# miR-638, a Novel Tumor Suppressor for Triple-Negative Breast Cancer

by Jin Peng

B.S. in Biotechnology, June 2009, Shandong University, P.R. China

A Thesis submitted to

The Faculty of
Columbian College of Arts and Sciences
of The George Washington University
in partial fulfillment of the requirements
for the degree of Master of Science

January 31st, 2014

Thesis directed by

Sidney W. Fu
Professor of Medicine

**Acknowledgement**

Firstly I would like to give my sincere acknowledgement to my mentor, Dr. Sidney W. Fu for always giving me his support. I thank him for his great guidance and patience during the whole project and thesis writing. Additionally, I thank Dr. Kazufumi and my colleague, Liang Chen for kindly teaching me all the required experimental skills. I am deeply grateful to Dr. Jack Vanderhoek for his meticulous reviewing my thesis and all the support he provided for my graduate studies. Meanwhile, I want to thanks my friends and classmates, Yebo Fu, Xi Chen, Qi Yang and Zhao Wang, who helped me when I encountered difficulties, both in my experiments or in my personal life. I especially want to thank my parents and my family, without their selfless devotion and love, I would not have had this treasured time to study and chase my dreams.

## Abstract

miR-638, a Novel Tumor Suppressor for Triple-Negative Breast Cancer

The Triple-negative breast cancer (TNBC) is known to be associated with poor outcome. Aberrantly expressed microRNAs (miRNAs) have emerged as an important set of biomarkers for breast cancer diagnosis and treatment. In our previous studies, we analyzed 8 breast cancer patient samples using microdissected Formalin-Fixed, Paraffin-Embedded (FFPE) tissues and miRNA microarray technologies. The miRNA expression profile was compared during the progression of breast cancer from normal, atypical ductal hyperplasia (ADH), ductal carcinoma *in situ* (DCIS) to invasive ductal carcinoma (IDC). Among the differentially expressed miRNAs during breast cancer progression, miR-638 is one of the differentially expressed miRNAs. In this study, we first sought to verify the expression of miR-638 in microdissected FFPE samples using the real-time qRT-PCR. Then we analyzed the expression of miR-638 in different breast cancer cell lines, which showed low expression in MDA-MB-231, Hs578T, MCF-7 and T47D compared to immortalized MCF-10A cells. We hypothesize that miR-638 may function as a tumor suppressor during breast cancer progression. To determine the role of miR-638 in both ER+ and ER- breast cancer cells, we transfected miR-638 mimic or scrambled oligos into MDA-MB-231 (ER-) , Hs578T (ER-), MCF-7 (ER+) and T47D (ER+) cells. We found that the proliferation rate was much lower in ER- cells compared to that in ER+ cells by MTT assays. In addition, Matrigel invasive analysis indicated that overexpressing miR-638 decreased invasion by 3~4 fold in TNBC cell lines, MDA-MB-231 and Hs578T. To elucidate the mechanism of its tumor suppressor activity, we used the TARGETSCAN-VERT to identify potential target genes of miR-638. Interestingly,

BRCA1 is one of the direct targets of miR-638 among the 30 conservative target genes. We found that overexpression of miR-638 resulted in upregulation of BRCA1expression in TNBC cells, but not in homornal positive cells. These findings show that miR-638 may function as a tumor suppressor in TNBC, likely by upregulating BRCA1 tumor suppressor gene. Further functional analysis is contemplated to decipher the exact role of miR-638 in TNBC, which could become a therapeutic target.

Keywords: breast cancer; miRNA; tumor suppressor; TNBC; BRCA1

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## List of Abbreviations

TNBC: triple negative breast cancer

FFPE: formalin fixed, paraffin-embedded

ADH: atypical ductal hyperplasia

DCIS: ductal carcinoma *in situ*

IDC: invasive ductal carcinoma

Real-time qRT-PCR: real time quantitative reverse transcription polymerase chain reaction

ER: estrogen receptor

PR: progesterone receptor

HER2: human Epidermal Growth Factor Receptor 2

BRCA1: breast cancer type 1 susceptibility protein

PAK2: P21 protein (Cdc42/Rac)-activated kinase 2

CCND1: Cyclin D1

3'UTR: three prime untranslated region

5'UTR: five prime untranslated region

# Chapter 1: Introduction

## Breast cancer

Breast cancer is one of the most frequently occurring cancers in women around the world, which rarely occurs to men [1]. A cancer fact sheet published by WHO indicated that 1.38 million new breast cancer cases were diagnosed in 2008(23% of all cancers), making it the second most common cancer in the world [2]. In 2013, an estimated 232,340 new cases of invasive breast cancer are expected to be diagnosed in women in the U.S., along with about 64,640 new cases of non-invasive (*in situ*) breast cancer. One out of eight women will develop invasive breast cancer during their lifetime. About 40,030 women in the U.S. are expected to die in 2013 from breast cancer. Although the mortality rate continues to decrease since 1990 due to early detection, treatment advances and increased awareness, breast cancer mortality still ranks high for women within all types of cancers [3].

Breast cancer arises from the uncontrolled growth of breast epithelial cells. Breast tissue is made up of ducts and lobes. The common breast cancer that originates from the inner lining of milk ducts is defined as ductal carcinoma and the one that stems from the lobules is called lobular carcinoma [4].

The major cause of breast cancer is a genetic abnormality. Yet inherited mutations in breast cancer susceptibility genes, such as BRCA1 and BRCA2, account for approximately 5%-10% of all female breast cancer. The rest of breast cancers are attributed to gene abnormalities that happen during women's lifetime as a result of the

aging, lifestyle, environment, etc [5]. The interaction between environmental factors and genetic susceptibility could lead to an accumulation of essential gene mutations, causing normal breast epithelium to gradually change from benign to malignant by losing the ability to stop dividing, immobility and apoptosis [6]. Therefore, genetic factors play a major role in the pathogenesis of breast cancer [7].

The development and progression of breast cancer can be considered from normal epithelial cells to atypical ductal hyperplasia (ADH), ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC) [8]. ADH is characterized by cellular proliferation (hyperplasia) within one or two breast ducts and (histomorphologic) architectural abnormalities, i.e. the cells are arranged in an abnormal or an atypical way but are considered precancerous cells [9]. For DCIS, the cells are cancer cells, however, they remain inside the breast ducts, which is the most common type of non-invasive breast cancer. When the cancer cells start to break through the duct walls and invade fatty breast tissues, they transform to IDC, which accounts for 80% of invasive breast cancer. DCIS stage can also be refered to as stage 0 breast cancer, while IDC can be classified into stage I, II and III based on the tumor size and other factors [10]. Lobular carcinoma can also be classified as atypical lobular hyperplasia (ALH), lobular carcinoma *in situ* (LCIS) and invasive lobular carcinoma (ILC).

Breast cancer is a molecularly heterogeneous disease. Hormone receptors such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor type 2 receptor (HER2/neu) can be used as molecular biomarkers for breast cancer classification and for prognostication [11]. Hormone receptors are a group of proteins activated by hormones such as estrogen or progesterone, then translocate into nucleus and

bind to DNA to regulate gene expression[12, 13]. There are two types of ER, the ERα and ERβ. Both are part of the steroid/thyroid nuclear receptor superfamily and ligand-dependent nuclear transcription factors. Progesterone also has two receptors, PRA and PRB [13]. Abnormal expression of ER and/or PR will lead to the development of cancers, such as breast cancer. Breast cancer tissues with high levels of ER and PR tend to grow and spread more quickly [4]. HER2 encoded by ERBB2, a known proto-oncogene located at the long arm of human chromosome 17, belongs to a class of proteins with high homology with epidermal growth factor receptor (EGFR or ERBB1). Overexpression of Her2/ERBB2 has been shown to be related to several aggressive types of breast cancer [14]. Hormone receptor-positive breast cancers are less aggressive compared with TNBC. They can be treated with hormonal therapeutic medications by decreasing the amount of estrogen in the body or block estrogen from supporting the growth and function of breast cells [15]. Breast cancer with negative expression of ER, PR and HER2 are called TNBC, which accounts for approximately 15% of all invasive breast cancer. TNBC tends to occur more often in younger women and in African-American women [16], and to grow and spread more quickly and is associated with poor survival rates [17]. Only approximately 20% of these tumors responds well to standard chemotherapy [18]. The lack of these hormone receptors also decreases the treatment efficiency. More than 75% BRCA1 mutations causing breast cancer are triple-negative phenotype, a basal-like phenotype, or both[19, 20].

Currently, the commonly used methods to diagnose breast cancer include physical exam, clinical breast exam (CBE), mammogram, ultrasound exam, Magnetic Resonance

Imaging (MRI), blood chemistry studies and biopsy (removal of tissue sample for testing) [4]. Physicians will generally require a combination of the above methods to make a diagnosis.

**MicroRNAs (miRNAs)**

MicroRNAs are small non-coding RNA molecules (18-25nt) encoded either in exons or introns, which are involved in the post-transcriptional regulation of gene expression [21]. miRNAs are transcribed by RNA polymerase II by binding to a promoter, generating about 70nt hairpin loop of primary miRNAs (or pri-miRNAs) in the nucleus. Pri-miRNAs then are cleaved by endonuclease RNase III Drosha, which is catalyzed by nucleus protein Pasha to form stem-loop precursor miRNAs (or pre-miRNAs). A single pri-miRNA may contain from one to six pre-miRNAs [22]. Pre-miRNAs are exported from the nucleus to cytoplasm by Exportin-5 and then are cleaved by the RNase III enzyme Dicer, yielding an imperfect miRNA: miRNA duplex of about 22 nucleotides in length [23]. Although either strand of the duplex may potentially act as a functional miRNA, only one strand is usually incorporated into the RNA-induced silencing complex (RISC) where the miRNA and its mRNA target interact [24].

Mature miRNA function via complementary base-pairing with the 3' untranslated regions (UTRs) of their target mRNAs, usually resulting in gene silencing through mRNA degradation or translational repression. There are 2578 mature miRNAs encoded in the human genome according to the most recent miRBase Release v.20 [25]. The aberrant expression of miRNAs may be involved in many human diseases, including cancer. Deregulated expression of miRNAs previously has been found in human chronic

lymphocytic leukemias, colorectal cancer, clinical prostate cancer and breast cancer by miRNA profiling. Since Iorio et. al.first reported that deregulation of miRNA expression was found in human breast cancer [26], a number of studies suggest that miRNA signatures are associated with clinicobiological features of breast cancer. As a regulatory molecule, miRNA can function as a tumor suppressor or oncogene. Abnormal expression of miRNAs has been detected in different types /stages of breast cancer. For example, miR-21 is an oncogene regulating cell apoptosis by interacting with BCL-2, PTEN, TPM1, MASPIN, and PDCD4 target genes, and is overexpressed in breast cancer patients [27, 28]. In contrast, miR-200b functions as a tumor suppressor in TGF-beta signaling pathway [29, 30, 31]. Furthermore, the aberrant expression of miRNAs may differentiate the the types and/or stages of breast cancer, such as miR-30, miR-213 and let-7c [29]. Therefore, miRNAs have the potential of being a new type of breast cancer classification and prognosis tool.

