adhesion 3: Protein digestion and absorption
6	SLC39A14	solute carrier family 39 member 14	-17.396	0.0013205	
7	CTDSPL	CTD small phosphatase like	-17.393	0.0068731	
8	HLX	H2.0 like homeobox	-17.37	0.0080663	
9	TAPT1	Transmembrane anterior posterior transformation 1	-17.175	0.0007844	
10	MAP3K20-AS1	MAP3K20 antisense RNA 1	-17.024	0.0030586	

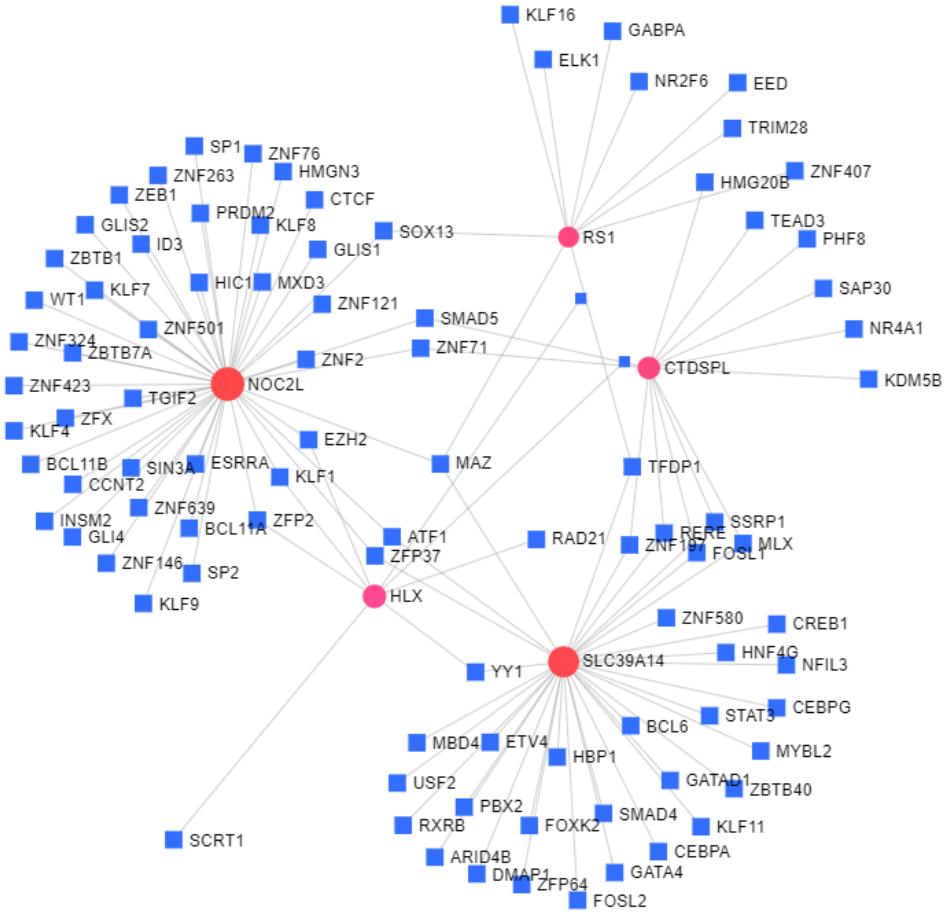


Figure 4.30. *The visual network analysis of top 10 down-regulated genes, from the class comparison analysis between control and cells transfected with "hsa-miR-99A-5p" mimic.*

The cancer-related pathways result, from the gene set overrepresentation analysis of the extracted genes that have the strongest relationships to the top 10 up-regulated genes was:

- Transcriptional misregulation in cancer
- TGF-beta signaling pathway
- Pathways in cancer
- Cell cycle
- Wnt signaling pathway
- Pancreatic cancer

Table 4.15 *The list of the top 10 up-regulated genes based on fold change values from the class comparison analysis between control and cells transfected with "hsa-miR-99A-5p" mimic.*

NO	Gene Symbol	Gene Name	Fold Change	p-value	Pathways
1	ITGA7	Integrin subunit alpha 7	28.044	0.0081103	KEGG Pathways: 1: ECM-receptor interaction 2: Focal adhesion 3: Regulation of actin cytoskeleton
2	CA4	Carbonic anhydrase 4	23.912	0.0089284	KEGG Pathways: Nitrogen metabolism
3	FAM167A	Family with sequence similarity 167 member A	23.238	0.0052625	
4	ARSB	Arylsulfatase B	22.378	0.0046701	KEGG Pathways: 1: Glycosaminoglycan degradation 2: Lysosome 3: Metabolic pathways
5	PTPN11	Protein tyrosine phosphatase, non-receptor type 11	16.993	0.0034512	BioCarta Pathways: 1: Insulin Signaling Pathway 2: IGF-1 Signaling Pathway 3: IL 6 signaling pathway KEGG Pathways: 1: Jak-STAT signaling pathway 2: Natural killer cell mediated cytotoxicity
6	PCDHB5	Protocadherin beta 5	16.517	0.0005951	
7	HID1	HID1 domain containing	15.618	0.0070489	
8	TAGAP	T cell activation RhoGTPase activating protein	15.155	0.007184	
9	FCGR2C	Fc fragment of IgG receptor IIc (gene/pseudogene)	14.902	0.0055777	KEGG Pathways: Phagosome
10	RAG1	Recombination activating 1	13.943	0.0062856	KEGG Pathways: Primary immunodeficiency

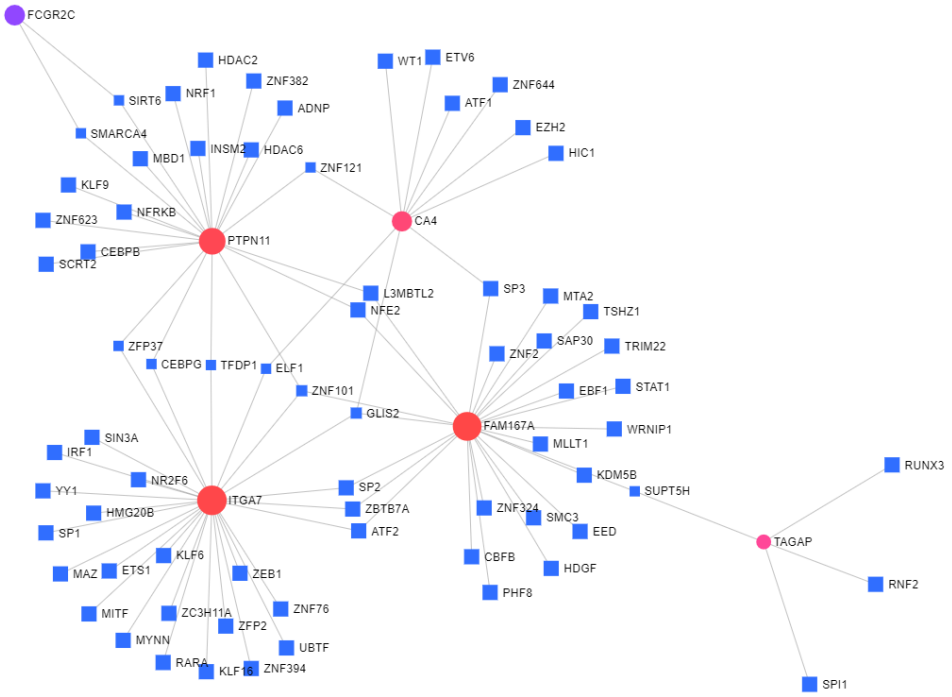


Figure 4.31. *The visual network analysis of top 10 up-regulated genes, from the class comparison analysis between control and cells transfected with "hsa-miR-99A-5p" mimic.*

