MicroRNA-125a-5p controls the proliferation, apoptosis, migration and PTEN/MEK1/2/ERK1/2 signaling pathway in MCF-7 breast cancer cells

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Abstract. MicroRNA (miR)-125a-5p has shown the potential for suppressing tumorigenesis and development; however, the effects of miR-125a-5p on breast cancer cells remains unknown. The aim of this study was to evaluate the effects and underlying mechanisms of miR-125a-5p in MCF-7 breast cancer cells. MCF-7 cells were transfected with miR-125a-5p mimic or miR-125a-5p small interfering RNA to produce miR-125a-5p overexpressing/knockdown cells. Cell proliferation was assessed by an MTT assay, and cell migration ability was determined by an in vitro scratch assay. Hoechst 33258 staining and flow cytometry were performed to assess the effects of miR-125a-5p on MCF-7 apoptosis. Western blotting and reverse transcription-quantitative polymerase chain reaction were used for measuring phosphatase and tensin homolog (PTEn), phosphorylated (p)-mitogen-activated protein kinase kinase (MEK)1/2/ERK1/2, p-ERK1/2, B-cell lymphoma-2 (Bcl-2), cleaved caspase-3, and miR-125a-5p expression. miR-125a-5p overexpression inhibited the proliferation and migration, but promoted the apoptosis of MCF-7 cells. These effects were associated with increases in PTEn and cleaved caspase-3 expression, and decreases in p-MEK1/2/MEK1/2, p-ERK1/2/ERK1/2, and Bcl-2. Silencing of miR-125a-5p exhibited opposing effects on MCF-7 cells. These observations suggested that miR-125a-5p participates in the regulation of multiple functions of MCF-7 cells by promoting the expression of PTEN tumor suppressor genes, activating MEK1/2/ERK1/2 signaling, and regulating caspase-3/Bcl-2 signaling. Thus, it may be a suitable target for breast cancer gene therapy.

Introduction

Breast cancer is one of the most common malignant tumors in females. It has the highest incidence among this population, and the age of onset is decreasing. The lifetime risk of having breast cancer is 10% in women (1). Approximately 500,000 people succumb to mortality each year from breast cancer, and the mortality rate increases by 2-3% annually (2). Early diagnosis and treatment have been particularly important in breast cancer as the options for primary prevention are limited. Identifying and studying the genes responsible for the growth and malignancy of breast cancer is critical for disease therapy.

MicroRNAs (miRs) bind to target genes involved in such pathologic processes as cell proliferation, differentiation and apoptosis, and affect their expression in the tissues and cells (3). miR-125a-5p has been reported to be abnormally expressed in prostate carcinoma (4). Various studies have shown miR-125a-5p to be closely related to tumor cell growth, differentiation and metastasis (5-7). Nevertheless, its effects on the proliferation, migration, and apoptosis of breast cancer cells and the underlying mechanisms remain unknown.

Phosphatase and tensin homolog (PTEN) is a well-characterized tumor suppressor, and it plays a key role in cell proliferation, migration and cell renewal by regulating the mitogen-activated protein kinase kinase (MEK)1/2/ERK1/2 pathway (8). The MEK1/2/ERK1/2 signal pathway and caspase-3 activation have been shown to be involved in breast cancer cell proliferation, migration and apoptosis processes (9). Studies revealed that miR-125a-5p could regulate the MEK1/2/ERK1/2 signaling pathway in multiple cell types (10,11). However, whether these proteins and pathways are involved in the mechanisms underlying miR-125a-5p regulation in breast cancer cells are unknown.

Thus, we aimed to evaluate the effects of miR-125a-5p on the biological behaviors of MCF-7 breast cancer cells and to investigate the underlying regulatory mechanisms.
Materials and methods

Cell culture and grouping. Human MCF-7 breast cancer cells were purchased from Shanghai BioLeaf Biotech. The MCF-7 breast cancer cell line was cultured in RPMI-1640 medium containing 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin, and 100 µg/ml streptomycin in an incubator with 5% CO₂ and 95% humidity at 37°C. Trypsin (0.25%) was used to digest the cells when 80% of the cells adhered to the culture dish and for the subculture, which was performed every 2-3 days.