Using microdissection, a miRNA microarray profiling of FFPE breast cancer samples of 8 patients was recently reported by Chen et al in our lab [29]. Thirty five miRNAs were found to be differentially expressed during the progression of breast cancer, some of which could be potential biomarkers for early stage breast cancer with further validation. miR-638 was one of the significantly down-regulated miRNAs in ADH and IDC compared to normal tissue.

miR-638 is located on chromosome 19. The stem-loop miR-638(Fig. 1) is cleaved by enzyme Dicer to form a 25nt mature miR-638 (Fig. 2). miR-638 has been implicated in the pathogenesis of lupus nephritis [32]. It has also been reported to be highly differentially expressed with emphysema and to contribute to the progression of this

disease by affecting fibroblast function [33]. Li et al. suggested that miR-638 was involved in the benzo(a)pyrene-induced carcinogenesis by targeting the BRCA1 gene [34]. Masami Tanaka et al. reported that miR-638 is stably present in human plasmas and the ratio of miR-92a/miR-638 in plasma can be used as a novel biomarker for detection of leukemia [35]. Although miR-638 has been reported to be deregulated in several diseases and may contribute to the pathological processes, to my knowledge, there is no report on the role of miR-638 in breast cancer.

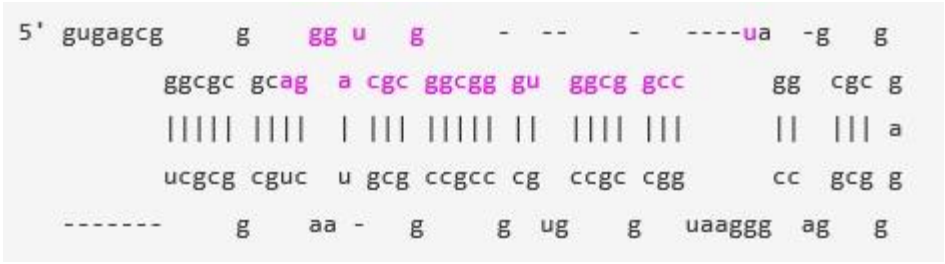
In my research, miR-638 expression was further verified in additional breast cancer FFPE samples and breast cancer cell lines (Table 1). We found that overexpresssion of miR-638 was associated with crucial tumor-suppressive biological features in TNBC cell lines, such as MDA-MB-231and Hs578T, possibly via upregulating BRCA1, one of the target genes of miR-638. Therefore, We hypothesize that miR-638 may function as a tumor suppressor in TNBC and could potentially serve as a therapeutic target.

**Figure 1. Stem-loop sequence of mir-638**

Sequence of mir-638 was acquired from miRBase (Release 20: June 2013, http://www.mirbase.org/)

**Figure 2. Mature sequences of miR-638**

Sequence of miR-638 was acquired from miRBase (Release 20: June 2013, http://www.mirbase.org/)




MIMAT0003308 16-AGGGAUCGCGGGCGGGUGGCGGCCU-40

**Table 1. Clinicopathological features of breast cancer cell lines.**

The table was cited from the paper *Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery*[11].

AC: Adenocarcinoma;  B: Basal B subtype

C Sar: carcinoma sarcoma;  F: fibrocystic disease

IDC: invasive ductal carcinoma;  L: Luminal subtype

Met AC: metastatic adenocarcinoma;  PE: pleural effusion;  PT: primary tumor

| Cell Line | Subtype | ER | PR | ERBB2/HER2 | Source | Tumor Type |
|---|---|---|---|---|---|---|
| **MCF-10A** | B | - | - | - | RM | F |
| **MDA-MB-231** | B | - | - | - | PE | Met AC |
| **Hs578T** | B | - | - | - | PT | C Sar |
| **MDA-MB-468** | A | - | - | - | PE | Met AC |
| **MCF-7** | L | + | + | - | PE | Met AC |
| **T47D** | L | + | + | - | PE | IDC |

## Chapter 2: Materials and Methods

### Breast cancer cell lines and cell culture

The human breast cancer cell lines, MDA-MB-231, Hs578T, MCF-7 and T47D were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza) supplemented with 10% fetal bovine serum (FBS) and 1% penicillum and streptomycin antibiotics. MCF-10A cells were cultured in MEGM medium (CC-3150, Lonza) containing 100 ng/ml of cholera toxin to make a complete growth culture medium. All cell lines were grown in a 37°C humidified incubator with 5% $CO_2$.

### Transient transfection in human breast cancer cell lines

For transient transfection, $2.4x10^5$ cells of each cell line were trypsinized and seeded in a 6-well plate, cultured in DMEM medium supplemented with 10% FBS in a 37°C humidified incubator with 5% $CO_2$. After overnight incubation, MCF-7, T47D, MDA-MB-231 and Hs578T cells reached 30%-50% confluency and were transiently transfected with miR-638 mimic (Ambion) or negative control (Ambion 4464060) by Lipofectamine RNAiMAX (Life Technologies) using the Optimum Medium (Life Technologies). miR-638 mimic and negative control were used to transfect cellsin a 37°C humidified incubator with 5% $CO_2$. After 4 hrs, the medium was replaced with DMEM medium. Cells are harvested for further analysis after 48 hrs transfection.

### Total RNA extraction from cell lines and FFPE breast cancer samples

Total RNA was isolated from the breast cancer cells, including the transfected ones using Trizol reagent (Life Technologies) following the manufacturer's instructions. The RecoverAll Total Nucleic Acid Isolation Kit ( AM1975, Ambion) was used to isolate

total RNA from the FFPE samples. . Briefly, 1ml of xylene was added to 4 pieces of 20 μm FFPE sections to remove paraffin. The tissue was digested with proteinase K at 55°C overnight and then treated with DNase I. After washing, total RNA, including the small miRNA fraction, was reconstituted in distilled water. Quantity and quality of the total RNA samples were assayed by the NanoDrop1000 Spectrophotometer (Thermo Scientific).

**miRNA real-time qRT-PCR analysis**

The Taqman MiRNA Reverse Transcript Kit (Applied Biosystem) features a stem-loop RT primer which specifically hybridizes with a miRNA. The reverse transcription was performed using the MultiScribe Reverse Transcriptase. Specifically, 10 nanogram of the total RNA was used to start the RT step following the manufacture's protocol. The RT reactions were carried out at 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes and then held at 4°C. To verify miRNA expression, a final volume of 20 μl for each PCR reaction mixture consisted of 10μl TaqMan Universal Master Mix II with no UNG (Applied Biosystems), 1 μl of 20xTaqman miR-638 PCR primer (Ambion), 2 μl of 1:1 diluted RT products and 7 μl nuclease-free water. QPCR was performed using the ABI 7300 Real-Time PCR System (Applied Biosystems). The conditions for QPCR were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The mean quantity values of the miRNA expression were normalized by U6 snRNA.

**miRNA target analysis**

The potential target genes of miR-638 were analyzed using the TARGETSCAN-VERT 6.2 (http://www.targetscan.org/), which helps identify targets based on comparative

sequence analysis, seed match complementation and Z-score for assigned untranslated regions (UTR). A group of selected target genes were further analyzed.

**mRNA quantitative real-time RT-PCR (QPCR)**

Two hundred ng of total RNA was used for reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad). To verify gene expression in transfected cell clones, QPCR was performed using the ABI 7300 real-time PCR System (Applied Biosystems). A final volume of 25 µl for each reaction consisted of 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 0.5 µl (20 µM) of each primer (IDT, Coralville, IA, USA), 2 µl of 10-fold diluted cDNA and 9.5µl nuclease-free water. The conditions for QPCR were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Dissociation curves were generated for each primer set to confirm the specificity of amplifications. The mean quantity values of target gene expression were normalized by 18S rRNA. The primer sequences are: for BRCA1, forward, 5'-GTGTCCCATCTGTCTGGAGT-3'and reverse, 5'-CGCTGCTTTGTCCTCAGAGT-3'; for CCND1, forward, 5'-CCTCCCCCAATTTGTCCTACTC-3' and reverse, 5'-GGTTGCTGGCAGGACAGGTA-3'; for 18S, forward, 5'-GCCGCTAGAGGTGAAATTCTTG-3'and reverse, 5'-CATTCTTGGCAAATGCTTTCG-3'.

The real-time qRT-PCR analysis for each miRNA and mRNA in all cell lines and FFPE samples were performed in quadruplicates and duplicates respectively. The comparative CT(threshold cycle) method was used to evaluate the relative expression changes and

presented the CT value as the mean fold change($2^{-\Delta\Delta CT}$) in the expression of genes normalized to internal control [36].

**MTT assays**

After 48 hrs, the miR-638 mimic or negative control transfected cells including MCF-7, T47D, MDA-MB-231 and Hs578T, were trypsinized and seeded in a 96-well micro-titer culture plate at a density of $10^4$ cells/well in a 37°C humidified incubator with 5% $CO_2$. 24 hrs later, cells in 96-well plate were washed with PBS. 100 µl of MTT working solution (5 mg/ml stock MTT diluted in optiMEM to 0.5 mg/ml working solution) was added to each well and incubated at 37℃ with 5% $CO_2$ for 3 hrs. The MTT solution was carefully removed and 100 µl DMSO was added to each well and incubated in a 37°C humidified incubator with 5% $CO_2$ for 30 min. Color development was measured spectrophotometrically at 490 nm on a plate reader (BIO-TEK Instruments) and quantified as per the manufacturer protocol (Promega, USA). Each sample was seeded in eight of the 96-wells and each experiment was repeated twice.

**Protein extraction and Western blot analysis**

Proteins were extracted from cell lines using RIPA Buffer (Thermo) according to the manufacturer's protocol. Complete, Mini Protease Inhibitor Cocktail (Roche) was added to the extraction solution prior to lysis to prevent proteolytic activity. Cell protein lysate was prepared with SDS gel-loading buffer containing β-mercaptoethanol and heated at 95 ºC for 5 minutes. Proteins were separated by SDS-PAGE using a 4–15% Mini-PROTEAN TGX™ Precast Gel (Bio-Rad, -456-1084) and transferred overnight at 30V in a 4°C cold room. The membrane was blocked prior to the addition of the primary

antibody with 5% milk in Tris-buffered saline (TBS) with 0.05% Tween. The membrane was incubated overnight with either BRCA1 rabbit polyclonal antibody (9010S, Cell Signaling), CCND1 rabbit polyclonal antibody (2922C, Cell Signaling) at or PAK2 rabbit polyclonal antibody (2608S, Cell Signaling), at a dilution of 1:1000 in TBS buffer with 0.05% Tween and 5% milk, or GAPDH (MA5-15738) mouse monoclonal antibody (Sigma) at a dilution of 1:2,000 in TBS buffer with 0.05% Tween. The membrane was washed 3 times with TBS/0.05% Tween and incubated with an anti-rabbit IgG conjugated to horseradish peroxidase (7074S, Cell Signaling) for BRCA1, CCND1 and PAK2, anti-mouse IgG (7076S, Cell Signaling) for GAPDH at a 1:2,000 dilution in TBS/0.05% Tween (and 5% milk). Super Signal WestFemo Maximum Sensitivity Substrate (Thermo) was used according to the manufacturer's protocol to visualize proteins and quantify band intensity.