The cancer-related pathways result, from the gene set overrepresentation analysis of the extracted genes that have the strongest relationships to the top 10 up-regulated genes was:

- Transcriptional misregulation in cancer
- Dorso-ventral axis formation
- Pathways in cancer
- Cell cycle
- Renal cell carcinoma
- TGF-beta signaling pathway

Specifically, to analyze the effect of the transfection of "hsa-miR-99A-5p" into MDA-MB-231 cell lines at the molecular mechanisms level and specially to understand the anti-metastatic mechanism of the transfected miRNA, through analyzing its effect on the whole genome expression profile, and obtain the regulated signaling pathway, gene set overrepresentation analysis was done for the differentially expressed gene set that extracted from the "class comparison analysis" based on univariate test's (p-value), the cut off value was (0.01).

Table 4.16. *The pathway enrichment result of the differentially expressed gene set from the class comparison analysis between control and cells transfected with "hsa-miR-99A-5" miRNA mimic.*

Enrichment Results	Significant regulated Genes in the enrichment Results	Fold change
Type II interferon signaling (IFNG)	Cytochrome b-245 beta chain (CYBB) protein tyrosine phosphatase, non-receptor type 11 (PTPN11)	10.8 16.99
Methylation Pathways	Indolethylamine N-methyltransferase (INMT)	-15.164
MFAP5-mediated ovarian cancer cell motility and invasiveness	inositol 1,4,5-trisphosphate receptor type 3 (ITPR3)	-3.069
Hereditary leiomyomatosis and renal cell carcinoma pathway	Egl-9 family hypoxia inducible factor 1 (EGLN1)	-1.868
Angiogenesis	TIMP metallopeptidase inhibitor 3 (TIMP3)	1.134
Apoptosis	caspase 10 (CASP10) nterferon regulatory factor 5 (IRF5)	13.64 4.65

4.4. Validation of the Microarray Result at the Protein Level

Differential expression of selected genes (*p-mTOR, MMP2,* and *MMP9*) were validated at the protein level by western blot and zymogram.

4.4.1. Western blot result

Western blot analysis was done for the gene of interest *p-mTOR*, and *B-Actin* as a control protein.

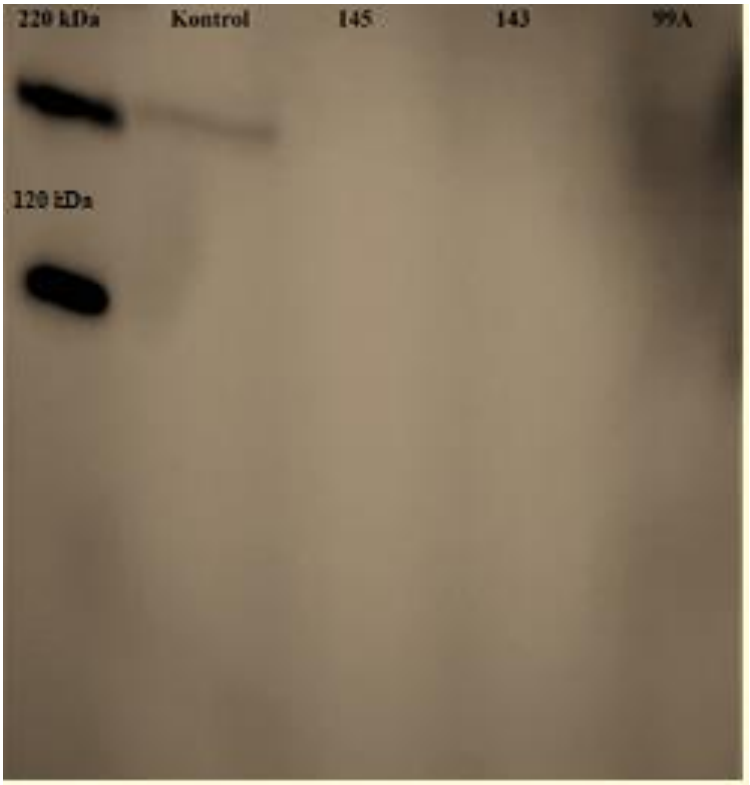


Figure 4.32. *The western blot result for (p-mTOR) protein of the control and the transfected samples. (control, hsa-miR-145-5p, hsa-miR-143-3p, and hsa-miR-99A-5p samples respectively).*

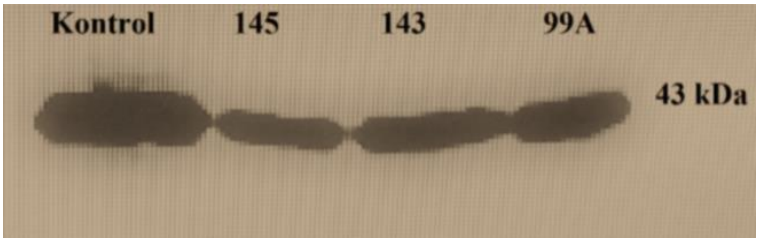


Figure 4.33. *The western blot result for the control protein (β-actin) of the control and the transfected samples. (control, hsa-miR-145-5p, hsa-miR-143-3p, and hsa-miR-99A-5p samples respectively).*

In the western blot result, *mTOR* protein is detected in the control (untransfected cells sample), and there were no band revealed for the samples that were transfected with hsa-miR-145-5p, hsa-miR-143-3p, and hsa-miR-99A-5p separately. Accordingly, at the protein level, the *mTOR* is down-regulated after the transfection of the three metastasis suppressors miRNAs separately. To interpret this result with the microanalysis result, *mTOR* was also significantly down-regulated in the comparison analysis of the

transfection of “hsa-miR-143-3p” with the fold change value = (-3.91), and p-value = (0.0024607). In the transfection of “has-miR-145-5p” the down-regulation of *EIF4G3*, as we assumed above, it correlates with the down-regulation of *mTOR*.

4.4.2. Gelatin zymography result

Gelatin zymography analysis were done for the genes of interest *MMP2*, and *MMP9* as a control protein.

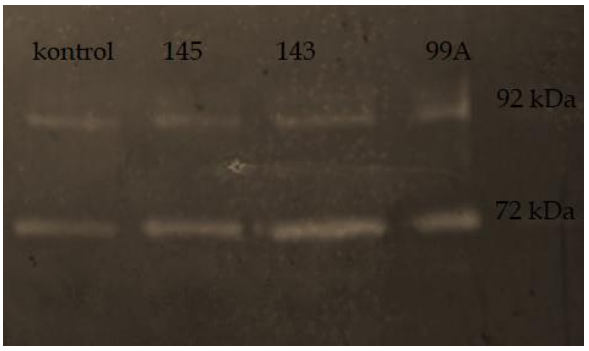


Figure 4.34. *The Gelatin zymography result for the (MMP2: 72 kDA) , and (MMP9: 92 kDa) proteins of the control and the transfected samples. (control, hsa-miR-145-5p, hsa-miR-143-3p, and hsa-miR-99A-5p samples respectively).*

In the gelatin zymography result, *MMP9* and *MMP2* proteins were detected in all the transfection experiments the same as the control, the *MMP2* of miR-143 sample is detected with high intensity.