Lentiviral-mediated transfection to establish stable MCF-7 cell lines with miR-125a-5p overexpression or reduced miR-125a-5p expression. MCF-7 cells were infected with green fluorescent protein (GFP)-expressing lentivirus (1x10⁶ multiplicity of infection) that also contained miR-125a-5p overexpression vector (LV-miR-125a-5p), miR-125a-5p silencing small interfering (si)RNA (siRNA-LV-miR-125a-5p), and a scrambled sequence (LV-NC). The medium was replaced 24 h after transfection, and the GFP-labeled cells were counted 72 h after transfection. The positive cells were screened with puromycin (final concentration of 1 mg/l) to eventually produce MCF-7 cells with miR-125a-5p overexpression (MCF-7miR-125a-5p cells), MCF-7 cells with miR-125a-5p knockdown (MCF-7SimiR-125a-5p cells), and MCF-7 stable LV-scramble infected MCF-7 cells (MCF-7NC), which served as the control.

Detection of miR-125a-5p expression in MCF-7 cells. The MCF-7miR-125a-5p, MCF-7SimiR-125a-5p, and MCF-7NC cells were seeded in 6-well plates. Once the MCF-7miR-125a-5p, MCF-7SimiR-125a-5p, and MCF-7NC cells reached 90% confluence, total RNAs of the harvested cells were extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.) a hairpin-it™ miRs reverse transcription-quantitative PCR (RT-qPCR) kit was then used to synthesize miR-125a-5p cDNA. The RT primer sequences were human miR-125a-5p-RT (5'-GTCGTATCCAGTGCGAGGTGTCGACACTAGAGAGATGCAACA-3'). The reaction conditions were 25°C for 30 min, 42°C for 30 min, and subsequently 85°C for 5 min. The products of RT were used as templates for PCR. The primer sequences of qPCR were 5'-ACACTCCAGTGGCCTCCCTAGACCTTAAA-3' (miR-125a-5p forward primer), 5'-CTCACTAGTGTTGAGT-3' (miR-125a-5p reverse primer), 5'-CTCGCTTCGACAGACGGA-3' (internal reference gene U6 forward primer), and 5'-AACGCTTCACAGAATTTGCGT-3' (internal reference gene U6 reverse primer). The PCR conditions were 95°C denaturation for 3 min, 40 cycles of 95°C denaturation for 12 sec and 60°C annealing and elongation for 40 sec. The RT-qPCR procedures were performed in strict accordance with the manufacturer's instructions. Six replicate wells were performed in each group, and each experiment was performed six times. The relative expression levels of target genes were calculated using the 2-∆∆Cq method (12).

Assessment of MCF-7 cell proliferation. The harvested MCF-7miR-125a-5p, MCF-7SimiR-125a-5p and MCF-7NC cells were independently inoculated in six wells in 96-well plates (each well contained 100 µl complete cell culture medium with 2x10⁵ cells and incubated at 37°C for 5 days. Cells were incubated in RPMI-164 + 10% FBS (HyClone; GE Healthcare Life Sciences). The viability of cells in different groups was measured directly via an MTT assay for 5 days. For the MTT assay, 20 µl MTT solution was added to each well of cells for incubation at 37°C for 4 h, followed by removal of the solution and addition of 150 µl dimethyl sulfoxide, followed by incubation at 37°C for 20 min; the absorbance was then measured at 490 nm using a microplate reader. This experiment was performed six times.

