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EXPERIMENTAL STUDY

# Micro RNA-126 coordinates cell behavior and signaling cascades according to characteristics of breast cancer cells

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**ABSTRACT**
BACKGROUND: Micro RNA-126 is known to enhance apoptotic processes and also plays a role in vascular growth through the regulation of vascular endothelial growth factor-mediated signaling, angiogenesis, and vascular integrity.
OBJECTIVES: We aimed to determine the role of miR-126 in breast cancer cell lines with a variety of different characteristics to evaluate its interaction with certain cancer-related molecules and mechanisms.
METHODS: To determine the effect of presence and absence of miR-126 in MCF-7 and MDA-MB-231 breast cancer cells, miR-126 mimics and inhibitor were transfected. miRNA and gene expressions were observed by using RT-PCR. Viability, proliferation, adhesion, invasion and lateral motility assays were performed to determine cell behavior changes.
RESULTS: miR-126 is more effective on MDA-MB-231 cells on cell behavior. We observed an increase in miR-126 expression when miR-126 mimics was transfected to MCF-7 and MDA-MB-231 cells. Also, there was a decrease in miR-126 expression when MCF-7 and MDA-MB-231 cells were transfected with miR-126 inhibitor. Furthermore, presence and absence of miR-126 modulated the gene expressions of VEGF/PI3K/AKT and MAPK signaling in MCF-7 and MDA-MB-231.
CONCLUSION: Our study showed that miR-126 is in a state of interaction with a multitude molecules playing a role in breast cancer. According to obtained data, we can say that miR-126 may be more effective in inhibition of metastatic breast cancer *(Tab. 4, Fig. 3, Ref. 46)*. Text in PDF *www.elis.sk.*
KEY WORDS: breast cancer, Egfl-7, metastasis, micro RNA-126, motility, signaling pathways.

**Abbreviations:** miRNA – Micro RNA; miR-126 – Micro RNA-126; miR-126* – Complementary Micro RNA-126; VEGF – Vascular Endothelial Growth Factor; 3'UTR – 3' Untranslated Region; Egfl-7 – Epidermal Growth Factor Like 7; pre-miR-126 – Precursor-miR-126; mat-miR-126 – Mature-miR-126; SPRED-1 – Sprouty-Related EVH1 Domain-Containing Protein 1; $PIK_3R_2$ – Phosphatidylinositol 3-Kinase Regulatory Subunit Beta; FBS – Fetal Bovine Serum; RT-PCR – Real Time Polymerase Chain Reaction; GAPDH – Glyceraldehyde-3-Phosphate Dehydrogenase; ELISA – Enzyme-Linked Immunosorbent Assay; PBS – Phosphate-Buffered Saline; NSCLC – Non-Small Cell Lung Cancer; IRS-1 – Insulin Receptor 1

## Introduction

Micro RNAs (miRNA), which are formed through a two-stage procedure involving endonucleases named as Drosha and Dicer as well as hairpin shaped precursors, regulate the target gene expression, and play an important role in physiological and pathological processes involving cell growth, differentiation, apoptosis, and stress response (1). Precursor-miR-126 (pre-miR-126) is the hairpin precursor miRNA, and its 3'-Poly A region formed in the nucleus during miRNA biogenesis is trimmed by the Dicer enzyme (2, 3). After trimming of pre-miR-126 in the cytoplasm by a second Dicer enzyme, the mature-miR-126 (mat-miR-126) is generated (4). miRNAs regulate the angiogenic signals through targeting angiogenic factors and protein kinases by repressing genes through alteration of gene activation (5). They can modulate pro-angiogenic signals induced by vascular endothelial growth factor (VEGF) and anti-angiogenic signals induced by thrombospondin-1 (TSP-1), and therefore promote or inhibit tumour angiogenesis. miRNAs play a role in cellular differentiation and growth using mRNA breakdown and translational suppression mechanisms (6–11). Receptor tyrosine kinases (RTKs) and hypoxia inducible factor (HIF) are also targeted by miRNAs. Furthermore, miRNAs crosstalk with reactive oxygen species (ROS) influences tumor angiogenesis (5). Due to the results obtained by the previous studies, miRNAs are considered to have a potential to be utilized for the diagnosis and therapy of cancers (6, 10, 12).

MicroRNAs are regulators of gene expression that have been shown to be essential elements in the coordination of complex regulatory pathways. One of these short non-coding RNAs, microRNA-126 (miR-126), is highly enriched in the vascular endothelium and was shown to play distinct roles in angiogenesis, vasculogen-

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esis and endothelial inflammation. Lack of miR-126 leads to severe complications in the response in vascular development as well as vital repair mechanisms carried out by endothelial cells (13).

In vertebrates the epidermal growth factor like 7 (*Egfl-7)* gene codes the biologically active miRNAs (7). miR-126 is one of these active miRNAs, which is found in intron 7 at 9p34.3 region of chromosome 9 (6), and is considered to be specific for endothelial cells. miR-126 is controlling the fate and/or function of a variety of cells differentiating from the hematopoietic lineage, including megakaryocytes and erythrocytes. Furthermore, reports have suggested a protective role of circulating microRNA-126 in murine models of organ ischemia (13).

The growth sprouting and the growth sprouty-related proteins observed during the angiogenesis step are regulators of cell growth (14). The activity of sprouty-related EVH1 domain-containing protein 1 (SPRED-1) is primarily regulated by tyrosine phosphorylation, facilitated by hematopoietic factors. SPRED-1 plays a vital role in the tumorigenesis and metastasis of a stable tumor since it is an inhibitor of Ras-Mitogen-Activated Protein Kinase (MAPK) and RhoA cell signaling pathways (15). In particular, SPRED-1 binds to and inactivates the RAF-1, which is involved in the MAPK signaling and which represents the upstream kinase of the pathway (16, 17). miR-126 downregulation was associated with increased SPRED-1, leading to decreased activation of RAF (phosphorylated RAF/RAF) and MAPK (phosphorylated MAPK/MAPK), thus inhibiting the vascular endothelial growth factor pathway (18).