In microarray result, in the transfection of “hsa-miR-143-3p”, the gene *LILRA4* as previously mentioned, has a role of inhibiting *TIMP3*, as it is down-regulated therefore MMPs will not be inhibited. And this supports the zymography result in the miR-143, in miR99A result, *TIMP3* gene was up-regulated with a fold change value = (1.134), so possibly this mechanism still doesn’t affect the protein level.

5. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. Discussion

Despite the developments in cancer treatment, it's still among one of the most mortality causing diseases, especially when it is invading the other parts of the body (Metastasis), and there is a need to find a promising biological therapy to treat metastasis cancer without affecting the healthy cells.

miRNA based therapy is a promising strategy in cancer treatment in the current biomedical researches, particularly miRNA replacement therapy, which aim to restore the function of a repressed endogenous miRNA in the cancerous cells, through inserting a chemically synthesized miRNA mimic. Therefore, it is important to identify the tumor suppressive miRNAs in every type of cancer. Many studies in the literature presenting the effect of one miRNA in a type of cancer, through studying some samples to identify the repressed miRNA. This study identified specific metastasis suppressor miRNA in breast cancer through meta-analysis method, which can be used as a therapeutic target in miRNA-based breast cancer therapy to prevent or delay breast cancer metastasis. As meta-analysis integrates results from different studies and from different centers, time, and platforms, increases the statistical power of the results, so a statistically significant Meta-miRNA list is obtained. It is based on ranking according to two features (the t-test probability (p-value), and the logFC value), additionally, two types of comparison were applied (normal Breast tissue vs breast cancer) and (Breast cancer vs metastatic breast cancer).

The second objective of the study is to present the therapeutic ability of the three identified metastasis suppressor miRNAs, in preventing or delaying cancer metastasis in breast cancer, through in-vitro miRNA replacement therapy. Many studies in the literature present the anti-tumor and the anti-metastatic effect of the identified miRNAs. In a review study about miR-145 and its effect on cancer metastasis, it was presented the ability of mir-145 to inhibit metastasis by targeting of multiple oncogenes including *MUC1, FSCN1, Vimentin, Cadherin, Fibronectin, Metadherin, GOLM1, ARF6, SMAD3, MMP11, Snail1, ZEB1/2, HIF-1α and Rock-1*, this review presented many studies reported the effect of one or two oncogenes in several types of cancer (Zeinali, et. al., 2019). Also it was reported to act as tumor suppressor in in gallbladder cancer (Letelier, et. al., 2014), in lung adenocarcinoma (Zhao, et. al., 2013) and its association of metastasis in colorectal cancer (Yuan, et. al., 2014). Moreover, it was reported to

suppress metastasis by targeting Mucin1 gene in TNBC cell lines (Sachdeva, and Mo, 2010), also a study on the same cell line, it was reported that it inhibits proliferation and migration by regulating *TGF-β1*(Ding, et.al., 2017). Another study found that targeting of *MTDH* by miR-145-5p or "miR-145-3p" is associated with prognosis and regulates the growth and metastasis of prostate cancer cells (Pan, et. al., 2019). Regarding miR-143, it was presented to be suppresses in gastric cancer and has reported to induce apoptosis by targeting cox-2 (Wu, et. al., 2013). Also in oral squamous cell carcinoma, miR-143 was reported to inhibit indirectly p53 (Manikandan, et. al., 2015). In Breast cancer, miR-143 regulates tumor proliferation and the apoptosis by targeting *MYBL2* (Chen, and Chen, 2018), In addition, through targeting LIM domain kinase 1 suppresses the progression of triple-negative breast cancer cells (Li, et. al., 2017). "miR-99a-5p" act as tumor suppressor, via targeting *mTOR* in human urinary bladder urothelial carcinoma (Tsai, et. al. 2018), and in Lung adenocarcinoma (Gu, et. al., 2013). furthermore, it was reported to suppress tumor metastasis by regulating *IGF1R* in oral squamous cell carcinoma (Yen, et. al.,2014). In breast cancer, the over expression of miR-99a suppress the proliferation through regulating "insulin-like growth factor 1" (Xia, et.al. 2016). In (TNBC) breast cancer cells, it was reported to inhibit proliferation by regulating HOXA1 (Wang, et. al., 2015), and through targeting *mTOR* (Hu, Zhu, and Tang, 2014). According to studies in the literature presented above of the identified miRNAs, the anti-tumor and anti-metastatic effect was reported in breast cancer and in many other cancer types. But all of the literature was studying the effect in one gene or two through quantitative real time PCR, dual luciferase reporter assay, or western blot, the molecular mechanism is not presented. Furthermore, since cancer metastasis is a complex and multistep process, the only way for successful treatment might be in targeting multiple genes in different steps simultaneously, some miRNAs may have more than 100 target genes (Ibrahim, et al., 2011), this is emphasizing the therapeutic potential of the miRNAs as unique regulators of multiple target genes. In this context, it is worthwhile to investigate the effect of the transfected miRNAs on the whole genomes expression profile of the cells in order to understand the biological molecular mechanism they regulate, and to investigate if they have unknown negative effects on the cells. Accordingly, it can be considered as a therapeutic target. In this respect, gene expression microarray analysis was done. In the microarray comparison analysis result, the differentially expressed genes between control cells and the cells transfected with "hsa-miR-145-5p", "hsa-miR-

143-3p", and "hsa-miR-99A-5p" were extracted. The top 10 down and up regulated genes obtained from the class comparison analysis were shown separately in tables (4.8, 4.9, 4.11, 4.12, 4.14, and 4.15). Then aiming to extract the genes that have the strongest relationships (interactions with transcription factor genes) in the top 10 up or down-regulated lists a network visual analysis was done for each table, represented in figures (4.20, 4.21, 4.25, 4.26, 4.30, and 4.31) respectively. In order to analyze the network results, "gene set overrepresentation analysis" was done for the extracted genes (the strongly related genes), in the results the cancer-related pathways were considered, the pathway lists result have pathways involved in cancer and cancer metastasis in all the Network analysis results.

Specifically, to analyze the effect of every miRNA, from the general aspect, and to understand its effect in the whole genome expression profile, moreover, to obtain the regulated signaling pathway, and to understand the anti-metastatic mechanism of the transfected miRNAs, gene set overrepresentation analysis was done, for the differentially expressed gene sets that extracted from the "class comparison analysis", the gene set filtering, based on univariate test's (p-value) with the cut off value of (0.01). In the GO enrichment result from the gene set overrepresentation analysis, each of the biological process, cellular component, and molecular function categories are represented by a red, blue and green bar, respectively in figures (4.19, 4.24, and 4.29) for the three miRNAs comparison analysis, this means that all the differentially expressed gene sets that were affected after the transfection of the three miRNAs separately "hsa-miR-145-5p", "hsa-miR-143-3p", and "hsa-miR-99a-5p" relatively have the same molecular functions, involved in the same biological process, and from the same cellular components.