**Matrigel invasion assays**

Matrigel invasion assays were performed using the BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences). BD BioCoat Matrigel Invasion Chambers provide cells with the conditions that allow assessment of their invasive property *in vitro*. It consists of a BD Falcon™ TC Companion Plate with Falcon Cell Culture Inserts containing an 8 micron pore size PET membrane with a thin layer of matrigel basement membrane matrix. Briefly, prior to the start of each experiment, 500 µl of warm (37°C) serum-free DMEM medium was added to the upper and lower chambers and allowed to rehydrate for 2 hrs in a 37°C cell culture incubator. After 2 hrs rehydration, the medium was removed from the upper and lower chambers, then750 µl of DMEM with 10% fetal bovine serum and 0.1% BSA was added to the pre-wetted lower chambers. Then 2.5 x $10^4$ cells for

MDA-MB-231, 3 x $10^4$ for Hs578T, 5 x $10^4$ for MCF-7, 8 x $10^4$ for T47D, transfected by either miR-638 or negative control for 24 hrs were seeded onto the top chamber of pre-wetted inserts, cultured in 500 μl serum-free DMEM with 0.1% BSA in the top chamber. Cells were incubated in a matrigel chamber in a 37°C humidified incubator with 5% $CO_2$ for 24 hrs for MDA-MB-231 and 48 hrs for Hs578T, MCF-7 and T47D. Next the non-invasive cells were removed from the upper surface of the membrane by scrubbing with a cotton swab and the invasive cells present on the bottom of the membrane were fixed, stained with the Diff-Quick staining solution and counted (five microscope fields under the 10X lens). Experiments were done in duplicate for each cell line twice. Cell counts were performed on five nonoverlapping random fields for each chamber and four chambers were counted for each experimental point, and the percentage of invasive cells was normalized to the corresponding control.

**Statistical Analysis**

Data were expressed as mean ± standard error (S.E.). Permutation test was performed for MTT assay between control groups and miR-638 transfected groups. The student's t test (two tailed) was applied to matrigel assay between control groups and miR-638 transfected group. *p*-value less than 0.05 and 0.01 were considered statistically significant and presented with one and two asterisks respectively.

## Chapter 3: Results

### Down-regulation of miR-638 during breast cancer progression

In previous work from our group, miR-638 was observed to be down-regulated in ADH and IDC from FFPE tissue samples of the breast cancer patients[29]. We analyzed additional 9 breast cancer FFPE samples following microdissection. The miR-638 expression in these four different lesions, including normal, ADH, DCIS and IDC, was assayed by real-time qRT-PCR.

Figure 3 shows the expression of miR-638 in each individual patient sample. Patient-matched histological types: ADH vs. Normal, DCIS vs. Normal and IDC vs. Normal was analyzed by the paired t-test. Based on the results shown in Figure 3, 8 of the 9 patients was showed miR-638 down-regulation in IDC compared to normal($p$ = 0.03991) and the same in 7 of 9 patients in DCIS ($p$ = 0.00074) compared with normal. The expression deregulations are all statistically significant ($p$<=0.05).

### Decreased expression of miR-638 in breast cancer cell lines

To analyze the expression of miR-638 in breast cancer cell lines, we used the following, MDA-MB-231(ER-, PR-, HER2-), Hs578T (ER-, PR-, HER2-), MDA-MB-468(ER-, PR-, HER2-), MCF-7(ER+, PR+, HER2-) and T47D (ER+, PR+, HER2-). In addition, MCF-10A(ER-, PR-, HER2-) was used as a control.

To determine whether miR-638 exhibits different expression among different breast cancer subtypes, real-time qPCR was performed. The data shown in Figure 4 indicates

that the expression of miR-638 in all breast cancer cell lines was low compared to the normal control MCF-10A.

**Overexpression of miR-638 in four breast cancer cell lines**

Having established that miR-638 was down-regulated during progression of breast cancer and in breast cancer cell lines, we over-expressed miR-638 in four breast cancer cell lines, MDA-MB-231, Hs578T and MCF-7, T47D to investigate the function of miR-638 in both ER- and ER+ breast cancer. Non-sense random oligo was tranfected into cells as negative control (MOCK). After 48 hrs of transfection, miR-638 expression was detected by real-time qRT-PCR(Fig. 5). The expression of miR-638 was significantly increased compared with the MOCK.

**Overexpresssion of miR-638 inhibited the proliferation in triple negative cell lines**

The genetically abnormal expression of miR-638 is associated with cell proliferation, invasion and apoptosis [37, 38]. To elucidate the biological function changes, we examined the cellular proliferation activity between miR-638 overexpressed and negative control (MOCK) transfected cells of MDA-MB-231, Hs578T, MCF-7 and T47D breast cancer cell lines by MTT assay. The data showed that overexpression of miR-638 inhibited the proliferation in triple negative cell lines MDA-MB-231 and Hs578T, while increased cell growth in hormone positive cell lines, such as MCF-7 and T47D compared with the control MOCK (Fig. 6).

**Figure 3. miR-638 expression in 9 breast cancer FFPE samples by real-time qRT-PCR.**

Relative quantificative expression of miR-638 in four stages of breast cancer FFPE samples was determined by real-time qRT-PCR method. Data were normalized to snU6 expression and were given as mean data±relative to the Normal . Values represent the average±SE of two independent experiments in duplicates. Paired t-test was performed to compare Normal with ADH, DCIS or IDC.

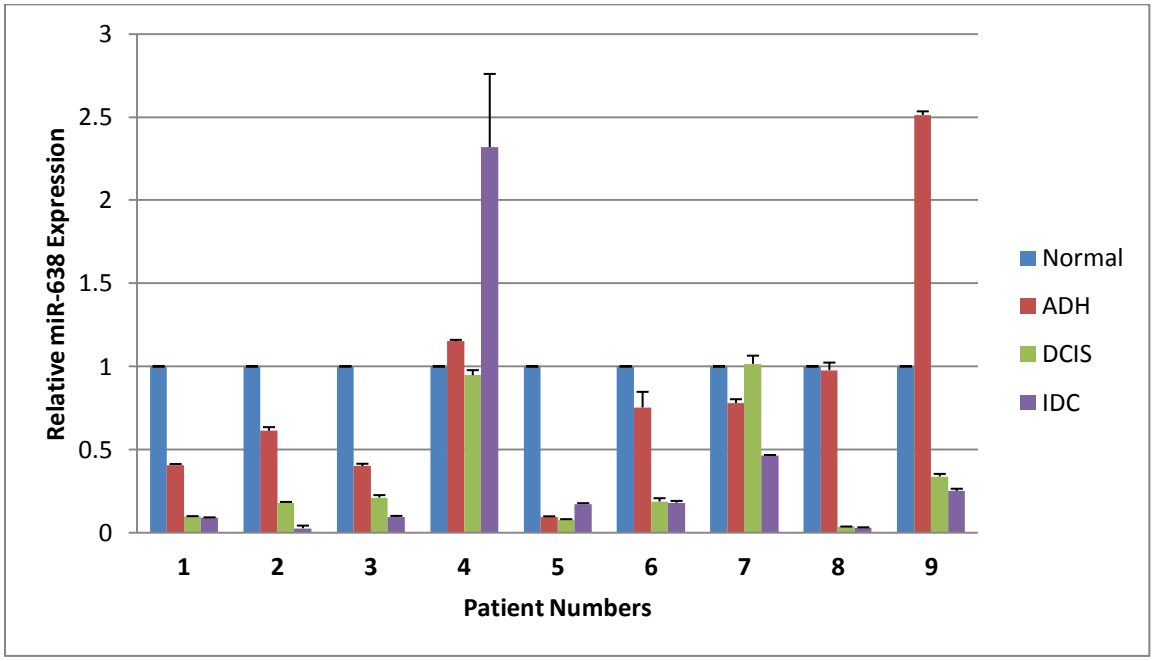

Relative miR-638 Expression
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
Patient Numbers
Normal
ADH
DCIS
IDC

**Figure 4. miR-638 expression in breast cancer cell lines by real-time qRT-PCR.** Quantitative analysis of miR-638 expression in breast cancer cell lines, including MDA-MB-231, Hs578T, MDA-MB-468, MCF-7 and T47D is shown. MCF-10A cell line was used as a negative control and data were normalized to snU6 . Values represent the average ± SE of two independent experiments in duplicates.

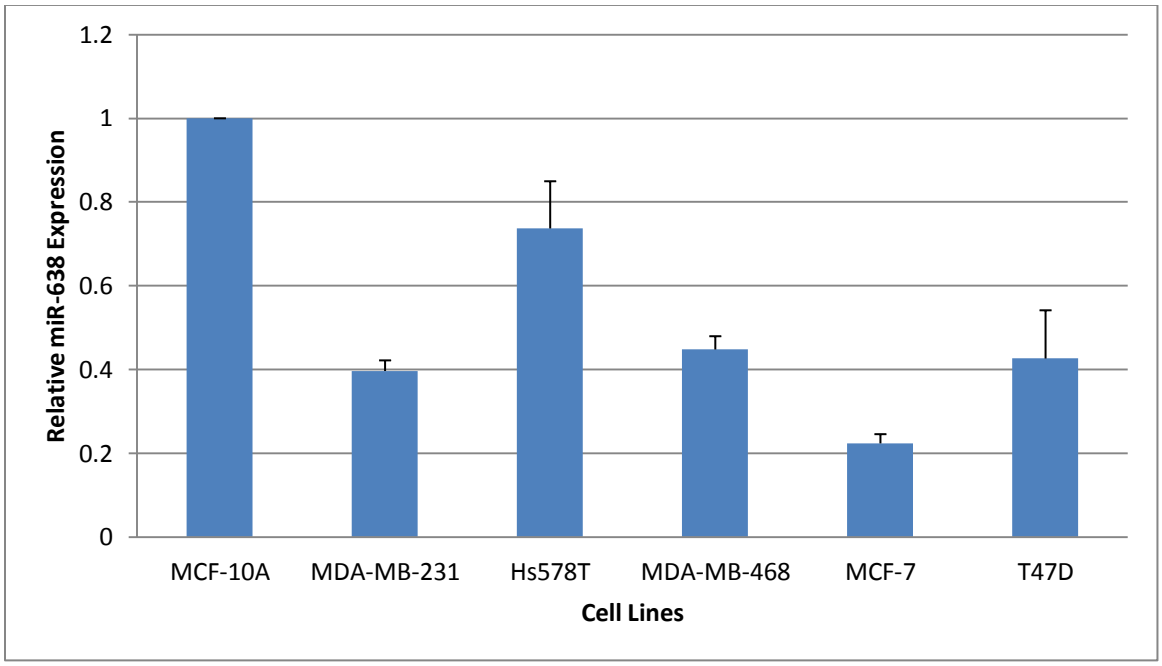

1.2
1
0.8
0.6
0.4
0.2
0
Relative miR-638 Expression
MCF-10A
MDA-MB-231
Hs578T
MDA-MB-468
MCF-7
T47D
Cell Lines

**Figure 5. Transfection efficiency of miR-638 in breast cancer cell lines.**

miR-638 mimic or MOCK was transfected into MDA-MB-231, Hs578T, MCF-7 and T47D cell lines for 48 hrs. Total RNA was isolated to assess transfection efficiency. miR-638 transfected cells are show significantly overexpressed compared with the MOCK.