In vitro scratch assay to detect MCF-7 cell migration. The harvested MCF-7miR-125a-5p, MCF-7SimiR-125a-5p, and MCF-7NC cells were independently inoculated and incubated in six-well plates (each well contained 2 ml complete cell culture medium with 5x10⁵ cells). Each group of cells was transferred into serum-free medium once they reached 90% confluence. Then, a 200-µl pipette tip was used to scratch a straight line in the middle of the well. The scratch width in each well was measured under an inverted microscope and the measurement points were recorded. The scratch width at the same position was measured again after incubated at 37°C for 16 h. The difference between the two measurements in each well was expressed in terms of cell migration distance after 16 h. This experiment was performed six times. Quantitative analysis of migration was calculated using the following equation: (Cell-free area at 0 h-cell-free area at 16 h)/cell area at 0 h x100%.

MCF-7 apoptosis analysis. Cell apoptosis was analyzed by Hoechst 33258 staining and an Annexin V-phycocerythrin (PE)/7-aminonitochondrion (7-AAD) apoptosis detection kit (BD Biosciences). The harvested MCF-7miR-125a-5p, MCF-7SimiR-125a-5p, and MCF-7NC cells were independently inoculated and incubated in six-well plates (each well contained 2 ml complete cell culture medium with 5x10⁵ cells). Once the cells reached 90% confluence, the cells were transferred to serum-free medium for 24 h incubation at 37°C to stimulate apoptosis, which was assessed using an apoptotic cell Hoechst 33258 dye kit according to the manufacturer's protocol (Beyotime Institute of Biotechnology) and observed under a fluorescence microscope (Leica Microsystems GmbH; magnification, x400). The apoptotic cells per well were counted in five different fields of vision. The percentage of apoptotic cells per field of vision divided by the total number of cells per field of vision was obtained as the apoptotic rate.

For Annexin V-PE/7-AAD apoptosis detection, MCF-7 cells after different treatments were collected by using 0.25% trypsin and centrifuged at 200 x g at room temperature for 3 min. Cells were then washed with PBS and resuspended in 100 µl 1X annexin-binding buffer, which was included in the kit. Subsequently, 5 µl PE-conjugated Annexin V and 5 µl 7-AAD were added to the cell suspension, and incubated at room temperature for 15 min. The rate of apoptosis in MCF-7 cells was detected by flow cytometry. The cells stained only with Annexin V were considered as MCF-7 cells in early apoptosis, and those stained for both Annexin V and 7-AAD were considered as late apoptotic cells. In this study, we defined the apoptotic cells as both early and late apoptotic cells.
Western blotting analysis of protein expression

**Protein extraction.** After collecting the MCF-7 cells by centrifugation (200 x g and 4°C for 3 min), the MCF-7 cells were lysed with RIPA buffer (Applygen Technologies, inc.) containing protease inhibitor and subsequently centrifuged at 13,000 x g and 4°C for 10 min to collect the supernatant (total protein) for later use.

**Western blotting.** The extracted proteins were separated using 12% SDS-PAGE and subsequently transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked at room temperature with Tris-buffered saline (TBS) containing 5% fat-free dry milk (i.e., 50 mmol/l Tris, 150 mmol/l NaCl, pH 7.6, and 5% fat-free dry milk) for 1 h. After a few washes in TBS containing 0.5% Tween-20 (TBST), the protein blots were incubated with β-actin (1:1,000; cat. no. E02102001; EarthOx), PTEN (1:1,000; cat. no. 9188S; Cell Signaling Technology, Inc.), MEK1/2 (1:500; cat. no. 4694S; Cell Signaling Technology, Inc.), ERK1/2 (1:1,000; cat. no. 4695S; Cell Signaling Technology, Inc.), B-cell lymphoma-2 (Bcl-2, 1:500, cat. no. 3498S; Cell Signaling Technology, Inc.), and caspase-3 (1:400; cat. no. 9664S; Cell Signaling Technology, Inc.) primary antibodies at 4°C overnight. After a few washes in TBST, the protein blots were incubated with 1:50,000 horseradish peroxidase (HRP)-conjugated goat-anti-mouse secondary antibodies (cat. no. 7076S; Cell Signaling Technology, Inc.) or HRP-conjugated goat-anti-rabbit secondary antibodies (cat. no. 7074S; Cell Signaling Technology, Inc.) at room temperature for 1 h. After three washes in TBST, the protein blots were developed in enhanced chemiluminescence (Amersham) substrate, and then results were analyzed under Quantity one 4.6 software (Bio-Rad Laboratories, Inc.). β-actin was used as an internal reference protein. Relative protein expression was measured based on the ratio of the target protein absorbance to the β-actin protein absorbance. This experiment was performed six times.
Statistical analysis. Data were presented as the mean ± standard deviation. Statistical analyses were performed using a t-test, one-way ANOVA followed by a least significant distance post-hoc test. GraphPad Prism 5 software (GraphPad Software, Inc.) was used for analyzing the data. P<0.05 was considered to indicate a statistically significant difference.