Phosphatidylinositol 3-kinase regulatory subunit beta ($PIK_3R_2$) is a regulatory component of PI3K, which is located on the upstream of AKT (19). $PIK_3R_2$ binds and inactivates PI3K, which is involved in PI3K/p-AKT signal pathway and inactivation of PI3K has an impact on PI3K/p-AKT pathway functioning (20). The PI3K/p-AKT pathway is well-known to be responsible for regulating cell growth, proliferation, survival and angiogenesis in the development of cancer. Therefore, molecules altering the expression of $PIK_3R_2$ may be an effective way to lower the malignancy of cancer types. Several previous reports have demonstrated that miR-126 reduces the expression of $PIK_3R_2$ by directly targeting its 3'-untranslated region (20, 21).

Breast cancer is highly resistant to chemotherapeutic approach and hence, alternative strategies have been developed to fight against this heterogeneous group of disease (22). One of these alternative strategies is gene treatment. Although many studies have demonstrated the effect of various drugs for the treatment of breast cancer, we aimed to demonstrate the effect of gene treatmentin breast cancer. In our study, we assessed the activities of micro RNA-126 in MCF-7 and MDA-MB-231 breast cancer cell lines.

**Tab. 1. The sequences are transfected into cells.**

| | Transfected sequence |
|---|---|
| Pre-miR-126 | 5'-UCGUACCCGUGAGUAAUAAUGCG-3' |
| Mat-miR-126 | 5'-GTGTAACACGTCTATACGCCCA-3' |
| Anti-miR-126 | 5'-GCATTATTACTCACGGTACGA-3' |
| Scrambled (non-target) | 5'-GTGTAACACGTCTATACGCCCA-3' |

(Pre – precursor; Mat – mature)

## Material and methods

### *Cell Culture and Transfection*

MCF-7 and MDA-MB-231 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and the cells were incubated at 37 °C with 5 % $CO_2$ in RPMI 1640 (Biological Industries, Israel) containing 100 U/ml penicillin/streptomycin (Gibco, UK) and 10 % fetal bovine serum (FBS) (Gibco, UK). Before transfection, MCF-7 and MDA-MB-231 cells were incubated in 6-well plates (Greiner, Germany) to make sure the cells grow to 80 % confluence. Precursor (pre-), mature (mat-), anti-miR-126 (precursor/mimic/inhibitor) and the small interfering RNA (siRNA) that acted on siRNA control transcripts (scrambled or non-target) were obtained from Alpha DNA (Montreal, Quebec). siPORT Transfection kit (Ambion, Carlsbad, USA) was used to transfect cells as per the instruction of the manufacturer. The transfected sequences are shown in Table 1.

### *RNA isolation and real time polymerase chain reaction (RT-PCR)*

Total RNA was isolated from cells using Paris Total RNA Isolation Kit (Ambion, Carlsbad, USA) as per the instructions of the manufacturer. Primer sets for amplification of miR–126, miR-126*, *Egfl–7, VEGFR$_2$, SPRED-1, PIK$_3$R$_2$, PI3K, AKT, RAF-,1 ERK* and *GAPDH* were designed and supplied by Alpha DNA, MontReal, Quebec. RT-PCR was performed in Stratagene MxPro3000 (Stratagene, UK). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Alpha DNA, Montreal, Quebec) was used as an internal control,

**Tab. 2. Forward and reverse sequences of the primers used in RT-PCR.**

| Primer | Forward Sequence | Reverse Sequence |
|---|---|---|
| miR-126 | 3'- GTCCGCTCGTACCGTGAGTAATA-'5 | 3'-CCAGTCTCAGGGTCCGAGGTATTC-'5 |
| miR-126* | 3'-CGCGCTCATTATTACTTTTGGTA–5' | 3'-CCAGTCTCAGGGTCCGAGGTATTC-'5 |
| Egfl-7 | 5'- TGCGACGGACACAGAGCCTGCA-3' | 5'-CAAGTATCTCCCTGCCATCCCA-3' |
| VEGFR$_2$ | 5'-CACCACTCAAACGCTGACATGTA-3' | 5'-GCTCGT TGGCGC ACTCTT-3' |
| SPRED-1 | 5'-CTGATCCCTGTTCGTGTGACA-3' | 5'-AGACAAAGCTACCAGGGCTAACC-3' |
| PIK$_3$R$_2$ | 5′-CGAGACCAGTACCTCGTGTG-3′ | 5′-TAATCCCCAGCCACTCGTT-3′ |
| PI3K | 5′-CCAAAATTACTGCTGTCAAT -3′ | 5′- TAGGCCAAATCTGAAGCA -3′ |
| AKT | 5'-TGGGCCTGGCAAAACCT-3' | 5'-CGGTCTGAATCTGGTTCATTCA-3' |
| RAF-1 | 5′-GGATTGGGTCAGGCTCTT-3′ | 5′-GGGTCGACAACCTTTAGGA-3′ |
| ERK | 5′-GTTCCCAAATGCTGACTC-3′ | 5′-CAGAGCCTGTTCTACTTCAA-3′ |
| GAPDH | 5'-CGAGGGGGGAGCCAAAAGGG-'3 | 3'-GAAACTGCGACCCCGACCGT-'5 |

**Tab. 3. Viability, proliferation and adhesion data in MCF-7 and MDA-MB-231.**

| | | | VIABILITY | PROLIFERATION | ADHESION |
|---|---|---|---|---|---|
| MCF–7 | SCRAMBLED | | 75 (69.050-90) | 24694 (22040-26292) | 0.725 (0.660-0.750) |
| | miR-126 MIMICS | PRE | 62.5 (53.57-100) | 13490 (12765-14640)* | 0.620 (0.550-0.680) |
| | | MAT | 80 (62.502-87.498) | 32940 (29942-35665) | 0.625 (0.510-0.690) |
| | miR-126 INHIBITOR (ANTI) | | 91.67 (65.002-100) | 69440 (59040-72290)*** | 0.335 (0.260-0.370)*** |
| MDA-MB–231 | SCRAMBLED | | 94.02 (90.835-97.825) | 81743 (70743-89457) | 0.425 (0.380-0.580) |
| | miR-126 MIMICS | PRE | 100 (81.665-100.000) | 29171 (28457-29743)*** | 0.515 (0.470-0.600) |
| | | MAT | 75 (70.715-89.735)… | 23028.5 (18886-25600)*** | 0.510 (0.470-0.600) |
| | miR-126 INHIBITOR (ANTI) | | 91.61 (88.240-100.000) | 42957 (38457-50457) | (0.310-0.380)*** |

PRE – precursor, MAT – mature, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$

and the expressions of miRNA and mRNA were normalized to the expression of *GAPDH*. Gene expression changes were quantified using the delta-delta CT method. The primer sequences used in RT-PCR are shown in Table 2.