1. After transfection with "hsa-miR-145-5p" mimic:

To summarize the results of the gene set obtained from the class comparison analysis, between control and cells transfected with "hsa-miR-145-5p" mimic; we will analyze the top 10 up-regulated, and top 10 down-regulated gene tables and the pathway enrichment result.

<u>According to top 10 down-regulated gene list (Table 4.8)</u>:

SLC22A16 is a transport protein, it is suppression means fewer proteins are transported, the death of some cells after the transfection, may lead to the decrease of its expression in comparison with the control cells. The *KCKN13* and *PIEZO2* genes are

components in ion channels, they may have a role in signaling functions, as ion channels help in generating second messenger or they can function as effector by responding to such messenger (Berridge, 2012). *ZNF506, POU4F2*, and *EIF4G3* are transcription factors and their suppression mean suppress of the transcription of their target genes, in the network analysis their strongly related genes are mainly also transcription factors. In addition, *EIF4G3* has a role in the initiation of the protein translation process in eukaryotes through *mTOR* signaling pathway and it is expression stimulated by the phosphorylated *mTOR* (Showkat, Beigh, and Andrabi, 2014), as a result of this, it can be assumed that *mTOR* also down-regulated. *TNFRSF4* is a member of the TNF-receptor superfamily, the summary of this gene in "NCBI database" mentioned that "this receptor activates NF-kappaB through its interaction with adaptor proteins TRAF2 and TRAF5, knockout studies in mice suggested that this receptor promotes the expression of apoptosis inhibitors *BCL2* and *BCL2lL1/BCL2-XL* and thus suppresses apoptosis (NCBI Gene Database). Accordingly, the suppression of this receptor prevents apoptosis suppression.

P4HA2 which has an effect on the synthesis of some metabolic components is the key enzyme in collagen synthesis (NCBI Gene Database). Collagens are essential for tumor angiogenesis. Inhibition of collagen metabolism has been demonstrated to have an antiangiogenic effect (Fang. et al. 2014). *TRO* is an invasion promoter and it is studied in testicular, colon and lung cancers (Fukuda, Sugihara, and Nakayama, 2008). *TJP2* is a tight junction protein and the loss of the tight junctions leads to metastasis (Martin, and Jiang, 2009).

In the top 10 up-regulated genes (Table 4.9):

It includes transcription factors like *TBX1, ZNF709* and *SIN3A* which is a member of a transcription regulatory family. *ARG3* and *DERL3* are member of endoplasmic reticulum proteins, they have a role in protein folding and modifications. *KCNMB2* gene is a member of Potassium Calcium-Activated Channel Subfamily M and *NPY2R* protein function in "G protein-coupled receptor activity and calcium channel regulator activity" (GeneCards database), they both have signaling functions. *PCDH11X* is potential calcium-dependent cell adhesion protein (UniProtKB database). The up-regulation of these genes implies that some signaling is triggered and new proteins are transcribed and translated in the cells.

The pathway enrichment analysis result (Table 4.10):

The main pathways that deregulated after the transfection of "hsa-miR-145-5p", shows that it suppresses the metastasis through the suppression of the Hedgehog Signaling Pathway (HSP). HSP is an important pathway in embryonic development, it has known of its role in cancer metastasis and it has many transcriptional targets involved in cell proliferation, invasion, migration, angiogenesis and EMT (Bailey, Singh, and Hollingsworth, 2007). "hsa-miR-145-5p" mimic suppresses the cell proliferation and angiogenesis through suppression of *TGFB2*. In the pathway results also chemokine signaling pathway is mentioned, the main genes included *CXCL12* are down-regulated, this is emphasizing the suppression of metastasis because it has been proved that *CXCL2* promotes metastasis in breast cancer (Ahirwar, et al., 2018).

2. After transfection with "hsa-miR-143-3p" mimic:

In the results of the gene set obtained from the class comparison analysis between control and cells transfected with "hsa-miR-143-3p" mimic;

In the top 10 down-regulated genes (Table 4.11):

PLCB4 protein has a role in the production of the second messenger molecules diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3) (UniProtKB database). *TAPT1* gene is annotated in GeneCards database as "growth hormone-releasing hormone receptor activity" and *ACE2* gene which is annotated at the same database as "metallopeptidase activity and peptide binding". According to these functions, the down-regulation of these genes can be a result of cell growth stoppage. *RPS2P45* is ribosomal protein S2 pseudogene 45, a non-coding RNA gene, and *SOX21-AS1* also is a non-coding RNA gene which associated with Oral Squamous Cell Carcinoma and Colorectal Cancer (GeneCards database), the down-regulation of these genes may inhibit some non-coding RNAs which is playing a role in gene regulation.

SLC5A12 is protein transporter, another protein transporter gene is also down-regulated, in the transfection of "hsa-miR-145-5p" results. *LILRA4* functions "as a negative regulator of *TLR7* and *TLR9* signaling cascades" (UniProtKB database), *TLR9* has been considered as invasion inducer in many types of cancer, it induces invasion by inhibiting *TIMP-3*, the inhibitor of matrix metalloproteinases (Sandholm, and Selander, 2014). Considering this, the downregulation of *LILRA4* may inhibit *TIMP3* gene, and

this could not affect the matrix metalloproteinases, but the up-regulation of *TLR7* and *TLR9* may have a role in antitumor immunity because, in the functional test (migration assay) the transfection of miR-143-3p inhibited the invasion and migration level.

TN4RL1 gene's related pathways are metabolism of proteins and post-translational modification, synthesis of GPI-anchored proteins" (GeneCards database). *DPYSL4* is necessary for signaling by class 3 semaphorins and subsequent remodeling of the cytoskeleton (UniProtKB database). *RNF152* gen's related pathways from (GeneCards database) are metabolism of proteins and mTOR signaling pathway. Considering the information of these genes from the databases, their down-regulation is presenting anti-tumor effects.

In the top 10 up-regulated genes (Table 4.12):

MEX3C is an RNA-binding ubiquitin E3 ligase and *LINC00445* is a non-coding RNA class gene, the up-regulation of both genes may present the role of "has-miR-143-3p" in gene regulation by regulating other non-coding RNAs. *FARS2* and *POLRMT* genes, are coding mitochondrial proteins. PTPRE and CABP2 both proteins have a role in the signaling processes. *TEAD2* is a transcription factor, *FAM19A5* a member of chemokine (C-C motif)-like family expressed in the brain, acts as a regulator of the immune and nervous cells (NCBI Gene database). *SHANK2* gene is a tumor suppressor in neuroblastoma (Conkrite, et al., 2015). *MYH7B* is a myosin stimulate cell-cell interaction in epithelial cells, the deficiency in this protein can lead to losing the contact inhibition of the cells, and promoting invasion, further investigations are needed on the role of myosin in cancer, it may differ depending on its isoform, and on the cell type (Ouderkirk, and Krendel, 2014).