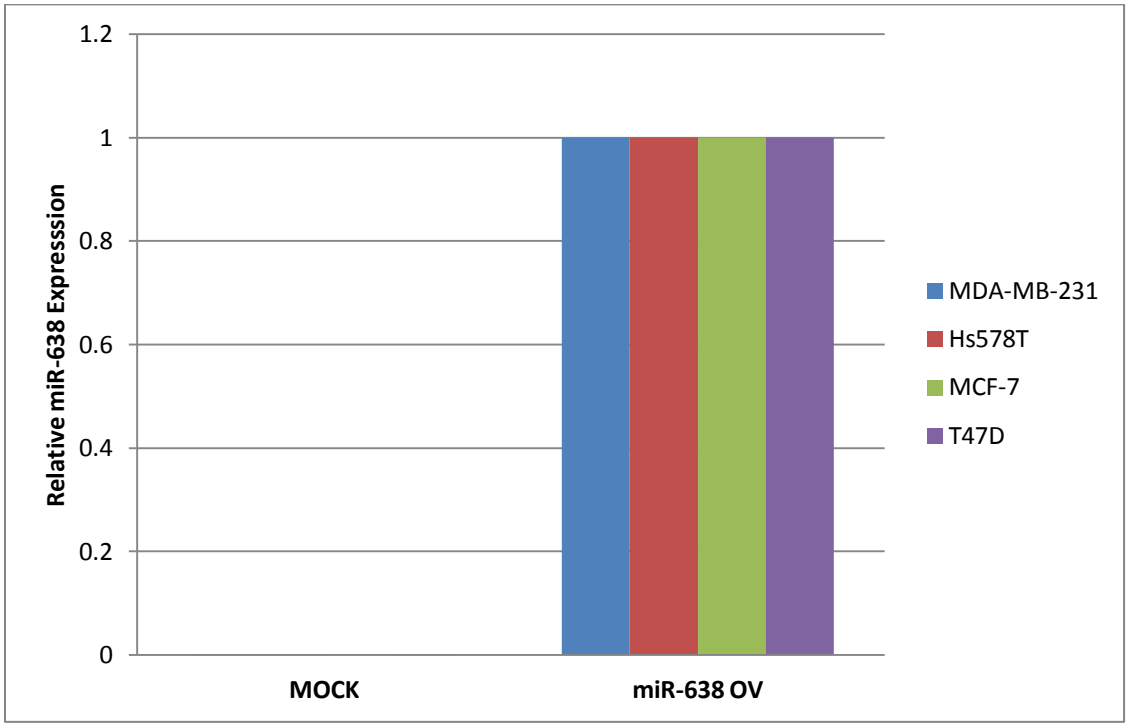
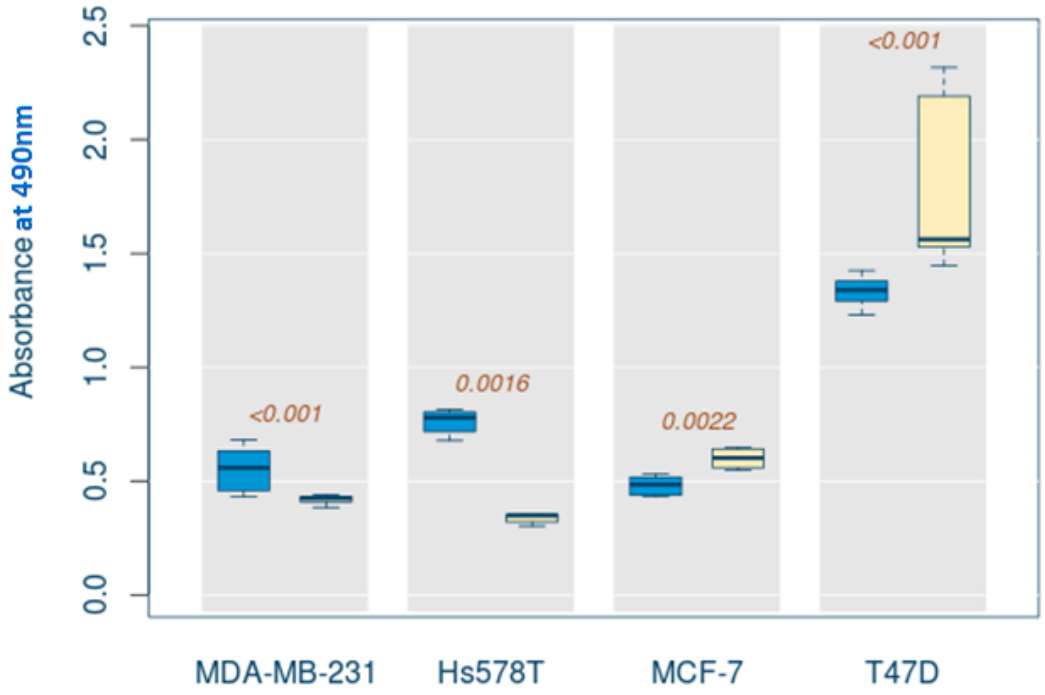

1.2
1
0.8
0.6
0.4
0.2
0
Relative miR-638 Expresssion
MOCK
miR-638 OV
MDA-MB-231
Hs578T
MCF-7
T47D

**Figure 6. The proliferation of miR-638 transfected breast cell lines by MTT assays.** Proliferative rate of four breast cancer cell lines, MDA-MB-231, Hs578T, MCF-7 and T47D after miR-638 overexpression was assayed by MTT assay. The blue bars on left are data of miR-638 overexpressed samples while the yellow bars on right are for the MOCK. the proliferation was decreased after overexpression of miR-638 compared to MOCK in both MDA-MB-231($p<0.001$) and Hs578T($p=0.026$) cell lines. On the contrast, the proliferation rate of MCF-7($p=0.0022$) and T47D($p<0.001$) cell lines was increased.


Absorbance at 490nm
0.0
0.5
1.0
1.5
2.0
2.5
<0.001
0.0016
0.0022
<0.001
MDA-MB-231
Hs578T
MCF-7
T47D

**Over-expression of miR-638 inhibited invasive ability in TNBC cells**

To determine if overexpression of miR-638 affects the invasive ability of breast cancer cell lines, a cell invasive assay was used in miR-638 transfected breast cancer cells, such as MDA-MB-231, Hs578T, MCF-7 and T47D using BD Matrigel. Since MDA-MB-231 is a highly invasive and metastatic adenocarcinoma, $2.5 \times 10^4$ cells were seeded on matrigel for 24 hrs before staining. For H578T, MCF-7 and T47D cell lines, $5 \times 10^4$ cells were seeded for 48 hrs.

miR-638 over-expressed MDA-MB-231 exhibited significantly lower invasiveness (almost 60%) than the control ($p<0.001$) (Fig.7). Hs578T shows a 70% decreased invasive activity after overexpression of miR-638 ($p=0.06$). For hormone positive cell lines, miR-638 over-expressed MCF-7 cell line exhibited no invasiveness change compared to the control ($p=0.90$) and T47D decreased its invasion activity after miR-638 overexpresion ($p=0.47$). These data suggest that miR-638 decreased TNBC cell lines invasive ability but had no or minimum effect on hormonal positive cells.

**miR-638 target genes identification**

Usually miRNAs regulate their target genes by binding to the 3'UTR of target genes. In order to explore how miR-638 function in breast cancer development and malignancy, it is crucial to identify the target genes of the miR-638. We used TARGETSCAN-VERT 6.2 (http://www.targetscan.org/) to predict target genes of miR-638.

Table 2 shows the 30 conserved target genes of miR-638. We then focused on the following target genes for further analysis, including breast cancer type 1 susceptibility protein (BRCA1), p21 protein (Cdc42/Rac)-activated kinase 2 (PAK2), LIM domain only 4 (LMO4) and V-Myc Myelocytomatosis Viral Oncogene Homolog 1(MYCL1), as being crucial to cancer development. In my thesis, I focused on BRCA1 and PAK2 which are possibly related to breast cancer proliferation and invasion.

BRCA1 is a multifunctional tumor suppressor protein, which interacts with other proteins to form a complex related to cell cycle, transcription, DNA damage response and chromatin remodeling [39]. BRCA1 has been reported to regualte oncogene CCND1 which is overexpressed in about 35% of all breast cancer cases. Kehn K, et. Al. identified Ser 632 of BRCA1 as a cyclin D1/cdk4 phosphorylation site *in vitro*, indicating that cyclin D1/cdk4-mediated phosphorylation of BRCA1 inhibits the ability of BRCA1 to be recruited to particular promoters *in vivo* [39]. Therefore CCND1 was also investigated to help understand the function of miR638 on BRCA1.