*Cell viability assays*

Cells were stained with trypan blue (Biological Industries, Israel) and were counted under the microscope to determine the number of viable/non-viable cells.

*Cell proliferation assays*

The XTT Kit (Biological Industries, Israel) was used to determine proliferation of MCF-7 and MDA-MB-231 cells. The cells were cultured into 96-well plates (Greiner, Germany). miRNA analogues or suppressors were used to transfect cells. After transfection, XTT was pipetted to wells and cultured for 2 h. The microplate spectrophotometer (Lab Systems, Finland)) was used to determine the absorbance at 450 nm.

*Cell adhesion assays*

After transfection, wells were flushed with phosphate-buffered saline (PBS; Sigma, Saint Louis, USA) three times. Then XTT was pipetted to all wells and cultured for 2 h. The microplate spectrophotometer (Lab Systems, Finland)) was used to determine the absorbance at 450 nm.

*Lateral motility assay*

The lateral motility of MCF-7 and MDA-MB-231 cells was analyzed in petri dishes. A total of $2x10^5$ transfected cells were suspended in petri dishes with medium. After 24 hours, the width of three wound areas generated at the base of the petri dish by the tip of a 1000 µl pipette was measured. Following incubation for 0, 24, 48 and 72 h, the width of three wound areas was measured. The motility in this area was assessed using measurements performed at 45 different locations for each group and for each hour.

*Cell invasion assay*

The invasion was analyzed in 24-well Boyden chambers with 8 µm pore size polycarbonate membranes (Corning, Inc., Corning, NY, USA). A total of $2x10^5$ transfected cells were suspended in the upper chamber with serum-free medium, while the lower chamber was filled with medium. In the invasion assays, the membranes were covered with Matrigel (BD Biosciences, San Diego, CA, USA) to form matrix barriers. Following incubation for 48 h, the cells on the upper surface were removed by wiping with a cotton swab and the cells on the lower surface of the membrane were fixed in methanol, stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and counted. The number of cells invading to the lower zone was measured in at least 8 locations randomly determined.

*Statistical analysis*

A normal distribution of the continuous variables was performed using the Kolmogorov-Smirnov test. Comparisons between groups of normally distributed variable were evaluated by One-Way variance analysis (ANOVA). The Tukey HSD test was used for multiple comparisons. Comparisons between groups of not normally distributed variables were evaluated by the Kruskal-Wallis test. Multiple comparisons of these groups were evaluated by the Dunn test. All analyses were performed using IBM SPSS Statistics 21.0 software package. The experiments were repeated independently three times.

## Results

*Effect of pre-, mat- and anti-miR-126 transfections on MCF-7 and MDA-MB-231 breast cancer cell behavior*

*Viability:* To assess the potential role of miR-126 in breast cancer, miR-26 mimics (pre-miR-126 and mat-miR-126) were used to overexpress the level of miR-126 in MCF-7 and MDA-MB-231 cells. Furthermore, miR-126 inhibitor (anti-miR-126) was used to repress the level of miR-126 in each breast cancer cell lines. Overexpression of mat-miR-126 significantly inhibited the viability in only MDA-MB-231 cells. Among MCF-7 cells, there was a decrease in the number of viable cells in the scrambled transfected cells as compared to cells transfected with pre-miR-126. In comparison with the scrambled transfected cells, an increase was detected in the viability of cells transfected with mat-miR-126 and miR-126 inhibitor (anti-miR-126) (Tab. 3) ($p > 0.05$). Although pre-miR-126 transfection induced the viability of MDA-MB-231 cells and mat-miR-126 transfection reduced the viability of MDA-MB-231 cells as compared to the scrambled transfected cells, no statistically significant difference was found between the groups (Tab. 3) ($p > 0.05$). Furthermore, viability of MDA-MB-231 cells was decreased significantly in miR-126 inhibitor as compared to the scrambled transfected cells (Tab. 3) ($p < 0.05$).