The pathway enrichment analysis result (Table 4.13):

The main pathways that deregulated after the transfection of "hsa-miR-143-3p" are activating cytokines and inflammatory response pathway that can stimulate apoptosis. Additionally, the Epithelial to mesenchymal transition pathway is shown to be inhibited through the suppression in *WNT6* and *CLDN3*. The target of rapamycin (TOR) signaling also is down-regulated through the down-regulation of *mTOR* gene.

3. After transfection with "hsa-miR-99a-3p" mimic:

Results of the gene set obtained from the class comparison analysis, between control and cells transfected with "hsa-miR-99A-5p" mimic;

In the top 10 down-regulated genes (Table 4.14):

In this result, a protein transporter *SLC39A14* is suppressed. *MAPK3K20-AS1* is a non-coding RNA gene (GeneCards database). *TAPT1* gene is also down-regulated in the "hsa-miR-143-3p" result. In "has-miR-145-5p" miRNA result, the gene responsible for collagen synthesis is down-regulated. In this list, *COL3A1* is down-regulated and as it mentioned above this inhibits angiogenesis, also as collagen is the major component of the ECM, and because ECM can mediate dual roles as tumor suppressors at the early stages, but as tumor promoters at the later stages of tumor progression (Fang, et al., 2014). This emphasizes that the down regulation of collagen associated with inhibiting cancer metastasis process.

Also, *NMRK1* gene which is responsible for some metabolic components synthesis is suppressed in this analysis. *SLIT3* gene act as molecular guidance cue in cellular migration (UniProtKB database). *HLX* is a transcription factor (UniProtKB database). *RS1* encodes an extracellular protein which has a role in cell-cell adhesion processes (UniProtKB database), it means the decreasing in this gene's expression will inhibit the migration process. *NOC2L* is a gene play a major role in transcriptional regulations through histone modification by histone acetyltransferases (HAT) and histone deacetylases (HDAC), (NCBI Gene database). *CTDSPL* gene negatively regulates RNA polymerase II transcription (UniProtKB database) which is the enzyme responsible for the transcription of miRNAs, it means that some miRNAs will be suppressed after the transfection of "hsa-miR-99A-3p" miRNA.

In the top 10 up-regulated list (table 4.15):

ITGA7 integrin alpha chain family, which has a role in cell-cell and cell-matrix interactions (NCBI Gene database). *CA4* is involved in nitrogen metabolism pathway. Also, *ARSB* is related to the metabolism of proteins pathway (GeneCards database). *PCDHB5* is the other family member of this gene and also up-regulated after transfection with "has-miR-145-5p" miRNA whereas *FAM167A* in "has-miR-143-3p" miRNA result.

PTPN11 is a signaling molecule that plays a role in many signaling pathways like, JAK-STAT signaling pathway and natural killer cell mediated cytotoxicity" (KEGG database). *HID1* gene reported being down-regulated in many human cancers (Harada, et al., 2001). Therefore, the upregulation of *HID1* after the miRNA transfection causes the gaining of its function back like in the non-cancerous cells. *TAGAP* encodes a member of the Rho GTPase-activator protein superfamily, this gene has a role in T cell activation related to many signaling pathways. *FCGR2C* is annotated as a transmembrane signaling receptor activity and IgG binding" (GeneCards database). *RAG1* gene is involved in primary immunodeficiency pathway. The up-regulation of these genes associated with the anti-tumor effect of the transfected miRNA.

The main pathways (Table 4.16) that deregulated after the transfection of "hsa-miR-99A-5p" are:

The activation of Type II interferon signaling (IFNG) and activation of apoptosis through the up-regulation of *CASP10* and *IRF5* genes. The inhibition of methylation pathways and angiogenesis is through the activation of *TIMP* metallopeptidase inhibitor3.

In order to validate the microarray results, selected genes were validated at the protein level by western blot and zymogram. The selected genes are *p-mTOR*, *MMP2*, and *MMP9*. *MMP2* and *MMP9* are members of the matrix metalloproteinase gene family, of zinc and calcium dependent endopeptidase (Li, et al., 2017), they are associated in degrading collagen and elastin components of the ECM, during cancer invasion and metastasis processes. Regarding *mTOR*, this gene is involved in the regulation of many cellular processes like, protein synthesis, growth, and survival (Lee, 2017). And because relevant results regarding those genes were in the microarray results. Also, because those genes have relevant results in the microarray analysis, they selected to be validated. In the western blot result, *mTOR* protein is detected in the control (untransfected cells sample) and there were no band revealed for the samples that were transfected with hsa-miR-145-5p, hsa-miR-143-3p, and hsa-miR-99A-5p, respectively. Accordingly, the protein level of *mTOR* is down-regulated after the transfection of the three metastasis suppressors miRNAs. To interpret this result with the microarray analysis result, *mTOR* was also significantly downregulated in the comparison analysis of the transfection of "hsa-miR-143-3p" with the fold change value; -3.91 and p-value; 0.0024607.

In the transfection of “has-miR-145-5p”, the down-regulation of *EIF4G3* correlates with the down-regulation of *mTOR*. Regarding the gelatin zymography, *MMP9* and *MMP2* proteins were detected in all the transfection experiments the same as the control, the *MMP2* of miR-143 sample is detected with high intensity. In the microarray result, after the transfection of “hsa-miR-143-3p the downregulation of *LILRA4* may inhibit *TIMP3* gene, and this could not affect the matrix metalloproteinases. These data support the zymography result in the miR-143. In miR99A result, *TIMP3* gene was up-regulated with a fold change value; 1.134, so possibly this mechanism still doesn’t affect the protein level. Western blot and zymography analysis validate the microarray experiment result.

5.2. Conclusion

In conclusion, as miRNA based cancer therapy have a promising potential, this study has identified a specific metastasis suppressor miRNA through meta-analysis method, which can be used as a therapeutic target in miRNA-based breast cancer therapy to prevent or delay breast cancer metastasis, and also this study is presented the therapeutic ability of the three identified metastasis suppressor miRNAs “hsa-miR-145-5p”, “hsa-miR-143-3p”, and “hsa-miR-99a-5p” in preventing or delaying cancer metastasis in breast cancer through in-vitro miRNA replacement therapy. Result of in-vitro cell migration assay was revealed to functional validation of the effect of the transfection and replacement therapy. Additionally, since a single miRNA can regulate many genes involved in different signaling pathways, we analyzed the effect of the transfected miRNAs at the transcription level with microarray analysis, through exploring the effect of the transfected miRNAs on the whole genome’s expression profile of the cells, in order to understand the biological molecular mechanisms that they regulate, the top 10 up and down regulated genes have been investigated, and the regulated signaling pathways lists has obtained. Differential expression of selected genes is validated at the protein level by western blot and zymogram. Our results suggest that each of the three metastatic suppressor miRNAs is affecting different biological pathways in tumorigenesis. Also, the therapeutic potentials of “hsa-miR-145-5p”, “hsa-miR-143-3p”, and “hsa-miR-99a-5p” in breast cancer are observed, showing the tumor-suppressive functionalities.

5.3. Recommendations

Metastasis is a complex and multistep process, so successful treatment might be in targeting multiple genes in different steps simultaneously. Thus, for miRNA-based therapy it could be more effective if the identified three miRNAs used together as a therapeutics complex for the suppression of tumorigenesis and metastasis. Additionally, a further investigation of the identified metastasis suppressor miRNAs transcription mechnism, could help to understand their endogenous transcription mechanism and a therapy targeting their transcription factors could be used.