**Target gene expression in miR-638 overexpressed breast cancer cell lines**

miRNAs inhibit mRNA expression or lead to mRNA degradation via binding to their target sequences. After successful transfection of miR-638 or mimic controls into breast cancer cells, we performed mRNA quantitative analysis of miR-638 target genes such as BRCA1 and PAK2 expression by real-time qRT-PCR. Overexpression of miR-638 resulted in BRCA1 up-regulation in TNBC cell lines, MDA-MB-231 and Hs578T while

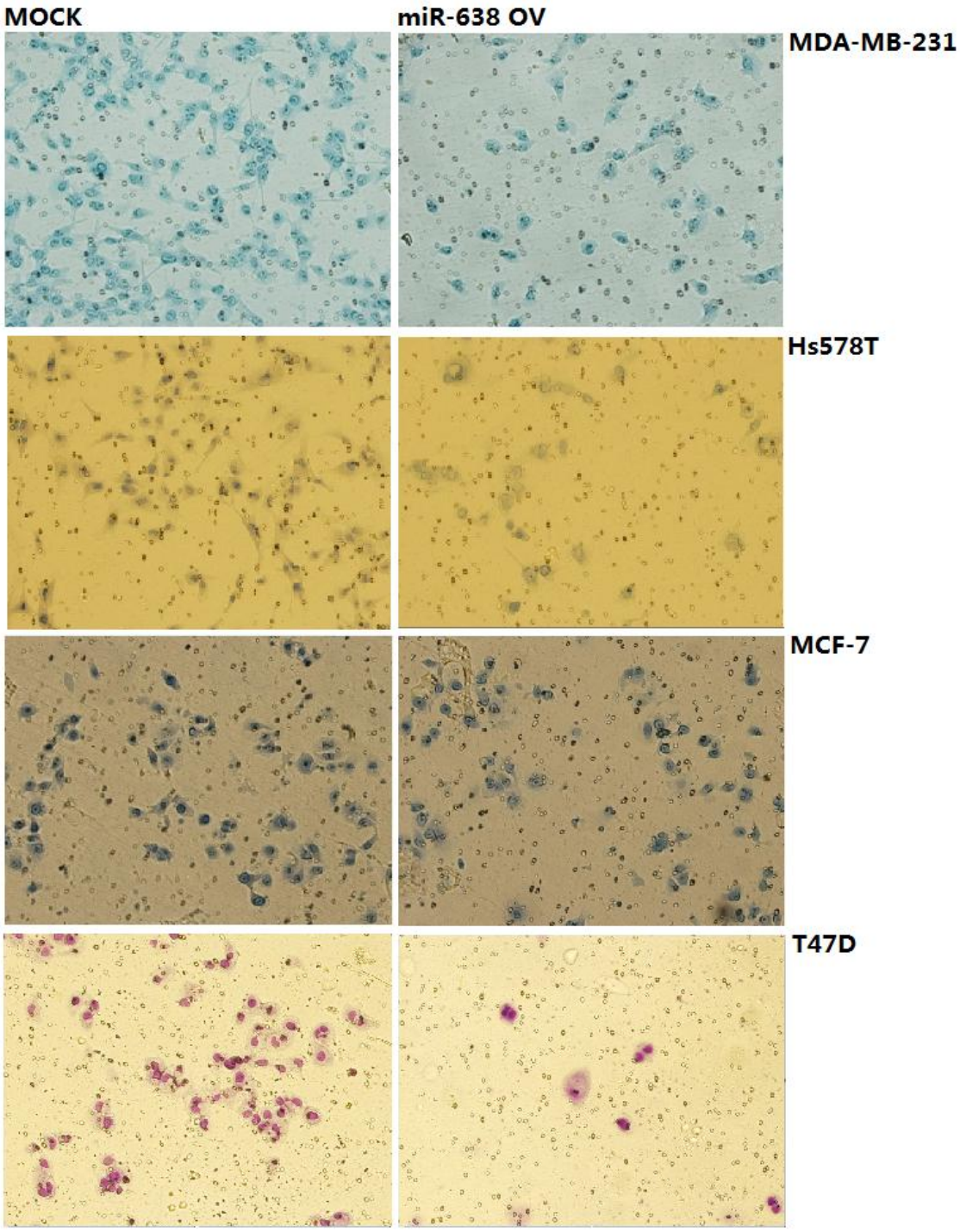
down-regulation in hormone positive cell lines, MCF-7 and T47D (Fig. 9a). Overexpression of miR-638 up-regulated PAK2 expression in MDA-MB-231 but showed no effect in Hs578T. PAK2 was down-regulated in hormone positive cell lines MCF-7 and T47D after miR-638overexpression (Fig. 9b). In addition, CCND1 was upregualted in Hs578T and down-regulated in hormone positive cell lines after miR-638overexpression ( Fig. 9c).

At the protein level, BRCA1 was significantly up-regulated in MDA-MB-231, Hs578T and MCF-7 cell lines after miR-638 transfection and no change in T47D cell line (Fig. 10a). Protein expression of PAK 2 decreased in Hs578T by about 24%. However, no significant expression changes were observed in three other cell lines (Fig. 10b). CCND1 protein was up-regulated in hormone positive cell lines by 1.45 fold in MCF-7 cells, 4 fold change in T47D cells and5 fold CCND1 in Hs578T; while there is no change in MDA-MB-231 cells (Fig. 10c).

**Figure 7. Cell invasion analysis in miR-638 overexpressed breast cancer cell lines.**

A. From left to right, cells treatment with negative control MOCK or miR-638 overexpresssion; from top to bottom, invasion assay results of MDA-MB-231, Hs578T, MCF-7 and T47D cell lines. B. Invasion ability of four cell lines was displayed as a percentage of the absolute cell numbers. Results are displayed as mean data±SE. ***p* <0.01 with comparison to MOCK. Five fields of unit area on each membrane or whole membrane were counted for cell numbers, and the experiments were repeated three times with triplicate.

**A.**



**B.**

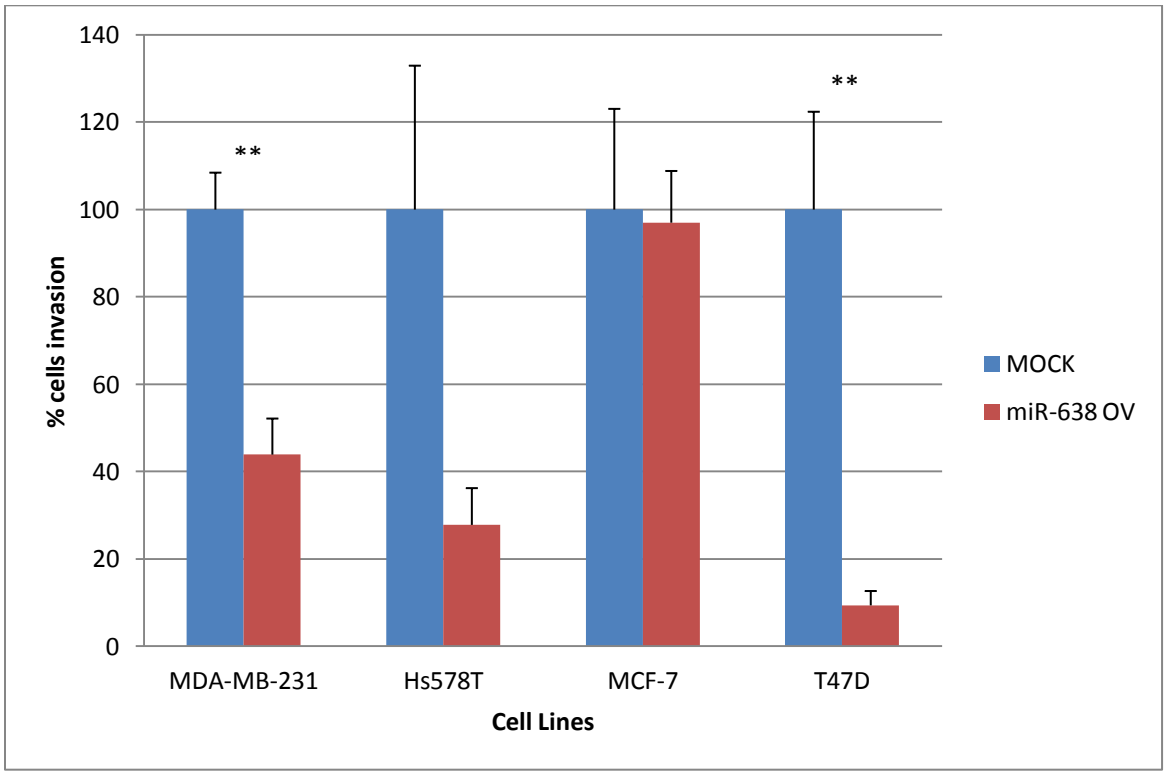

140
120
100
80
60
40
20
0
% cells invasion
**
**
MDA-MB-231
Hs578T
MCF-7
T47D
Cell Lines
MOCK
miR-638 OV

**Table 2. The representive 30 conserved target genes of miR-638 sorted by total context score.**

TARGETSCAN-VERT 6.2 (http://www.targetscan.org/) was used to predict the target genes of miR-638. Each predicted target gene is sorted based on the predicted efficacy of targeting as calculated using the context+ scores of the sites. The conserved targeting has also been detected within open reading frames.

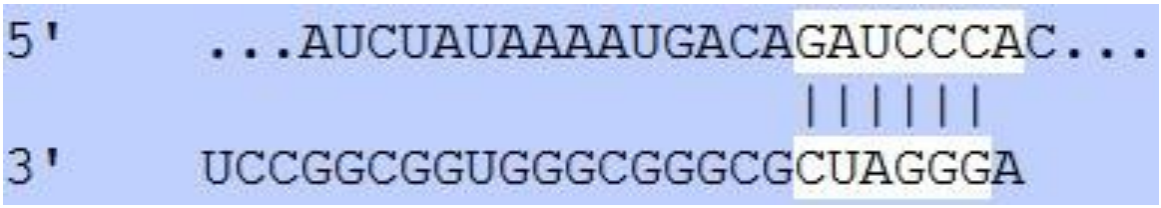
| Target gene | Representatie transcript | Gene name | Conserved sites | | | | Total context + score |
|---|---|---|---|---|---|---|---|
| | | | total | 8mer | 7mer -m8 | 7mer -1A | |
| **STARD10** | NM_006645 | StAR-related lipid transfer (START) domain containing 10 | 1 | 1 | 0 | 0 | -0.53 |
| **NPAS4** | NM_178864 | neuronal PAS domain protein 4 | 1 | 1 | 0 | 0 | -0.44 |
| **MKLN1** | NM_001145354 | muskelin 1, intracellular mediator containing kelch motifs | 1 | 0 | 0 | 1 | -0.27 |
| **CCDC92** | NM_025140 | coiled-coil domain containing 92 | 1 | 0 | 0 | 1 | -0.26 |
| **SHPK** | NM_013276 | sedoheptulokinase | 1 | 0 | 0 | 1 | -0.26 |
| **RIMKLB** | NM_020734 | ribosomal modification protein rimK-like family member B | 1 | 0 | 1 | 0 | -0.24 |
| **PGK1** | NM_000291 | phosphoglycerate kinase 1 | 1 | 0 | 0 | 1 | -0.22 |
| **HP1BP3** | NM_016287 | heterochromatin protein 1, binding protein 3 | 1 | 0 | 0 | 1 | -0.21 |
| **LMO4** | NM_006769 | LIM domain only 4 | 1 | 0 | 0 | 1 | -0.21 |
| **PAK2** | NM_002577 | p21 protein (Cdc42/Rac)-activated kinase 2 | 1 | 0 | 0 | 1 | -0.2 |
| **MARCKS** | NM_002356 | myristoylated alanine-rich protein kinase C substrate | 1 | 0 | 0 | 1 | -0.19 |
| **FRMPD4** | NM_014728 | FERM and PDZ domain containing 4 | 1 | 0 | 0 | 1 | -0.18 |
| **EMILIN3** | NM_052846 | elastin microfibril interfacer 3 | 1 | 0 | 0 | 1 | -0.18 |
| **MYCL1** | NM_001033081 | v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian) | 1 | 0 | 0 | 1 | -0.18 |
| **LOC1005** | NM_001195528 | hypothetical | 1 | 0 | 0 | 1 | -0.18 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **07050** | | LOC100507050 | | | | | |
| **ISOC2** | NM_001136201 | isochorismatase domain containing 2 | 1 | 0 | 0 | 1 | -0.18 |
| **EDN3** | NM_207032 | endothelin 3 | 1 | 0 | 0 | 1 | -0.18 |
| **BRCA1** | NM_007294 | breast cancer 1, early onset | 1 | 0 | 0 | 1 | -0.16 |
| **TSPAN1** | NM_005727 | tetraspanin 1 | 1 | 0 | 0 | 1 | -0.16 |
| **BUB3** | NM_004725 | budding uninhibited by benzimidazoles 3 homolog (yeast) | 1 | 0 | 0 | 1 | -0.15 |
| **SP8** | NM_182700 | Sp8 transcription factor | 1 | 0 | 0 | 1 | -0.15 |
| **ZNF24** | NM_006965 | zinc finger protein 24 | 1 | 0 | 0 | 1 | -0.14 |
| **KDSR** | NM_002035 | 3-ketodihydrosphingosine reductase | 1 | 0 | 0 | 1 | -0.14 |
| **KPNA6** | NM_012316 | karyopherin alpha 6 (importin alpha 7) | 1 | 0 | 0 | 1 | -0.13 |
| **SP7** | NM_001173467 | Sp7 transcription factor | 1 | 0 | 0 | 1 | -0.12 |
| **NOVA1** | NM_002515 | neuro-oncological ventral antigen 1 | 1 | 0 | 0 | 1 | -0.11 |
| **SENP1** | NM_014554 | SUMO1/sentrin specific peptidase 1 | 1 | 0 | 0 | 1 | -0.06 |
| **SRSF1** | NM_001078166 | serine/arginine-rich splicing factor 1 | 1 | 0 | 0 | 1 | -0.05 |
| **EPHA7** | NM_004440 | EPH receptor A7 | 1 | 0 | 0 | 1 | -0.04 |
| **TTC28** | NM_001145418 | tetratricopeptide repeat domain 28 | 1 | 0 | 0 | 1 | -0.04 |