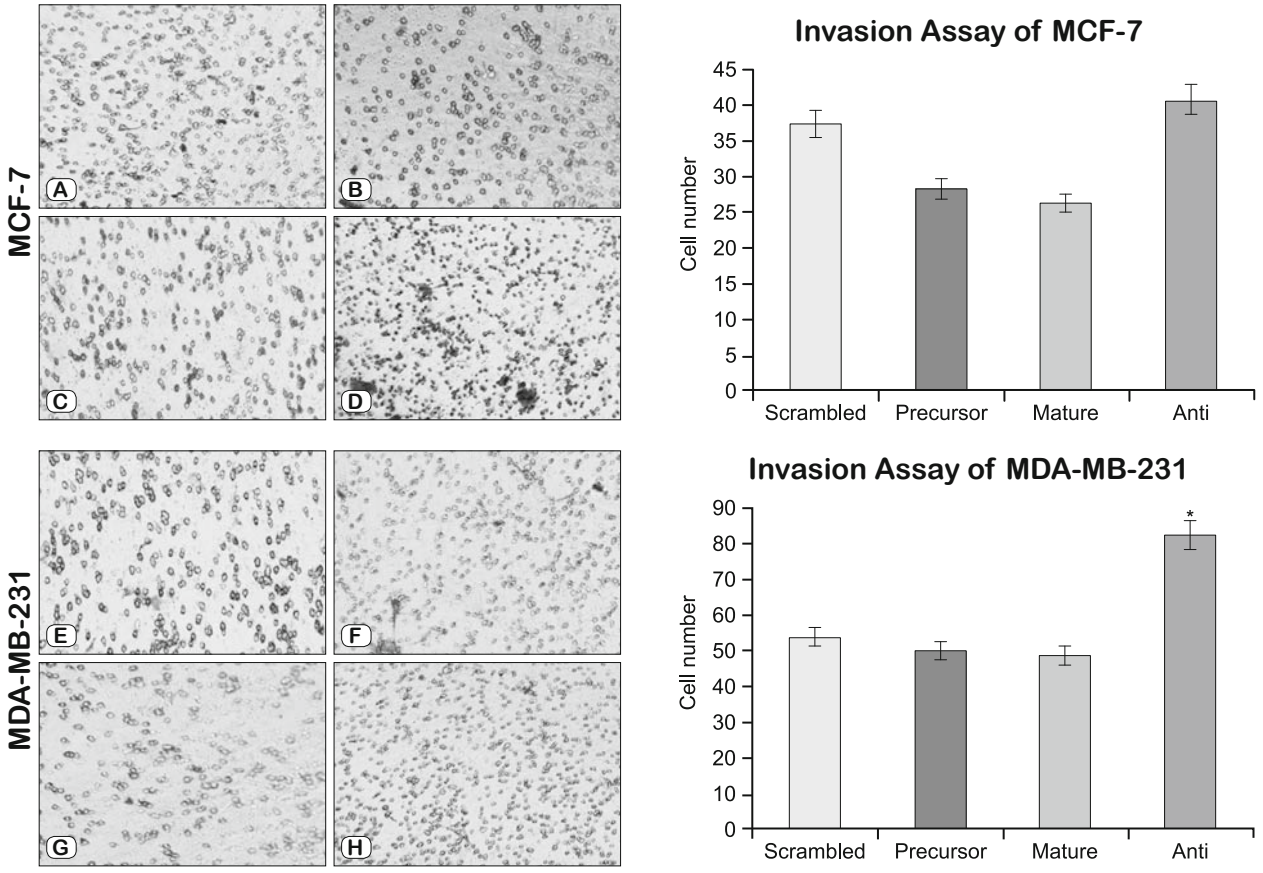



**Fig. 1. Invasion assay of breast cancer cells transfected with scrambled (non-target), miR-126 mimics or miR-126 inhibitor. The invasion assay revealed that the suppression of miR-126 induced the invasion in MDA-MB-231 cells (* $p < 0.001$). Representative images are shown [A: MCF-7 Scrambled (Non-Target) Group; B: MCF-7 pre-miR-126 Transfected Group; C: MCF-7 mat-miR-126 Transfected Group; D: MCF-7 anti-miR-126 Transfected Group; E: MDA-MB-231 Scrambled (Non-Target) Group; F: MDA-MB-231 pre-miR-126 Transfected Group; G: MDA-MB-231 mat-miR-126 Transfected Group and H: MDA-MB-231 anti-miR-126 Transfected Group].**

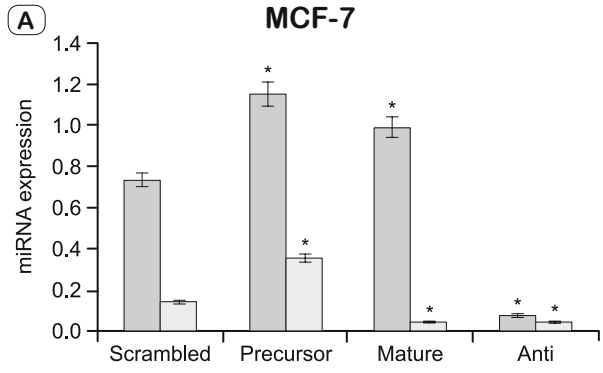
*Proliferation:* Proliferation is one of the characteristic aggressive properties of cancer cells. Overexpression of miR-126 significantly inhibited the growth rate of breast cancer cells. There was a decrease in proliferation in MCF-7 cells transfected with pre-miR-126 as compared to scrambled transfected cells (Tab. 3) ($p < 0.05$). There was an increase in cells transfected with mat-miR-126, but no statistically significant difference was found between the groups (Tab. 3) ($p > 0.05$). The transfection of miR-126 inhibitor increased the growth rate of MCF-7 cells (Tab. 3) ($p < 0.001$). In MDA-MB-231 cells, a significant induction was determined in cells transfected with miR-126 mimics as compared to the scrambled transfected cells (Tab. 3) ($p < 0.001$). However, miR-126 inhibitor transfection showed no statistically significant difference as compared to the scrambled transfected cells (Tab. 3) ($p > 0.05$).

*Adhesion:* There was a decrease in miR-126 mimic transfected with MCF-7 cells (Tab. 3) ($p > 0.05$). A significant inhibition of adhesion was observed as compared to the scrambled transfected cells when miR-126 inhibitor was transfected to MCF-7 cells (Tab. 3) ($p < 0.001$). Although overexpression of miR-126 by transfecting miR-126 mimics resulted in an increase of the adhesion in MDA-MB-231 cells as compared to the scrambled transfected cells, no statistically significant difference was found between the groups (Tab. 3) ($p > 0.05$). As compared to the scrambled transfected cells, a there was a statistically significant decrease in MDA-MB-231 cells when transfected with miR-126 inhibitor (Tab. 3) ($p < 0.001$).

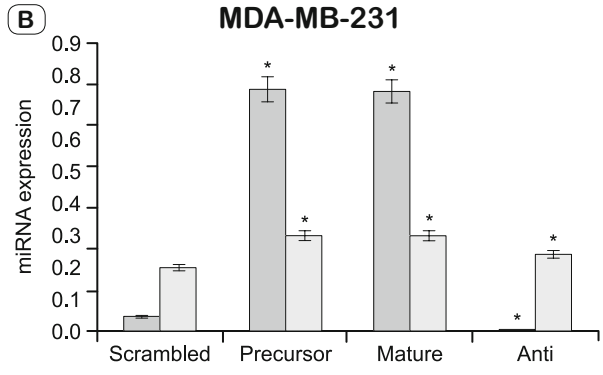
*Lateral motility (Wound healing):* Lateral motility and invasion are examples of the characteristic aggressive properties of cancer cells. These assays were used to measure the cell invasive and migrating capability. Therefore, we performed these assays to examine the metastasis potential of miR-126. There was a statistically significant inhibition in MCF-7 cells as compared to the scrambled transfected cells (0.421) when transfected with pre-miR-126 (0.347). However, transfection of mat-miR-126 (0.402) and miR-126 inhibitor (0.382) also inhibited the motility of MCF-7 cells; no significant difference between the two in the number of migrating cells was observed in the lateral motility assay ($p > 0.05$). Overexpression of miR-126 by transfecting with mat-miR-126 (0.129) was observed as compared to the scrambled group (0.182) ($p < 0.05$). Also, there was a significant increase in MDA-MB-231 cells transfected with miR-126 inhibitor (0.731) as compared to the scrambled transfected cells (0.182) ($p < 0.001$). Cells transfected

with pre-miR-126 (0.188) showed no statistically significant difference as compared to the scrambled transfected cells (0.182) in MDA-MB-231 cells ($p > 0.05$).