Results

Overexpression and knockdown of miR-125a-5p in MCF-7 cells. MCF-7 cells were labeled with green fluorescence after transfection with LV-scramble, siRNA-LV-mir-125a-5p, or LV-miR-125a-5p and selection of stable clones (Fig. 1A). The results of RT-qPCR showed that miR-125a-5p expression in the MCF-7mir-125a-5p cells was significantly higher than in the MCF-7NC cells (P<0.05; Fig. 1B); in addition, there miR-125a-5p expression was significantly reduced in the MCF-7Simir-125a-5p cells than in MCF-7NC cells (P<0.05; Fig. 1B).

miR-125a-5p is involved in MCF-7 cell proliferation and migration. The results of the MTT assay showed the viability of MCF-7 cells in different groups. Starting from the 3rd day of experiment, the proliferation rate of the MCF-7mir-125a-5p cells was significantly lower than that of MCF-7NC cells (P<0.05; Fig. 2), while the proliferation rate of the MCF-7Simir-125a-5p cells was significantly higher than that of MCF-7NC cells (P<0.05; Fig. 2). This suggested that high miR-125a-5p expression inhibited the proliferation of MCF-7 cells, and low miR-125a-5p expression promoted this ability within MCF-7 cells.

The results of in vitro scratch assay revealed that migration ability of MCF-7mir-125a-5p cells was significantly lower than the MCF-7NC cells (P<0.05; Fig. 3A and B), while that of the MCF-7Simir-125a-5p cells was significantly higher than the MCF-7NC cells (P<0.05; Fig. 3A and B). These results indicated that high miR-125a-5p expression inhibited the migration of MCF-7 cells. Conversely, low miR-125a-5p expression in MCF-7 cells resulted in enhanced migration of the cells.

miR-125a-5p increases PTEN/MEK1/2/ERK1/2 pathway protein expression in MCF-7 cells. PTEN protein expression in MCF-7mir-125a-5p cells was significantly higher than in MCF-7NC cells (P<0.05; Fig. 4A and B). In contrast, significantly reduced PTEN protein expression was exhibited by MCF-7Simir-125a-5p cells compared with MCF-7NC and MCF-7mir-125a-5p cells (P<0.05; Fig. 4A and B). In addition, we found that overexpression of miR-125a-5p significantly decreased the phosphorylation levels of MEK1/2 and ERK1/2 in MCF-7 cells compared with the MCF-7NC group (P<0.05; Fig. 4A,C and D). Additionally, silencing miR-125a-5p significantly increased the phosphorylation levels of MEK1/2 and ERK1/2 in MCF-7 cells compared with the MCF-7mir-125a-5p group (P<0.05, Fig. 4A,C and D). These data suggested that the PTEN/MEK1/2/ERK1/2 signaling pathway may have...
contributed to the effects of miR-125a-5p on inhibiting McF-7 cell proliferation and migration.