**Figure 8. The predicted binding sites of miR-638 target genes**

A. miR-638 has a predicted binding site of BRCA1 in the position 1103-1109 of 3' UTR with 7 mer-1A seed match. B. miR-638 has a predicted binding site of BRCA1 in the position 316-322 of 3' UTR, with 7mer-1A seed match. The binding sites are predicted by TARGETSCAN-VERT 6.2.

**A.**


5' ...AUCUAUAAAAUGACAGAUCCCAC...
3' UCCGGCGGUGGGCGGGCGCUAGGGA

**B.**

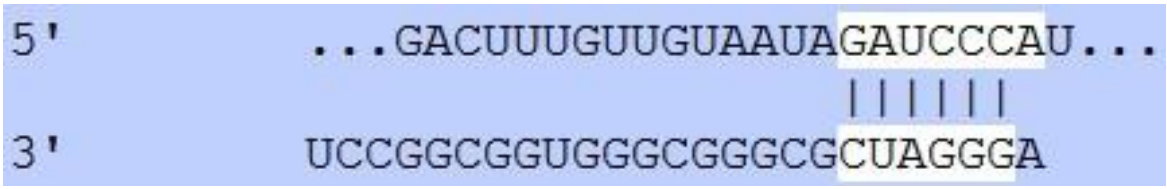
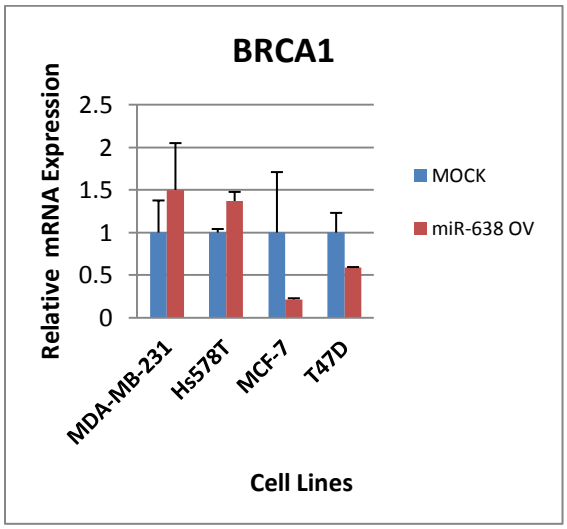

5' ...GACUUUGUUGUAAUAGAUCCCAU...
3' UCCGGCGGUGGGCGGGCGCUAGGGA

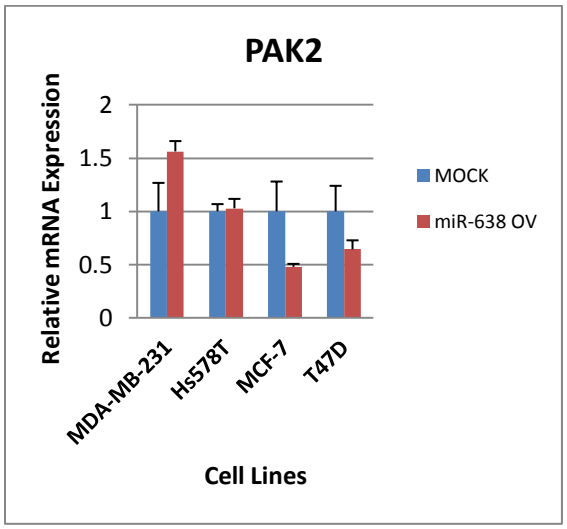
**Figure 9. Expression of miR-638 target genes, BRCA1 and PAK2 verified by real-time qRT-PCR in MDA-MB-231, Hs578T, MCF-7 and T47D breast cancer cell lines.**

Total RNAs were extracted from each cell line, MDA-MB-231, Hs578T, MCF-7 and T47D after 48 hrs transfected with miR-638 mimic or MOCK. The blue bars represent the target gene expression levels in MOCK control while the red bars are for miR-638 transfected cells. Panel A, B and C are for BRCA1, PAK2 and CCND1 respectively.

**A.**


BRCA1
Relative mRNA Expression
2.5
2
1.5
1
0.5
0
MDA-MB-231
Hs578T
MCF-7
T47D
Cell Lines
MOCK
miR-638 OV

**B.**


PAK2
Relative mRNA Expression
2
1.5
1
0.5
0
MDA-MB-231
Hs578T
MCF-7
T47D
Cell Lines
MOCK
miR-638 OV

**C.**

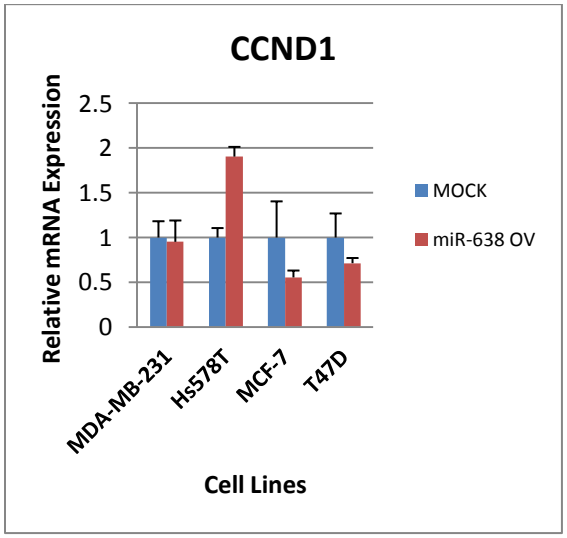
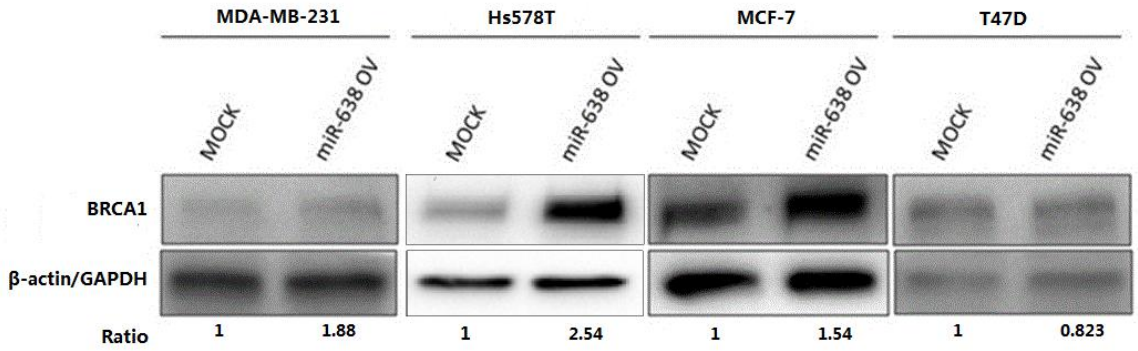
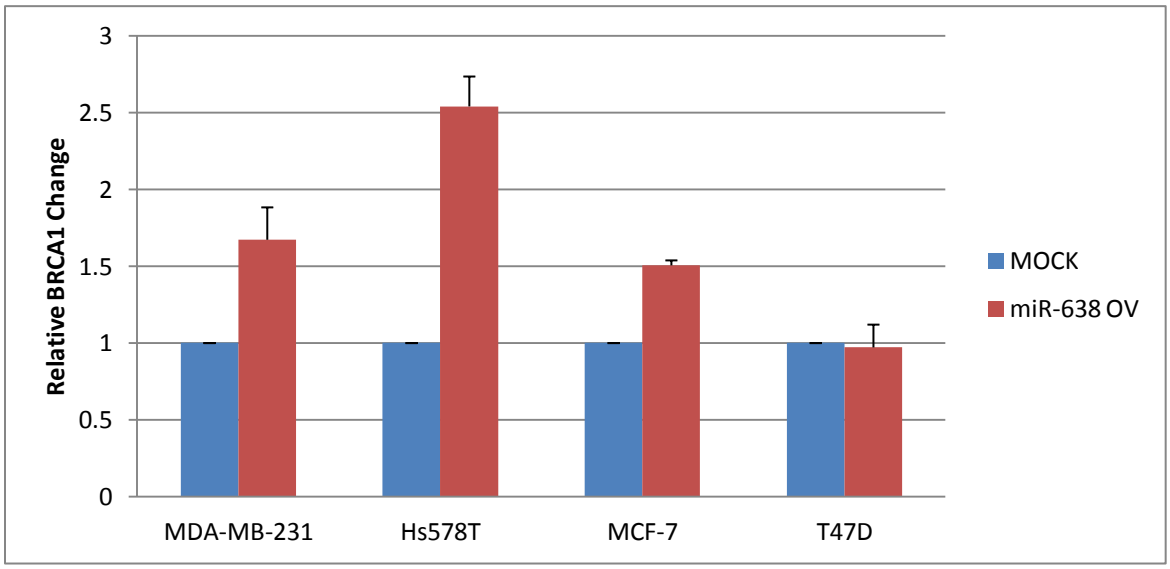