*Invasion:* Overexpression of miR-126 by transfecting pre-miR-126 (28.09 ± 2.38) and mat-miR-126 (26.09 ± 1.88) to MCF-7 cells resulted in a reduction of invasion compared to the scrambled group (37.18 ± 1.54). Repression of miR-126 by transfecting anti-miR-126 (40.45 ± 2.58) resultedins an induction of invasion compared to the scrambled group (37.18 ± 1.54). No statistically significant difference was found between the groups (Fig. 1) ($p > 0.05$). Transfection of miR-126 mimics [pre- (49.4 ± 2.58) and mat-miR-126 (47.9 ± 3.03)] to MDA-MB-231 cells reduced invasion compared to the scrambled group (53 ± 4.7); however no statistically significant difference was found between the groups (Fig. 1) ($p > 0.05$). There was also a statistically significant increase of invasion in MDA-MB-231 cells transfected with anti-miR-126 (81.2 ± 6.78) as compared to the scrambled group (53 ± 4.7) (Fig. 1) ($p < 0.001$).




| | Scrambled | Precursor | Mature | Anti |
|---|---|---|---|---|
| miR-126 | 0.7371 ± 0.00470 | 1.1567 ± 0.00055 | 0.9931 ± 0.00051 | 0.0786 ± 0.00026 |
| miR-126* | 0.1426 ± 0.00166 | 0.356 ± 0.00041 | 0.0487 ± 0.00059 | 0.0458 ± 0.00043 |




| | Scrambled | Precursor | Mature | Anti |
|---|---|---|---|---|
| miR-126 | 0.0454 ± 0.00018 | 0.7579 ± 0.00007 | 0.7526 ± 0.0002 | 0.0046 ± 0.00021 |
| miR-126* | 0.2 ± 0.00024 | 0.3 ± 0.00024 | 0.3 ± 0.00024 | 0.2415 ± 0.0002 |

**Fig. 2. Expressions of miR-126 and miR-126* in breast cancer cell lines. (A) miR-126/126* gene expressions were detected after transfecting scrambled (SCR), precursor-miR-126 (PRE), mature-miR-126 (MAT) and anti-miR-126 (ANTİ) in MCF-7 breast cancer cell lines. (B) miR-126/126* gene expressions were measured after transfecting scrambled (SCR), precursor-miR-126 (PRE), mature-miR-126 (MAT) and anti-miR-126 (ANTİ) in MDA-MB-231 breast cancer cell lines. * $p < 0.001$.**

*miR-126 mimics and inhibitor transfection modulates the expression of miR–126 and its complementary miR-126* in MCF-7 and MDA-MB-231 cells*

To assess the potential role of miR-126 in breast cancer, miR-126 mimics (precursor and mature-miR-126) were used to overexpress the level of miR-126 in MCF-7 and MDA-MB-231 breast cancer cells. Also miR-126 inhibitor (anti-miR-126) was used to suppress the level of miR-126 in MCF-7 and MDA-MB-231 cells. According to our results, a significant increase was observed when miR-126 mimics (pre- and mat-miR-126) was transfected to MCF-7 and MDA-MB-231 cells (Figs 2A and 2B) ($p < 0.001$). Also, there was a statistically significant decrease when MCF-7 and MDA-MB-231 cells were transfected with miR-126 inhibitor (anti-miR-126) (Fig. 2A) ($p < 0.001$).

miR-126* expression was statistically significantly increased when transfected with miR-126 mimics in MCF-7 and MDA-MB-231 cells (Figs 2A and 2B) ($p < 0.001$). But there was a statistically significant decrease in MCF-7 cells transfected with mat-miR-126 (Fig. 2A) ($p < 0.001$). miR-126 inhibitor transfection caused a statistically significant decrease in MCF-7 cells (Fig. 2A) ($p < 0.001$) while an increase was observed in MDA-MB-231 cells (Fig. 2B) ($p < 0.001$).

*Effect of miR-126 on Target Gene Egfl-7; VEGF/PI3K/AKT and MAPK signaling in MCF-7 and MDA-MB-231 cells*

Egfl-7 is one of the targets of miR-126 and also miR-126 is located in 3'-UTR of Egfl-7. According to our results, *Egfl-7* gene expression statistically significantly decreased according to scrambled group when miR-126 mimics was (pre- and mat-miR-126) transfected to MCF-7 cells (Tab. 4) ($p < 0.001$). Furthermore, transfection of miR-126 mimics to MCF-7 cells caused a reduction in *$VEGFR_2$, PI3K, ERK, AKT and RAF-1* gene expressions (Tab. 4) ($p < 0.001$). On the other hand, *SPRED-1 and $PIK_3R_2$* gene expressions increase was observed while miR-126 mimics have transfected to MCF-7 cells; but no statistically significant difference was found between the groups (Tab. 4) ($p > 0.05$). In MCF-7 cells transfected with miR-126 inhibitor (anti-miR-126), a statistically significant increase was observed in *$VEGFR_2$, SPRED-1, $PIK_3R_2$, PI3K, ERK, AKT* and *RAF–1* gene expressions (Tab. 4) ($p < 0.001$).