miR-125a-5p promotes the apoptosis of MCF-7 cells, which is accompanied with increased cleaved caspase-3 and decreased Bcl-2 levels. Hoechst 33258 staining and flow cytometry showed that the rate of apoptosis among MCF-7mir-125a-5p cells was significantly higher than that of the MCF-7nc cells (P<0.05; Fig. 5a-d), while the apoptotic rate of the MCF-7SimiR-125a-5p cells was significantly lower than that of the MCF-7nc cells (P<0.05, Fig. 5a-d). These results indicated that high levels of miR-125a-5p promoted, while low levels of miR-125a-5p reduced the apoptosis of McF-7 cells.

In addition, via western blotting, we monitored the levels of cleaved caspase-3 and Bcl-2, which are associated with induction of apoptosis (13). The results showed that overexpression of miR-125a-5p significantly increased cleaved caspase-3 expression, while decreased Bcl-2 expression was observed compared with the control (P<0.05; Fig. 6A-C). Silencing miR-125a-5p in MCF-7 cells revealed significantly decreased cleaved caspase-3 protein expression and increased Bcl-2 expression compared with the control and overexpression groups (P<0.05; Fig. 6A-C).

Discussion

In the present study, we found that miR-125a-5p inhibited MCF-7 migration and proliferation, but promoted apoptosis. The effects of miR-125a-5p on MCF-7 cell migration and proliferation may be associated with an increase in PTEN, and decrease in MEK1/2 and ERK1/2 protein expression, while the apoptotic effects could be associated with upregulated cleaved caspase-3 levels.

The survival time of breast cancer patients is closely related to the pathological stage of breast cancer: The earlier the pathological stage at the time of diagnosis, the higher the recovery rate (14). For this reason, and as there are currently no effective primary prevention methods, early diagnosis and treatment are particularly important in breast cancer (2,15). Mammography, ultrasonography, computed tomography, magnetic resonance imaging and biopsies are commonly used for the diagnosis of breast cancer (16). However, each diagnostic method has its limitations. Breast cancer treatments mainly include surgery, chemotherapy, radiotherapy, endocrine therapy, and biological targeted therapy (14). Although these treatments prolong patient survival, and the postoperative recurrent and metastatic rates are still relatively high, it is important to study the mechanism underlying the development and progression of breast cancer. Thus, novel therapeutic targets for breast cancer could be identified.

miRs are non-coding single-stranded small RNAs. They are ~18-25 nucleotides in length with high evolutionarily conservation (17). It binds to its target mRNAs to inhibit translation and induce degradation of the mRNA, altering the levels of expression of the target protein (18). miR-125a expression is low in breast cancer tissues, and was negatively correlated with
tumor size, lymph node metastasis and clinical stage of breast cancer (19). Previous studies have reported that miR-125a is closely related to tumor cell growth, differentiation, and metastasis (20,21). A study by Scott et al (20) has shown that miR-125a downregulates human epidermal growth factor receptor 2 (ERBB2) and ERBB3 protein and mRNA expression.

Figure 5. Effects of miR-125a-5p on serum deprivation-induced apoptosis of MCF-7 cells. (A and B) Representative images and summarized data of MCF-7 apoptosis as detected by flow cytometry. (C and D) Representative images and summarized data of MCF-7 apoptosis as detected by Hoechst 33258 staining. Scale bar=100 µm. *P<0.05, vs. MCF-7NC; **P<0.05, vs. MCF-7mir-125a-5p; n=6/group. 7‑AAD, 7‑amino‑actinomycin; Lv, lentiviral; miR, microRNA; NC, negative control; PE, phycoerythrin.