REFERENCES

Ab, H. (2010). Meta-analysis in medical research. *Hippokratia*, *14*(1), 29–37.

Abba, M., Patil, N., and Allgayer, H. (2014). Review MicroRNAs in the regulation of MMPs and Metastasis. Cancers Journal,6, 625-645; doi: 10.3390/cancers6020625.

Ahirwar, D. K., Nasser, M. W., Ouseph, M. M., Elbaz, M., Cuitiño, M. C., Kladney, R. D., … Ganju, R. K. (2018). Fibroblast-derived CXCL12 promotes breast cancer metastasis by facilitating tumor cell intravasation. *Oncogene*, *37*(32), 4428–4442. https://doi.org/10.1038/s41388-018-0263-7.

Ahmadzada, T., Reid, G., & McKenzie, D. R. (2018). Fundamentals of siRNA and miRNA therapeutics and a review of targeted nanoparticle delivery systems in breast cancer. *Biophysical Reviews*, 10(1), 69–86. https://doi.org/10.1007/s12551-017-0392-1.

Alizadeh, A. M., Shiri, S., & Farsinejad, S. (2014). *Metastasis review: from bench to bedside. Tumor Biology, 35(9), 8483–8523.*doi:10.1007/s13277-014-2421-z.

Almeida, M. I., Reis, R. M., & Calin, G. A. (2011). MicroRNA history: Discovery, recent applications, and next frontiers. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 717(1-2), 1–8.*doi:10.1016/j.mrfmmm.2011.03.009

American Cancer Society. (2016). Breast Cancer What is breast cancer ? *American Cancer Society. Cancer Facts and Figures Atlanta, Ga: American Cancer Society*, *5*, 127.

Anna, C., & Yee, Y. (2010). Comparison study of microarray meta-analysis methods. *BMC Bioinformatics*, *11*. Retrieved from http://www.doaj.org/doaj?func=abstract&id=584025.

Bader, A., G., Brown, D., and Winkler, M. (2010). The Promise of MicroRNA Replacement Therapy. Cancer Res,15; 70(18): 7027–7030. doi: 10.1158/0008-5472.CAN-10-2010.

Bailey, J. M., Singh, P. K., & Hollingsworth, M. A. (2007). Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, notch, and bone morphogenic proteins. Journal of Cellular Biochemistry, 102(4), 829–839. https://doi.org/10.1002/jcb.21509.

Bayraktar, S., & Glück, S. (2013). Molecularly targeted therapies for metastatic triple-negative breast cancer. Breast Cancer Research and Treatment, 138(1), 21–35. https://doi.org/10.1007/s10549-013-2421-5.

Berridge, M. J. (2012). Module 3: Ion Channels. Cell Signalling Biology, 6, csb0001003. https://doi.org/10.1042/csb0001003.

Bouyssou, J., M.C., Manier, S., Huynh, D., İssa, S., Roccaro, A., M., and Ghobrial, I., M. (2014). Biochimica et Biophysica Acta, 1845, 255-265.

Chang, L. (2018) Understanding Cancer, the Basics. WebMD Medical Reference from the American College of Physicians: "Oncology I Cancer Epidemiology and Prevention", https://www.webmd.com/cancer/understanding-cancer-basics.

Chen, J., & Chen, X. (2018). MYBL2 Is Targeted by miR-143-3p and Regulates Breast Cancer Cell Proliferation and Apoptosis. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics,* 26(6), 913–922. doi:10.3727/096504017x1513594118210

Christopher, A., Kaur, R., Kaur, G., Kaur, A., Gupta, V., & Bansal, P. (2016). MicroRNA therapeutics: Discovering novel targets and developing specific therapy. *Perspectives in Clinical Research, 7*(2), 68. https://doi.org/10.4103/2229-3485.179431.

Conkrite, K., Ferraro, N., McDaniel, L., Oldridge, D.A., … Diskin,S.J. Identification of SHANK2 as a tumor suppressor disrupted by recurrent somatic structural variation (SV) in neuroblastoma. [abstract]. In: Proceedings of the 106th Annual Meeting of the American Association for Cancer Research; 2015 Apr 18-22; Philadelphia, PA. Philadelphia (PA): AACR; Cancer Res 2015;75(15 Suppl): Abstract nr 475. doi:10.1158/1538-7445.AM2015-475 .

Ding, Y., Zhang, C., Zhang, J., Zhang, N., Li, T., Fang, J., … Sun, X. (2017). miR-145 inhibits proliferation and migration of breast cancer cells by directly or indirectly regulating TGF-β1 expression. *International Journal of Oncology, 50(5), 1701–1710*.doi:10.3892/ijo.2017.3945

Du, T., & Zamore, P. D. (2005). microPrimer: the biogenesis and function of microRNA. Development (Cambridge, England), 132(21), 4645–52. doi:10.1242/dev.02070.

Qomi Ekenel, E. (2012). Microarray Applications for Determination of the effects of Emodin on breast cancer cell lines. *A thesis submitted in partial fulfillment of the requirements for the degree of master of science, Middle East Technical University*.

Fang, M., Yuan, J., Peng, C., & Li, Y. (2014). Collagen as a double-edged sword in tumor progression. *Tumor Biology*, *35*(4), 2871–2882. https://doi.org/10.1007/s13277-013-1511-7.

Fukuda, M. N., Sugihara, K., & Nakayama, J. (2008). Trophinin: What embryo implantation teaches us about human cancer. *Cancer Biology and Therapy*, *7*(8), 1165–1170. https://doi.org/10.4161/cbt.7.8.6696.

Farazi, T. A., Spitzer, J. I., Morozov, P., Tuschl, T. (2011). miRNAs in human cancer. Journal of Pathology, 223: 102–115.

Feng, Y., Spezia, M., Huang, S., Yuan, C., Zeng, Z., Zhang, L., … Ren, G. (2018). Breast cancer development and progression: Risk factors, cancer stem cells, signaling

pathways, genomics, and molecular pathogenesis. Genes and Diseases, 5(2), 77–106. https://doi.org/10.1016/j.gendis.2018.05.001.

Griffiths-Jones, S. (2005). miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Research, 34(90001), D140–D144. https://doi.org/10.1093/nar/gkj112.

Gu, W., Fang, S., Gao, L., Tan, Y., & Yang, Z. (2013). Clinic significance of microRNA-99a expression in human lung adenocarcinoma. *Journal of Surgical Oncology*, *108*(4), 248–255. https://doi.org/10.1002/jso.23381

Habib, J. G., & O'Shaughnessy, J. A. (2016). The hedgehog pathway in triple-negative breast cancer. *Cancer Medicine*, *5*(10), 2989–3006. https://doi.org/10.1002/cam4.833.

Harada, H., Nagai, H., Tsuneizumi, M., Mikami, I., Sugano, S., & Emi, M. (2001). Identification of DMC1, a novel gene in the TOC region on 17q25.1 that shows loss of expression in multiple human cancers. Journal of Human Genetics, 46(2), 90–95. https://doi.org/10.1007/s100380170115.