In MDA-MB-231 cells transfected with miR-126 mimics (pre- and mat-miR-126), a statistically significant decrease in *Egfl-7* gene expression was observed as compared to the scrambled group (Tab. 4) ($p < 0.001$). Furthermore, a statistically significant increase of *Egfl-7* gene expression was determined in the MDA-MB-231 cells transfected with miR-126 inhibitor (anti-miR-126) (Tab. 4) ($p < 0.001$). According to our observed data, miR-126 mimic transfection (pre- and mat-miR-126) to MDA-MB-231 cells caused a statistically significant decrease in *ERK*, *RAF-1*, *PI3K* and *AKT* gene expressions (Tab. 4) ($p < 0.001$). miR-126 mimics couldn't affect *SPRED-1* gene expressions (Tab. 4) ($p > 0.05$), while miR-126 inhibitor transfection have caused a statistically significant increase in *SPRED-1* gene expressions (Tab. 4) ($p < 0.001$). Furthermore, there

**Tab. 4. Gene expressions of Egfl-7, $VEGFR_2$, SPRED-1, $PIK_3R_2$, PI3K, ERK, AKT and RAF-1 in MCF-7 and MDA-MB-231.**

| | MCF-7, SCRAMBLED | MCF-7, PRECURSOR | MCF-7, MATURE | MCF-7, ANTI |
|---|---|---|---|---|
| Egfl-7 | 2.4284±0.00739 | 1.1567±0.00055* | 2.0279±0.00025* | 12.9063±0.00029* |
| $VEGFR_2$ | 0.0988±0.00194 | 0.0237±0.00022* | 0.0723±0.00017* | 0.1174±0.00016* |
| SPRED-1 | 0.0448±0.00105 | 0.1539±0.00024 | 0.1672±0.00034 | 0.2449±0.00048* |
| $PIK_3R_2$ | 0.0769±0.00048 | 0.5176±0.00166 | 0.2698±0.00085 | 1.2311±0.00241* |
| PI3K | 14.5203±0.00179 | 8.7543±0.00098* | 0.8351±0.00109* | 36.7583±0.00028* |
| ERK | 18.8959 ±0.001 | 2.514±0.00024* | 0.6071±0.00047* | 36.7583±0.0003* |
| AKT | 9.6465±0.00018 | 6.4531±0.00076* | 5.579±0.00013* | 36.7583±0.0004* |
| RAF | 30.2738±0.00085 | 22.4711±0.00049* | 14.5203±0.0033* | 36.7583±0.00028* |
| | MDA-MB-231, SCRAMBLED | MDA-MB-231, PRECURSOR | MDA-MB-231, MATURE | MDA-MB-231, ANTI |
| Egfl-7 | 0.2774±0.00019* | 0.0477±0.0004* | 0.0575±0.00012* | 0.6199±0.00022* |
| $VEGFR_2$ | 0.0022 ±0.0002 | 0.0019±0.00022* | 0.0006±0.00005* | 0.0025±0.00013* |
| SPRED-1 | 8.5±0.00344 | 14±0.0226 | 17.3±0.00488 | 21.5557±0.00017* |
| $PIK_3R_2$ | 0.8011±0.00102 | 0.2774±0.00034* | 0.1487±0.00006* | 2.4±0.0216* |
| PI3K | 7.4127±0.00019 | 2.969±0.00198* | 2.6945±0.00015* | 10.1965±0.00025* |
| ERK | 0.9593±0.00094 | 0.6156±0.00016* | 0.2088±0.0002* | 1.9725±0.00013* |
| AKT | 0.2736±0.00015 | 0.133±0.00073* | 0.2161±0.00012* | 0.5249±0.00017* |
| RAF | 0.4147±0.00019 | 0.1497±0.00129* | 0.1044±0.00016* | 1.932±0.0001* |

* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$

was a statistically significant induction observed in the gene expression of *$VEGFR_2$* , *$PIK_3R_2$*, *PI3K, AKT, ERK* and *RAF-1* in MDA-MB-231 cells transfected with miR-126 inhibitor (anti-miR-126) as compared to the scrambled group (Tab. 4) ($p < 0.001$). *$VEGFR_2$* and *$PIK_3R_2$* and gene expressions showed a statistically significant decrease when miR-126 mimics (pre- and mat-miR-126) were transfected to MDA-MB-231 breast cancer cells (Tab. 4) ($p < 0.001$).

## Discussion

Breast cancer is one of the most frequent and malignant types of cancer in women with an increasing morbidity and mortality rate. miRNAs are hypothesized to be a highly involved in mediating malignant functions in cancer development (23, 24). Previous studies indicated that proliferation (25), viability (26), migration and invasion of epithelial cells (27) were decreased while miR-126 was transfected (28). In a study, reduction of miR-126 led various cancer cells to induce angiogenesis, tumor growth and metastasis (29). In another study involving proliferation, migration, and invasion assays, miR-126 was found to suppress the growth and migration of gastric cancer cells (7). miR-126 has been observed to inhibit cell growth by targeting VEGF-A in lung cancer cells (30). It was previously demonstrated that overexpression of miR-126 suppressed the proliferation and inhibited the metastasis in two prostate cancer cell lines, DU-145 and PC-3 (28). miR-126 has been reported to represent an active miRNA in breast cancer and its metastasis (31). In a study, it was determined that miR-126 influenced insulin receptor 1 (IRS-1) and caused a reduction of the growth of MCF-7 and MDA-MB-231 breast cancer cells (32). It was indicated that miR-126 is important for endothelial cell signaling and promotes angiogenesis (33) and reduces breast cancer cell growth and metastasis (34).

According to our results, transfection of miR-126 mimics (pre- and mat-miR-126) to MCF-7 breast cancer cells showed no statistically significant changes in cellular movement, except the results of pre-miR-126 transfection in proliferation and lateral motility. Nevertheless, pre-miR-126 transfection inhibited proliferation and lateral motility of MCF-7 cells. Induction of proliferation and reduction of adhesion was determined while tumor suppressive function of miR-126 has been inhibited by using miR-126 inhibitor (anti miR-126) in MCF-7 cells. Our results showed that miR-126 can be effective on cell proliferation of MCF-7 because of inhibiting the proliferation by using miR-126 mimics (pre- and mat-miR-126) and inducing the proliferation by using miR-126 inhibitor (anti-miR-126) and reducing the amount of miR-126 in MCF-7 cells. Furthermore, it was observed that MCF-7 cells may have lost their adhesive functions and caused the transformation to more aggressive cancer cells while there weren't adequate miR-126 in cells by inhibiting miR-126 using miR-126 inhibitor. This situation indicated that miR-126 may inhibit metastasis of breast cancer cells and so breast cancer cells are not aggressive anymore (Tab. 3 and Fig. 3).

miR-126 mimic (mat-miR-126) transfection to aggressive breast cancer cell line MDA-MB-231 inhibits these cells survival. Presence of excess miR-126 in cells may have caused a reduction the viability of MDA-MB-231 cells. Furthermore, inhibition of MDA-MB-231 proliferation by using miR-126 mimics (pre- and mat-miR-126) supported our hypothesis which is miR-126 mimics might have affected the survival of MDA-MB-231 cells negatively. According to our results, with miR-126 inhibitor (anti miR-126) became these cells more metastatic, with miR-126 mimic (mat-miR-126) became these cells more adhesive. As a result of miR-126 removal, MDA-MB-231 invasive breast cancer cells becoming more invasive of supported that miR-126 might have suppressed aggressiveness of metastatic breast cancer cells (Tab. 3 and Fig. 3). Using miR-126 inhibitor caused MDA-MB-231 cells to become more metastatic. This situation was also supported by observing that miR-126 mimic transfection caused MCF-7 adhesive breast cancer cells to become more adhesive.