CCND1
Relative mRNA Expression
2.5
2
1.5
1
0.5
0
MDA-MB-231
Hs578T
MCF-7
T47D
Cell Lines
MOCK
miR-638 OV

**Figure 10. miR-638 target gene expression in miR-638 overexpressed breast cancer cell lines by Western blot**.

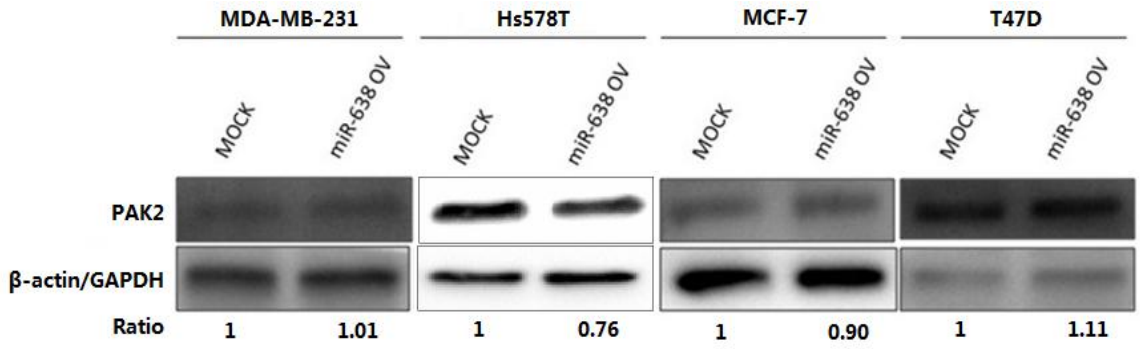
A. Western blot analysis of BRCA1 expression in miR-638 overexpressed MDA-MB-231, Hs578T, MCF-7 and T47D cell lines after 72 hrs transfection. B. Western blot analysis of PAK2 expression in miR-638 overexpressed MDA-MB-231, Hs578T, MCF-7 and T47D cell lines after 72 hrs transfection. C. Western blot analysis of CCND1 expression in miR-638 overexpressed MDA-MB-231, Hs578T, MCF-7 and T47D cell lines after 72 hrs transfection. The western blot original pictures show one representative experiment. The bar figures represent two independent experiments result. Values represent the average $\pm$ SE of two independent experiments.

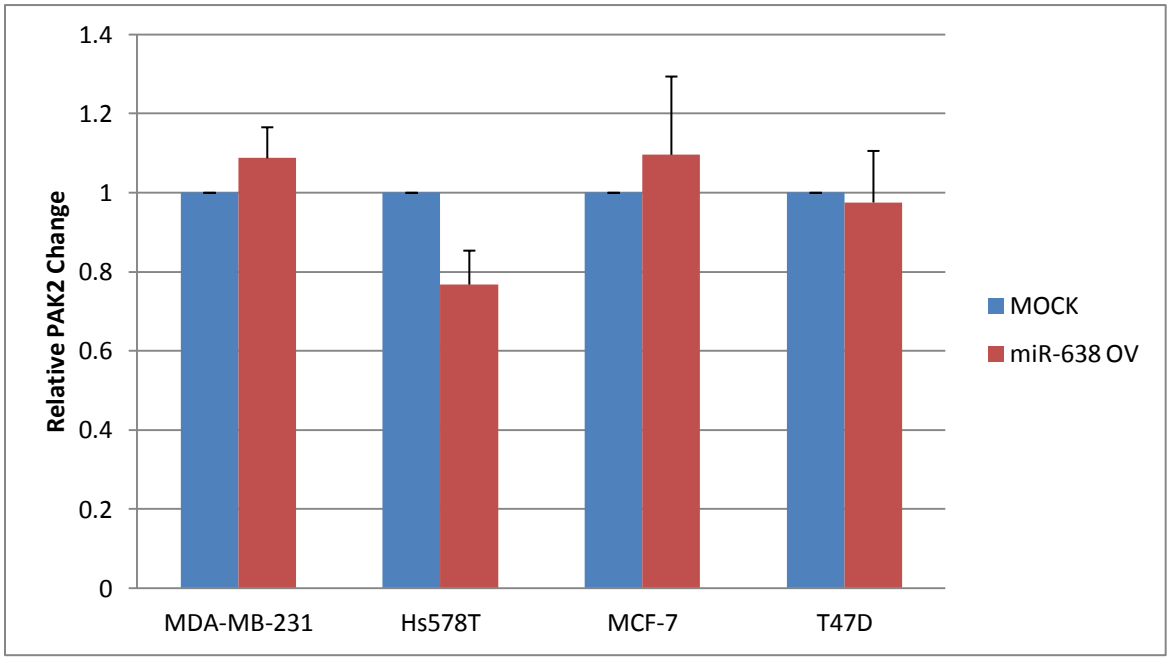
**A.**


MDA-MB-231
Hs578T
MCF-7
T47D
MOCK
miR-638 OV
MOCK
miR-638 OV
MOCK
miR-638 OV
MOCK
miR-638 OV
BRCA1
β-actin/GAPDH
Ratio
1
1.88
1
2.54
1
1.54
1
0.823

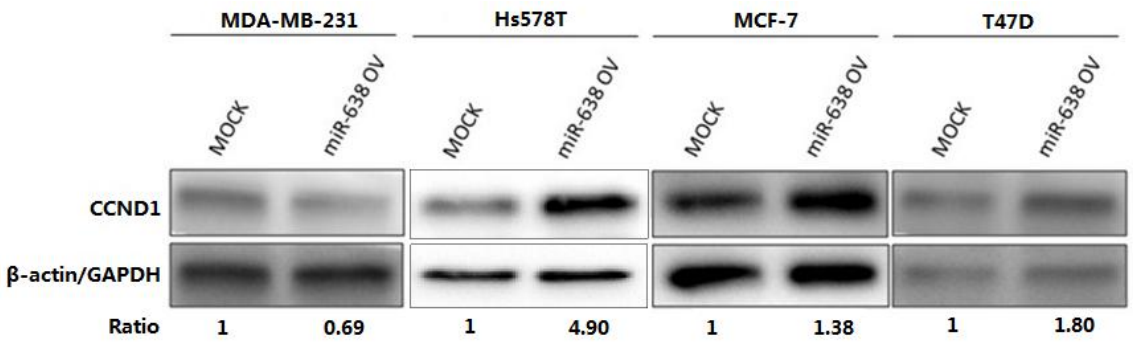

Relative BRCA1 Change
3
2.5
2
1.5
1
0.5
0
MDA-MB-231
Hs578T
MCF-7
T47D
MOCK
miR-638 OV

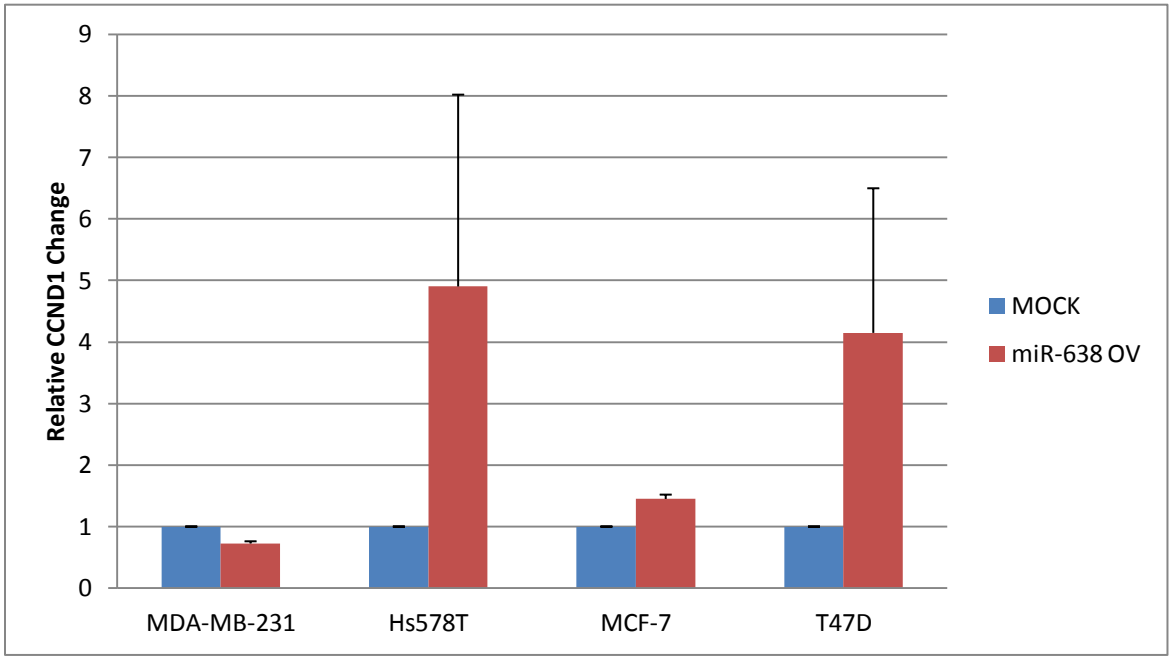
**B.**


MDA-MB-231
Hs578T
MCF-7
T47D
MOCK
miR-638 OV
MOCK
miR-638 OV
MOCK
miR-638 OV
MOCK
miR-638 OV
PAK2
β-actin/GAPDH
Ratio
1
1.01
1
0.76
1
0.90
1
1.11


Relative PAK2 Change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
MDA-MB-231
Hs578T
MCF-7
T47D
MOCK
miR-638 OV

**C.**


MDA-MB-231
Hs578T
MCF-7
T47D
MOCK
miR-638 OV
MOCK
miR-638 OV
MOCK
miR-638 OV
MOCK
miR-638 OV
CCND1
β-actin/GAPDH
Ratio
1
0.69
1
4.90
1
1.38
1
1.80


Relative CCND1 Change
9
8
7
6
5
4
3
2
1
0
MDA-MB-231
Hs578T
MCF-7
T47D
MOCK
miR-638 OV

## Chapter 4: Discussions and Conclusion

### Down-regulation of miR-638 in breast cancer FFPE samples and cell lines.

The objective of this study was to decipher the role of miR-638 in breast cancer tumorigenesis. An earlier study from our lab indicated that miR-638 is down-regulated in ADH and IDC breast cancer developing stages compared to normal controls and ADH stages [29]. In this study, we analyzed miR-638 expression in 9 additional breast cancer FFPE samples following microdissection by real-time qRT-PCR. The data shows that miR-638 was significantly down-regulated in DCIS and IDC stages in 8 of the 9 patient samples. Additionally, more significant change of miR-638 expression was observed in the transition of ADH to DCIS. Although we can not offer an exact explanation of the inconsistency of one patient's data, we speculate that possibly this particular patient's tumorigenesis has a different molecular / pathological feature. It could also be due to technical errors. I believe that analyzing more patient FFPE sampes will help understand the exact role of miR-638 in breast cancer development.