Harrington, K. J. (2016). The biology of cancer. *Medicine (United Kingdom)*, *44*(1), 1–5. https://doi.org/10.1016/j.mpmed.2015.10.005

Henry, J., C., Azevedo-Pouly, A., C. P., and Schmittgen, T., D. (2011). Expert Review, microRNA Replacement Therapy for Cancer. Pharm Res, 28:3030–3042, DOI 10.1007/s11095-011-0548-9.

Ibrahim, A., F., Weirauch, U., Thomas, M., Grüunweller, A., Hartmann, R., K., and Aigner, A. (2011). MicroRNA Replacement Therapy for miR-145 and miR-33a Is Efficacious in a Model of Colon Carcinoma. Cancer Res, 71:5214-5224.

Jewell, J. L., & Guan, K. (2014). NIH Public Access, 38(5), 233–242. https://doi.org/10.1016/j.tibs.2013.01.004.Nutrient.

Ji, S., Su, X., Zhang, H., Han, Z., Zhao, Y., & Liu, Q. (2019). MicroRNA-372 functions as a tumor suppressor in cell invasion, migration and epithelial-mesenchymal transition by targeting ATAD2 in renal cell carcinoma. *Oncology Letters*, *17*(2), 2400–2408. https://doi.org/10.3892/ol.2018.9871.

Kim, J., Yao, F., Xiao, Z.,Sun, Y., and Ma, L., (2018). MicroRNAs and metastasis: small RNAs play big roles**.** Cancer Metastasis Rev. 37(1): 5–15. doi:10.1007/s10555-017-9712-y.

Martin, T. A., & Jiang, W. G. (2009). Loss of tight junction barrier function and its role in cancer metastasis. *Biochimica et Biophysica Acta - Biomembranes*, *1788*(4), 872–891. https://doi.org/10.1016/j.bbamem.2008.11.005.

Lam, J. K. W., Chow, M. Y. T., Zhang, Y., & Leung, S. W. S. (2015). siRNA versus miRNA as therapeutics for gene silencing. *Molecular Therapy - Nucleic Acids*, 4(9), e252. https://doi.org/10.1038/mtna.2015.23.

Lee, H. (2017). Phosphorylated mTOR Expression Profiles in Human Normal and Carcinoma Tissues. *Disease Markers*, *2017*, 1–8. https://doi.org/10.1155/2017/1397063.

Letelier, P., García, P., Leal, P., Álvarez, H., Ili, C., López, J., … Roa, J. C. (2014). miR-1 and miR-145 act as tumor suppressor microRNAs in gallbladder cancer. *Int J Clin Exp Pathol.7*(5), 1849–1867.

Li, D., Hu, J., Song, H., Xu, H., Wu, C., Zhao, B., … Wu, T. (2017). miR-143-3p targeting LIM domain kinase 1 suppresses the progression of triple-negative breast cancer cells. *Am J Transl Res*, *9*(5), 2276–2285.

Li, H., Qiu, Z., Li, F., & Wang, C. (2017). The relationship between MMP-2 and MMP-9 expression levels with breast cancer incidence and prognosis. *Oncology Letters*, *14*(5), 5865–5870. https://doi.org/10.3892/ol.2017.6924.

Lin, X., Shang, X., Manorek, G., & Howell, S. B. (2013). Regulation of the Epithelial-Mesenchymal Transition by Claudin-3 and Claudin-4. PLoS ONE, 8(6). https://doi.org/10.1371/journal.pone.0067496.

Lu, P., Li, H., Li, N., Singh, R. N., Bishop, C. E., Chen, X., & Lu, B. (2017). MEX3C interacts with adaptor-related protein complex 2 and involves in miR-451a exosomal sorting. PLoS ONE, 12(10), 1–25. https://doi.org/10.1371/journal.pone.0185992.

Macgregor, P. F., & Squire, J. A. (2002). Application of microarrays to the analysis of gene expression in cancer. *Clinical Chemistry*, *48*(8), 1170–1177. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12142369

Malarkey, D. E., Maronpot, R. R., & Handbook, R. (2013). *Carcinogenesis Learn more about Carcinogenesis Practice of Toxicologic Pathology*.

Manikandan, M., Kuha, A., Magendhra, D., Rajkumar, K. S., Rajaraman, R., & Munirajan, A. K. (2015). *Down Regulation of miR-34a and miR-143 May Indirectly Inhibit p53 in Oral Squamous Cell Carcinoma : a Pilot Study*. *16*, 7619–7625.

Marson, L. P., Miller, W. R., & Dixon, J. M. (1998). Angiogenesis and breast cancer. *Breast*, *7*(6), 299–307. https://doi.org/10.1016/S0960-9776(98)90071-6

Martin, T. A., & Jiang, W. G. (2009). Loss of tight junction barrier function and its role in cancer metastasis. Biochimica et Biophysica Acta - Biomembranes, 1788(4), 872–891. https://doi.org/10.1016/j.bbamem.2008.11.005.

Mollaei, H., Safaralizadeh, R., & Rostami, Z. (2019). MicroRNA replacement therapy in cancer. *Journal of Cellular Physiology*, *234*(8), 12369–12384. https://doi.org/10.1002/jcp.28058.

Nagai, A., Nagai, Y., Qu, H., and Zhang, Sh. (2007). Dynamic Behaviors of Lipid-Like Self-Assembling Peptide A6D and A6K Nanotubes. Journal of Nanoscience and Nanotechnology, 7, 1–7.

Nussbaum, R. L., McInnes, R. R., Willard, H. F., Hamosh, A., & Thompson, M. W. (2007). Thompson & Thompson genetics in medicine. Philadelphia: Saunders/Elsevier.

Okegawa, T., Pong, R.-C., Li, Y., & Hsieh, J.-T. (2004). The role of cell adhesion molecule in cancer progression and its application in cancer therapy. *Acta Biochimica Polonica*, *51*(2), 445–457. https://doi.org/035001445.

Oskarsson, T. (2013). Extracellular matrix components in breast cancer progression and metastasis. *Breast*, *22*(S2), S66–S72. https://doi.org/10.1016/j.breast.2013.07.012

Ouderkirk, J.L., and Krendel, M., (2014). Non-muscle myosins in tumor progression, cancer cell invasion and metastasis. *Cytoskeleton (Hoboken),* 2014 August ; 71(8): 447–463. doi:10.1002/cm.21187.

Pan, D., Jia, Z., Li, W., & Dou, Z. (2019). The targeting of MTDH by miR-145-5p or miR-145-3p is associated with prognosis and regulates the growth and metastasis of prostate cancer cells. *International Journal of Oncology.* doi:10.3892/ijo.2019.4782 .

Pereira, D. M., Rodrigues, P. M., Borralho, P. M., & Rodrigues, C. M. P., (2013). Delivering the promise of miRNA cancer therapeutics. Drug discovery today, 18(5-6), 282–9. doi: 10.1016/j.drudis.2012.10.002.

Tungsukruthai S., Petpiroon N., Chanvorachote P., (2018). Molecular Mechanisms of Breast Cancer Metastasis and Potential Anti-metastatic Compounds. *Anticancer Research*, *38*(5), 2607–2618. https://doi.org/10.21873/anticanres.12502.