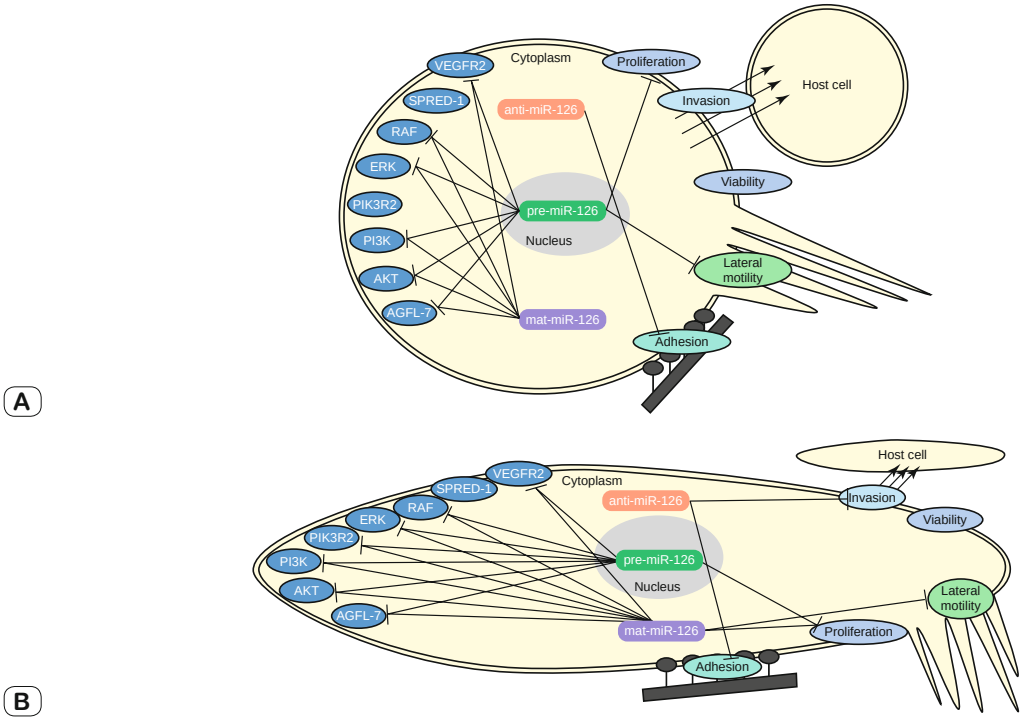



**Fig. 3. A. The effect of miR-126 on cell behaviour and signalling molecules in MCF-7. miR-126 mimics (pre- and mat-miR-126) transfection to MCF-7 cells inhibited the gene expressions of *Egfl-7, PI3K, AKT, ERK, RAF* and *VEGFR*$_2$. Cell proliferation and lateral motility of MCF-7 cells were inhibited after pre-miR-126 transfection. Furthermore, miR-126 inhibitor (anti-miR-126) transfection only affected adhesion of MCF-7 cells. B. The effect of miR-126 on cell behaviour and signalling molecules in MDA-MB-231. miR-126 mimics (pre- and mat-miR-126) to MDA-MB-231 cells inhibited the expressions of *Egfl-7, PI3K, AKT, RAF, ERK, PIK*$_3$*R*$_2$ and *VEGFR*$_2$. Cell proliferation of MDA-MB-231 cells was inhibited after pre-miR-126 transfection while mat-miR-126 transfection has a reduction of cell proliferation and lateral motility. Furthermore, miR-126 inhibitor (anti-miR-126) transfection inhibited invasion and adhesion of MDA-MB-231 cells.**

The overexpression of miR-126, therefore, may be an attractive therapeutic strategy for the treatment of cancer (34). Tumor suppressive miR-126 downregulation was observed in various cancerous tissues (7, 35). In a study, oncogene v-Scr transfected to Cx43KO mice embryonic brain cell lines resulted in an increase in miR-126 and miR-126* expressions (8). Increasing the amount of miR-126 in cells is aimed when transfecting miR-126 mimics. We also aimed to decrease the amount of miR-126 in cells while we have transfected miR-126 inhibitor. In our study, we observed miR-126 upregulation after miR-126 mimics were transfected to MCF-7 and MDA-MB-231 cells while a downregulation of miR-126 was observed after miR-126 inhibitor transfection.

In the biogenesis of miRNA, an uncertainty exists as to which double-stranded miRNA strands will remain following the trimming of the hairpin structure in the precursor. Either of the strands may have a role in the mechanism of miRNA. Also, these miRNAs have different nucleotide sequences and their potential different gene targets. Therefore, miRNAs and their complementary RNAs are needed to be considered as two different miRNAs. Compared to miR-126 results, miR-126* expression results must be assessed as a different micro RNA because of different sequences. Since transfected sequences are suitable for miR-126, in some expressions of miR-126* it was not possible to observe the expected results as miR-126.