Figure 6. Effects of miR-125a-5p on cleaved caspase-3 and Bcl-2 expression in MCF-7 cells. (A) Expression of cleaved caspase-3 and Bcl-2 in MCF-7 cells. (B) Summarized data of the cleaved caspase-3 expression. (C) Summarized data of the Bcl-2 level. *P<0.05, vs. MCF-7NC; **P<0.05, vs. MCF-7mir-125a-5p; n=6/group. Bcl-2, B‑cell lymphoma‑2; Lv, lentiviral; miR, microRNA; NC, negative control; Pe, phycoerythrin.
by targeting ERBB2 and ERBB3; furthermore, miR-125a inhibited the migration and invasion of SKBR3 breast cancer cells. To the best of our knowledge, our study, for the first time found that miR-125a-5p inhibited the proliferation and migration, and promoted the apoptosis of MCF-7 cells. Collectively, these findings demonstrated that miR-125a-5p is a candidate target for breast cancer therapy.

The MEK1/2/ERK1/2 pathway is extensively involved in the proliferation and the migration-associated protein expression of tumor cells (22), and serves an important role in tumor development and progression. A study by Hsieh et al (19) indicated that human aldo-keto reductase family 1 member B10 promotes the invasion and migration of MCF-7 cells by activating the MEK1/2/ERK1/2 pathway. Another study reported that the activation of ERK1/2/p38 MAPK signaling is involved in the regulation of invasion and metastasis of MDA-MB-231 breast cancer cells (23). These studies suggest that abnormal activation of the MEK1/2/ERK1/2 pathway may be an important mechanism underlying the development of breast cancer. In this study, MEK1/2/ERK1/2 signaling was inhibited in MCF-7 breast cancer cells with high miR-125a-5p expression. In contrast, we observed that knockdown of miR-125a-5p expression led to activation of MEK1/2/ERK1/2 signaling. PTEN, located on human chromosome 10, is a tumor suppressor gene that inhibits the growth and migration of various tumors by negatively regulating the PI3K/Akt/mTOR pathway (24,25). A recent study revealed that PTEN also inhibits the migration of type 2 endometrial cancer cells by negatively regulating the MEK1/2/ERK1/2 pathway (26). In this study, high levels of miR-125a-5p expression promoted PTEN expression in MCF-7 cells, while knockdown of miR-125a-5p expression significantly reduced PTEN expression in MCF-7 cells. This suggested that miR-125a-5p inhibited the protein expression of the MEK1/2/ERK1/2 pathway proteins by upregulating the expression of PTEN, playing a tumor suppressive role.

Caspase-3 is an important pro-apoptotic factor that plays an important role in the process of apoptosis. Bcl-2 is an important anti-apoptotic gene that serves a key role in the regulation of early apoptosis in cells (13). One recent study has shown that cyanidin-3-glucoside inhibits MDA-MB-231 breast cancer cells by promoting caspase-3 expression and downregulating Bcl-2 expression (27). In this study, we found that high miR-125a-5p expression promoted the apoptosis of MCF-7 cells, while knockdown of miR-125a-5p expression significantly lowered the rate of apoptosis among MCF-7 cells. Our study into the underlying mechanism showed that high levels of miR-125a-5p expression promoted the expression of pro-apoptotic protein, caspase-3 and inhibited the expression of anti-apoptotic protein Bcl-2. These results indicated that miR-125a-5p might be involved in regulating apoptosis in MCF-7 breast cancer cells by regulating the caspase/Bcl-2 pathway.

In summary, the present study demonstrated a potential role of miR-125a-5p in the pathogenesis of breast cancer via control of cancer cell proliferation, apoptosis and migration. In addition, our data indicated that miR-125a-5p exerted these functions in breast cancer cells by upregulating PTEN expression and inhibiting the activity of the MEK1/2/ERK1/2 pathway. However, further in-depth and in vivo investigations into the role, and the precise mechanisms underlying miR-125a-5p regulation in breast cancer should be conducted in the future.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

ZL, JL and QP designed the experiments. ZL, QP, ZZ, CH, ZY and YZ performed the data. ZL, JL, QP interpreted the data. ZL and QP drafted the manuscript. JL and QP revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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