Since miR-638 is down-regulated during breast cancer progression as demonstrated by our FFPE sample analysis, we sought to investigate the expression of miR-638 in a number of breast cancer cell lines, including MDA-MB-231, Hs578T, MDA-MB-468, MCF-7 and T47D with MCF-10A as a control (Table 1). The ER-, PR- and HER2-expression status of the cell lines is as shown in the table. MCF-10A is a fibrocystic disease derived cell line. Although it shows no sign of terminal differentiation or senescence, it is a non-tumorigenic mammary epithelial line[11, 40]. The MCF-10A cells have intact cell cycle checkpoints and normal proliferation controls. Therefore, it is

frequently used as a "normal" mammary epithelial cell line control in breast cancer research [41]. MDA-MB-231, Hs578T and MDA-MB-468 are highly invasive lines exhibiting features of epithelial mesenchymal transition(EMT) [11, 42]. The expression of miR-638 was very low in all 5 breast cancer cell lines compared with the MCF-10A (Fig. 4). From the FFPE sample as well as the breast cancer cell line analysis, we have demonstrated the low expression of miR-638. Therefore we hypothesize that miR-638 functions as a tumor-suppressor miRNA in breast cancer progression.

**miR-638 plays an important role in cell proliferation and invasion**

Since the expression of miR-638 is deregulated in breast cancer, we further evaluated which cellular behaviors may be regulated by miR-638. There are some recent publications indicating that miR-638 regulates cell growth and proliferation, involving in cancer progression. It is reported that miR-638 is a key molecule in regulating human vascular smooth muscle cell proliferation and migration by targeting NOR1/cyclin D pathway. Up-regulated miR-638 can inhibit the proliferation of the human aortic smooth muscle cells [43]. Christenson et al. reported that miR-638 contributed to emphysema progression including altered cell growth, through multiple effects on fibroblast function [33]. Interestingly, miR-638 could negatively regulate BRCA1 expression and increase ESCC cell proliferation *in vitro* [44]. miR-638 was reported to be involved in the BaP-induced human bronchial epithelial cell carcinogenesis by targeting BRCA1[34]. However, there is no report about the role of miR-638 in proliferation and invasion of breast cancer. We analyzed the functional consequences of overexpressing miR-638 in

TNBC cell lines, MDA-MB-231and Hs578T, as well as the hormonal positive cell lines, MCF-7 and T47D. Our data indicated that miR-638 has different functions on cell behaviors towards different breast cancer types. In TNBC cells, miR-638 inhibited cell proliferation, while in hormonal positive cells, miR-638 promoted cell proliferation. Importantly, miR-638 can not only affect cell proliferation but also alter cell invasive ability. After overexpression of miR-638, the invasiveness of the TNBC cell lines, MDA-MB-231 and Hs578T was suppressed. In hormonal positive cell lines, the invasion of T47D decreased but increased in MCF-7. These data indicate that the function of miR-638 may depend on hormonal receptor expression status. It may serve as a tumor suppressor in TNBC cell lines.

**miR-638 target gene analysis**

Recent data suggest that the significance of a post-transcriptional regulation mechanism involves the participation of miRNAs. miRNAs can regulate the genes in the transcriptional level via post-translational modifications through directly binding to the complimentary seed site of target genes or indirectly regulation by binding to other target genes. One miRNA can target multiple genes through perfect or imperfect complementarily bindings. One mRNA also can be regulated by several miRNAs [45]. Different target gene prediction tools are available, such as DIANA-MICROT, MICRORNA.ORG, MIRDB, TARGETMINER and TARGETSCAN-VERT in predicting potential miRNA target genes. We chose to use TARGETSCAN-VERT to predict target genes of miR-638. We then focused on the top 30 conservative target

genes as listed inTable 2. BRCA1 and PAK2 are among the the predicted target genes of miR-638. BRCA1 is a well known tumor suppressor which controls cell growth, apotosis and cell motility [46]. The expression of BRCA1 was determined to be hormone-dependent via direct activation through the estrogen (ERα) [47]. PAK2 also plays a role in cancerous signaling pathways including cytoskeleton regulation, cell motility, cell cycle progression, apotosis, proliferation and invasion [48, 49].

BRCA1 is a tumor suppressor which interacts with a number of transcription factors and regulatory proteins to form a BRCA1-associated genome surveillance complex (BASC) [50]. The mutation or loss of BRCA1 alleles can contribute to the development of tumor. Abnormal BRCA1 and BRCA2 genes may account for up to 10% of all breast cancers [51]. BRCA1 can bind with other tumor suppressors, such as binding to p53 to enhance its transcription activity [52]. The C-terminus of BRCA1 can fuse to a protein domain to bind to a promoter [46, 53]. The N-terminus of BRCA1 contains a RING finger motif and is involved in transactivation [53]. BRCA1 can also form complexes with a number of proteins directly involved in DNA damage repair. It can repair double strand breaks, DNA mismatch and transcription-coupled DNA damage [46, 53, 54]. BRCA1 is linked to cell- cycle arrest, associated with E2F, CDC2 and cyclins to control cell cycle progression [41]. It was reported that BRCA1 has an inhibitory effect on cell proliferation and growth, but the expression of BRCA1 only selectively inhibits the cell proliferation in specific cell types determined by retinoblastoma protein (RB)[41]. RB is a tumor suppressor activated in a hypophosphorylated state and is involved in cell cycle suppression [55]. Aprelikova et al. found that only cells with hypophosphorylated RB were sensitive to BRCA1-induced growth arrest [56]. BRCA1 binds to hyposphorylated

RB, which interacts with E2F to form an active complex that lead to histone deacetylation to block genes transcription [41]. Although RB is a tumor suppressor, the loss of RB in TNBC cell lines patients appears to become more sensitive to chemotherapy, which is favorable for the TNBC patients [57]. This study shows that overexpression of miR-638 supresses cell proliferation in MDA-MB-231 and Hs578T cell lines, possibly due to the activation of BRCA1.

Generally, miRNA targets are predicted by computational methods, which considers the binding site in the 3'UTR of the target genes. Yet more and more data suggest that miRNAs can not only bind the 3'UTR but also the 5'UTR or coding or promoter regions of the target genes [58]. Not all binding of miRNAs to their target genes will suppress or degrade their target gene expression, some binding might stabilize the target genes, and even increase their expression [59]. Additionally, the SNP in the binding region can also influence the binding ability of miRNAs [58]. miR-638 is predicted to have a 7 mer-1A binding with BRCA1 in the 3'UTR (Figure 8a). However one report indicates that the binding site of miR-638 with BRCA1 is not in the 3'UTR as predicted, instead it is in the coding region of BRCA1. Additionally, the SNP in BRCA1 binding region will also influence the binding ability of miR-638. The SNP of BRCA1 on rs799917 from [TT] to [CC] enhances the inhibition of BRCA1 protein expression after binding with miR-638 in MCF-7 and T47D cell lines [58]. Yet the SNP of XRCC1 from C to T on rs1799782 could strengthen the binding of miR-138, leading to an increase of XRCC1 expression [58]. In my study, I tested BRCA1 gene expression in breast cancer cell lines MDA-MB-231, Hs578T, MCF-7 and T47D after overexpression of miR-638. The mRNA expression

of BRCA1 increased in MDA-MB-231 and Hs578T cell lines but decreased in MCF-7 and T47D cell lines when miR-638 is overexpressed. When miR-638 is overexpressed, BRCA1 protein expression was increased in MDA-MB-231 and MCF-7, but decreased in Hs578T and no change in T47D cell line. The data support the notion that miR-638 can up-regulate BRCA1. We also discovered that miR-638 has different functions even in the same type of cancer but different cell lines. Our data is not in agreement with that of Nicoloso M.S et al. since we obtained an opposite BRCA1 protein expression result in MCF-7 and T47D compared with theirs [58]. The variant cell cultured environment may cause DNA sequence change in the binding site of miR-638 with BRCA1. All these differences may lead to unique changes in biological properties hence show different experiment results.

Germline mutations in BRCA1 confer a high risk of breast cancer and ovarian cancer. BRCA1 gene test is offered to women who are likely to have an inherited mutation. BRCA1 has 18 different alterations in the gene sequence in most of breast cancer cell lines. Different cell lines have different variants, such as MDA-MB-231 with variants 3 and 5, Hs578T with variant 3, and no variants for MCF-7 and T47D. The BRCA1 mutation phenotypes in these four cell lines are wild types [60]. Although BRCA1 displays wild type in these cell lines, the differentially expressed variants and the SNP may alter the binding of miR-638, which leads to the distinct expression of BRCA1 in these cell lines. Luciferase assay experiments are in progress to further verify that BRCA1 is a direct target if miR-638.

CCND1 is a cell cycle regulatory protein functioning in CCND1-CDK4 complex, which phosphorylates and inhibits members of the RB protein family including RB1 and regulates the cell-cycle during G1/S transition. Protein overexpression has been reported in 42–80% of primary breast tumours and breast cancer cell lines. Vaziri et al. reported that BRCA1 mutation carriers has low CCND1 amplification [61]. Several lines of evidence suggest that CCND1 is a component of the core cell-cycle machinery which is expressed in all proliferating cell types. In previous study, it was shown that induction of BRCA1 could reduce CCND1 expression [62]. In my experiment, CCND1 expression was decreased by 30% in MDA-MB-231, while increased in various degree in Hs578T, MCF-7 and T47D when miR-638 is overexpressed. CCND1 may not be regulated strongly by BRCA1 but by other proteins and factors.

PAK2 is one of the Pak kinases, which are thought to play critical roles in cell migration and invasion. Coniglio reported that PAK2-depleted breast cancer cells display an increase in focal adhesion size and enhance RhoA activity to inhibit cell invasion [48]. The transcript expression of PAK2 does not agree with its protein expression. PAK2 mRNA was upregulated in MDA-MB-231, with no change in Hs578T, but downregulated in MCF-7 and T47D cells. However, there is no protein change in MDA-MB-231, MCF-7 and T47D except Hs578T with slight decrease compared to MOCK. miRNA regulate target genes in post transcription level, which may not contribute to protein level since other post transcriptional modifications and regulations by other

proteins and factors may also alter the protein expression. No significant change of PAK2 in protein level indicates miR-638 may not directly target PAK2. Functional assays, such as luciferase assays, need to be done to verify whether PAK2 is a direct target of miR-638 as TARGETSCAN-VERT predicted.

In conclusion, miR-638 may be a tumor suppressor during breast cancer development and progression. In particular, miR-638 may play an important role in regulating cell proliferation and invasiveness in TNBC. The functional mechanism for miR-638 is likely through its binding to and activating the BRCA1 tumor suppressor. Therefore, miR-638 could be a potential biomarker for breast cancer diagnosis and serve as a therapeutic target for TNBC.

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