In a study, transfection of miR-126* to LNCaP prostate cancer cells resulted in a reduced prostein protein translation. Also, it has been reported that the increase in the prostein protein expression in prostate cells was due to the absence of miR-126* found in *Egfl-7* gene (36). In a non-small cell lung cancer (NSCLC) study, a significant reduction in CRK protein which is the functional target of miR-126 was observed while cells were transfected with pre-miR-126 (6). In another study, suppression of miR-126 with anti-miR-126 was associated with increased *Egfl-7* gene and its products in NSCLC cells (37). In our study, we observed an upregulation of *Egfl-7* gene expression in MCF-7 and MDA-MB-231 cells transfected with miR-126 inhibitor. Furthermore, transfection of miR-126 mimics caused a reduction in *Egfl-7* gene expression (Figure 3A and 3B). These results supported the opinion that Egfl-7 might be the target of miR-126 and presence and absence of miR-126 might affect Egfl-7 function in cancer progression. We suggest that if this case gets out of control, it may lead to vasculogenesis and angiogenesis in later periods.

miRNAs regulate vascular diseases such as atherosclerosis and cancer (34). miR-126 has a positive regulatory effect on response of vascular endothelial cells to Vascular Endothelial Growth Factor (VEGF) (20). Over stimulation of endothelial cells with VEGF leads to hyperactivity of the receptor (VEGFR$_2$). In cancer cells,

we can see resembled characteristics like response to VEGF as vascular endothelial cells. Cancer cells differentiate and grow from angioblastic precursors, mainly from vascular endothelial cells and this process is initiated by angiogenesis. Zhu et al. (2011) observed miR-126 downregulation in breast tumors where the VEGF/PI3K/AKT signaling pathway was activated. Furthermore, they also determined that introduction of miR-126 mimics into MCF-7 could effectively decrease VEGF/PI3K/AKT signaling activity (38). Yoshida et al. (2006) observed that phosphorylation of ERK and AKT by VEGF induction is increased in miR-126 knockout cancer cells (39). Our results showed that $VEGFR_2$, membrane receptor of VEGF, could be affected by miR-126 in both MCF-7 and MDA-MB-231 cells. Presence of miR-126 caused a reduction of receptor activity in MCF-7 and MDA-MB-231 cells. Furthermore, knockdown miR-126 in both cancer cell lines induce the receptor activity ($VEGFR_2$) and increase the response to VEGF of MCF-7 and MDA-MB-231 breast cancer cells relatively (Figs 3A and 3B).

Studies at the molecular level involving different types of cancer suggest significant roles for SPRED-1 and $PIK_3R_2$. SPRED-1 and $PIK_3R_2$ block the VEGF signal transduction in angiogenesis (15, 21, 40). While SPRED-1 inhibits the activation of MAPK signaling, $PIK_3R_2$ is associated with the inhibition of VEGF/PI3K/AKT signaling (15, 20). In several studies, miR-126 has been reported to regulate the VEGF signal transduction in MAPK and VEGF/PI3K/AKT signaling by using SPRED-1 and $PIK_3R_2$ (7, 41). A reduction in SPRED-1 and $PIK_3R_2$ levels is associated with increased expression of miR-126. When the impaired ERK phosphorylation can be salvaged by the blockade of SPRED-1, the damage in AKT phosphorylation-dependent VEGF may also be corrected through $PIK_3R_2$-blocked siRNA (38, 42). It has been proposed that miR-126 specific for breast cancer targets VEGFA and $PIK_3R_2$, resulting in reduced activity of these gene locations (43). In a study, pre-miR-126 was found to significantly reduce $PIK_3R_2$ gene expression while anti-miR-126 was observed to increase $PIK_3R_2$ gene expression in HeLA cells (40). According to obtained data, only absence of miR-126 affected the gene expressions of SPRED-1 and $PIK_3R_2$ in MCF-7 and MDA-MB-231 breast cancer cells while presence of miR-126 only has modulated $PIK_3R_2$ in MDA-MB-231 cells. These regions take important roles in angiogenesis and vasculogenesis of mainly epithelial cells and partly cancer cells. MDA-MB-231 cells are invasive, aggressive and metastatic breast cancer cells. Because of these functions, miR-126 is more effective to SPRED-1 and $PIK_3R_2$ in these cancer cells (Figure 3A and 3B).

Cell proliferation of MDA-MB-231 cells was inhibited after pre-miR-126 transfection while mat-miR-126 transfection has a reduction of cell proliferation and lateral motility. Furthermore, miR-126 inhibitor (anti-miR-126) transfection inhibited invasion and adhesion of MDA-MB-231 cells.

VEGF and FGF binding to endothelial cells initiate the activation of the MAPK signaling. miR-126 suppresses the expression of SPRED-1, which is a regulator for the MAPK signaling (44). Therefore, deficiency of miR-126 reduces MAPK signaling in response to VEGF and FGF found in accelerated angiogenic signaling (20). In a study about chronic ocular ischemia, the application of miR-126 inhibitor induced upregulation of SPRED-1 protein level and 72 hours after transfection, miR-126 inhibitor downregulated phosphorylated ERK, VEGF, and FGF (45). MAPK signaling regulates the transcription of the genes which play a role in angiogenesis and are located in the nucleus. Activation of MAPK and VEGF/PI3K/AKT signaling by growth factors like VEGF causes increased phosphorylation of ERK and AKT. The induction of ERK and AKT phosphorylation by VEGF is increased in miR-126 knockdown cells (39). In a study, it has been reported that when anti-miR-126 leads to increased $PIK_3R_2$ gene expression, it also causes inhibition of *AKT* gene expression in VEGF/PI3K/AKT signaling (40). Furthermore, Li et al demonstrated that the up-regulation of miR-126 contributes to the aberrant activation of the ERK signaling and inhibits cell proliferation and invasion through targeting KRAS in glioma (46). In cancer, both VEGF/PI3K/AKT and MAPK signaling pathways have been working very actively. Furthermore, previous studies indicated that miR-126 has a tumor suppressive function in breast cancer. Also, our obtained data indicated that presence and absence of miR-126 modulate the gene expressions of VEGF/PI3K/AKT and MAPK signaling in MCF-7 and MDA-MB-231. miR-126 mimics suppress the gene expressions of VEGF/PI3K/AKT and MAPK signaling via modulating the function of $VEGFR_2$ while absence of miR-126 caused increased activitz y of genes for VEGF/PI3K/AKT and MAPK signaling (Figs 3A and 3B).

Cancer is a complex disease that is associated with pharmacology, genetic, molecular biology etc. Our study indicated that miR-126 interacts with a variety of molecules that have role in cell signaling in breast cancer. Furthermore, miR-126 might be more effective in inhibition of metastatic breast cancer and may play a significant role in the metastasis of breast cancer.

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