Sachdeva, M., & Mo, Y. Y. (2010). MicroRNA-145 suppresses cell invasion and metastasis by directly targeting mucin 1. *Cancer Research*, *70*(1), 378–387. https://doi.org/10.1158/0008-5472.CAN-09-2021

Sandholm, J., & Selander, K. S. (2014). Toll-like receptor 9 in breast cancer. Frontiers in Immunology, 5(JUL), 1–6 https://doi.org/10.3389/fimmu.2014.00330.

Senchenko, V. N., Anedchenko, E. A., Kondratieva, T. T., Krasnov, G. S., Dmitriev, A. A., Zabarovska, V. I., … Zabarovsky, E. R. (2010). Simultaneous down-regulation of tumor suppressor genes RBSP3/CTDSPL, NPRL2/G21 and RASSF1A in primary non-small cell lung cancer. BMC Cancer, 10. https://doi.org/10.1186/1471-2407-10-75.

Showkat, M., Beigh, M. A., & Andrabi, K. I. (2014). mTOR Signaling in Protein Translation Regulation: Implications in Cancer Genesis and Therapeutic Interventions. *Molecular Biology International*, *2014*, 1–14. https://doi.org/10.1155/2014/686984.

Takebe, N., Warren, R. Q., & Ivy, S. P. (2011). Breast cancer growth and metastasis: Interplay between cancer stem cells, embryonic signaling pathways and epithelial-to-mesenchymal transition. Breast Cancer Research, 13(3). https://doi.org/10.1186/bcr2876.

Tan, W., Liu, B., Qu, S., Liang, G., Luo, W., & Gong, C. (2018). MicroRNAs and cancer: Key paradigms in molecular therapy (Review). *Oncology Letters*, *15*(3), 2735–2742. https://doi.org/10.3892/ol.2017.7638

Technologies, A. (2010). HiPerFect Transfection Reagent Handbook Sample & Assay Technologies QIAGEN Sample and Assay Technologies, (October).

Tétreault, N., & De Guire, V. (2013). miRNAs: their discovery, biogenesis and mechanism of action. Clinical biochemistry, 46(10-11), 842–5. doi: 10.1016/j.clinbiochem.2013.02.009.

Thiery, J. P. (2003). Cell adhesion in cancer. *Comptes Rendus Physique*, *4*(2), 289–304. https://doi.org/10.1016/S1631-0705(03)00031-8

Tsai, T.-F., Lin, J.-F., Chou, K.-Y., Lin, Y.-C., Chen, H.-E., & Hwang, T. I.-S. (2018). miR-99a-5p acts as tumor suppressor via targeting to mTOR and enhances RAD001-induced apoptosis in human urinary bladder urothelial carcinoma cells. OncoTargets and Therapy, Volume 11, 239–252.doi:10.2147/ott.s114276

Varghese, E., Samuel, S. M., Abotaleb, M., Cheema, S., Mamtani, R., & Büsselberg, D. (2018). The "Yin and Yang" of natural compounds in anticancer therapy of triple-negative breast cancers. Cancers, 10(10). https://doi.org/10.3390/cancers10100346.

Wang, X., Li, Y., Qi, W., Zhang, N., Sun, M., Huo, Q., … Yang, Q. (2015). MicroRNA-99a inhibits tumor aggressive phenotypes through regulating HOXA1 in breast cancer cells. *Oncotarget*, *6*(32). https://doi.org/10.18632/oncotarget.5355.

Wu, X., Cheng, B., Li, P., Huang, H., Zhao, Q., Dan, Z., … Zhang, P. (2013). *MicroRNA-143 suppresses gastric cancer cell growth and induces apoptosis by targeting COX-2*. *19*(43), 7758–7765. https://doi.org/10.3748/wjg.v19.i43.7758

Xia, M., & Hu, M. (2009). The role of microRNA in tumor invasion and metastasis. *Journal of Cancer Molecules*, *5*(2), 33–39.

Xia, M., Li, H., Wang, J., Zeng, H., & Wang, S. (2016). *MiR-99a suppress proliferation, migration and invasion through regulating insulin-like growth factor 1 receptor in breast cancer*. 1755–1763.

Yen, Y. C., Shiah, S. G., Chu, H. C., Hsu, Y. M., Hsiao, J. R., Chang, J. Y., … Chen, Y. W. (2014). Reciprocal regulation of MicroRNA-99a and insulin-like growth factor I receptor signaling in oral squamous cell carcinoma cells. *Molecular Cancer*, *13*(1), 1–16. https://doi.org/10.1186/1476-4598-13-6.

Yin,J. Q., Zhao, R. C., Morris, K. V. (2007). Profiling microRNA expression with microarrays. Trends in Biotechnology, 26/2.

Zeinali, T., Mansoori, B., Mohammadi, A., & Baradaran, B. (2019). Regulatory mechanisms of miR-145 expression and the importance of its function in cancer metastasis. *Biomedicine and Pharmacotherapy*, *109*(June 2018), 195–207. https://doi.org/10.1016/j.biopha.2018.10.037.

Zhang, Y., Wang, Z., and Gemeinhart, R. A. (2013). Review Progress in microRNA delivery. Journal of Controlled Release, 172, 962–974.

Zhang, Y., Yang, P., and Wang, X. (2014). Microenvironmental regulation of cancer metastasis by miRNAs. Trends in Cell Biology, 24, No.3.

Zhao, C., Xu, Y., Zhang, Y., Tan, W., Xue, J., Yang, Z., … Hu, X. (2013). Downregulation of miR-145 contributes to lung adenocarcinoma cell growth to form brain metastases. *Oncology Reports*, *30*(5), 2027–2034. https://doi.org/10.3892/or.2013.2728

http-1: http://www.mirbase.org/help/nomenclature.shtml,(last Visted: 14/04/2019).

http-2: http://www.mirbase.org/cgi-bin/browse.pl , (last Visted: 14/11/2018).

http-3:https://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/mirna/learning-center/mirna-introduction.html , (last Visted: 14/11/2018).

http-4: https://www.almrsal.com/post/644916 , (last Visted: 22/11/2018).

http-5: http://www.lgcstandards-atcc.org/products/all/HTB-26.aspx?geo_country=tr, (last visited 30/01/2019).

http-6: http://www.lgcstandards-atcc.org/products/all/HTB-26.aspx?geo_country=tr#characteristics, (last visited 30/01/2019).

http-7: https://www.ncbi.nlm.nih.gov/geo/, (last Visted: 20/11/2016).

http-8: http://www.ncbi.nlm.nih.gov/geo/geo2r/ , (last visited 20/11/2016).

http-9: https://paolo.shinyapps.io/ShinyVolcanoPlot/ (last visited 20.12.2018).

http-10: http://www.mirbase.org , (last visited 20/11/2016).

http-11: http://mirtarbase.mbc.nctu.edu.tw/php/index.php , (last visited 20/11/2016)

http-12: http://www.webgestalt.org/, (last Visted: 20/04/2019).

http-13: https://www.qiagen.com/jp/resources/molecular-biology-methods/transfection/ (last Visted: 20/01/2019).

http-14: https://brb.nci.nih.gov/BRB-ArrayTools/, (last visited: 20/04/2019).

http-15: https://www.networkanalyst.ca, (last visited: 20/04/2019).