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ARTICLE

Effect of miR-382 on triple negative breast cancer cell line 4T1 by targeting PGC-1α

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Breast cancer is one of the most frequent and malignant types of cancer in women, with an increasing morbidity and mortality rate. Treatment of triple negative breast cancer remains a challenge, since the efforts made with targeted therapies were ineffective. MicroRNAs (miRNAs) could play important roles in cancers via post-transcriptionally regulating target genes via binding to specific sequences in the 3'-UTR of downstream target genes. In the present study, the effect of miR-382 on the biological behaviors of triple negative breast cancer 4T1 cells is investigated. The expression of PGC-1α in the 4T1 cells transfected with miR-382 mimics was significantly decreased, while it was obviously increased after transfection with anti-miR-382. Moreover, the cell migration and proliferation activity were significantly increased in the miR-382+pCDNA-PGC-1α group when compared with the control+pCDNA3.1 group. However, the antitumor effect of miR-382 was blocked by overexpression of PGC-1α. Our results showed that miR-382 inhibits the proliferation and metastasis of 4T1 cells by down-regulating PGC-1α, suggesting miR-382 might be a therapeutic target in triple negative breast cancer.

Keywords: triple negative breast cancer; proliferation; metastasis; microRNA-382; PGC-1α

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Triple Negative Breast Cancer (TNBC) is a special type of breast cancer, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her-2) in TNBC) are negative expression. Such breast cancer has the characteristics of high recurrence rate, easy metastasis, and poor treatment and prognosis [1].

PGC-1α is one of the subtypes of the PGC-1 family and is a coactivator of peroxisome proliferator-activated receptor γ (PPARγ) [2]. It is mainly found in cells with high energy, high metabolism and containing more mitochondria. Studies have shown that PGC-1α promotes distant metastasis of cancer cells by enhancing mitochondrial function in invasive breast cancer [3]. Many microRNAs (miRNAs) upstream of PGC-1α are involved in the regulation of PGC-1α expression. We found through online software predictive analysis (www.micro-rna.org) that there are 11 potential miRNA binding sites in the 3'UTR untranslated region of mouse PGC-1α. miRNAs are small non-coding RNAs containing approximately 20-22 nucleotides that specifically promote or inhibit target messenger mRNA by specifically binding to the 3' untranslated region (3'-UTR) of the target messenger RNA (mRNA). Translation of 3'-UTR promotes and inhibits the action of cancer cells. Many studies have shown that miRNAs play an important role in the process of cell growth, including metabolism, homeostasis, proliferation and apoptosis [4].

Studies have found that the expression level of miR-382 is closely related to the prognosis of breast cancer [5]. Our previous study found that tumor-associated macrophages (TAMs) inhibit the expression of PGC-1α by intracellular miR-382, thereby preventing M2-type differentiation of

TAMs and subsequent PGC-1α-mediated oxidation. Inhibition of tumor environmental metabolic reprogramming and metastasis of cancer cells [6-7]. However, whether miR-382, which exists in breast cancer cells itself, regulates the transfer of breast cancer cells by regulating PGC-1α is still unclear. This study focused on the effects of miR-382 on PGC-1α and cell biological characteristics in TNBC 4T1 cell line, in order to provide more experimental data for the diagnosis and treatment of TNBC.

Materials and methods

Main reagents

Plasmid, 4T1 cell line, 293T cell line were purchased from the Shanghai Institute of Life Sciences Cell Bank of Chinese Academy of Sciences; pcDNA3.1 and pcDNA3.1- PGC-1α plasmid (#1026, addgene, USA); pMIR-REPORT (Applied Biosystems Inc); miR-382 and Anti-miR-382 (#4464066, Invitrogen) and Control miR (#4464058, Invitrogen); Reverse Transcription Kit and qRT-PCR Kit (TaKaRa); GAPDH (Biyuntian); Anti-pgc1 antibody (Abcam), Western blot other related reagents (Dingguo company); plasmid large endotoxin-free kit (QIAGEN), luciferase assay kit (promega), opti-MEM (TBD), TransIT-293 Transfection Reagent 2700 (Mirus Bio LLC), Transwell chamber (pore size 0.4 m) was purchased from Corning Corporation, PROMEGA 20/20 LUMINOMETER GLOMAX (promega). Cell culture flask, cell culture plate (Corning), DMEM high glucose medium, RPMI 1640 medium and fetal bovine serum (GIBCO); BB5060 UV carbon dioxide incubator (Heraeus), inverted phase contrast microscope (Leica).

Cell culture

4T1 cells were cultured in RPMI1640 containing 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin double antibody, and cultured in an incubator at 37 ℃, 5% CO_2, and saturated humidity. 293T cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and cultured in an incubator at 37 ℃, 5% CO_2, and saturated humidity.

Animal and grouping

40 SPF grade BALB/c female mice in 6-8 weeks old and 18~20 g were raised in the Animal Experimental Center of Gannan University. Experimental group design as following: control group: control miR transfected 4T1 cells; miR-382 experimental group: miR-382mimics transfected 4T1 cells; anti-miR-382 experimental group: miR-382 inhibitory probe anti-miR-382 Dye to 4T1 cells. Each set of experiments was repeated 3 times.

Cell luciferase assay

Wild control group: co-transfected 4T1 cells with control miR and 3'-UTR reporter plasmid containing core complementary sequence; wild experimental group: co-transfected miR-382 mimics with 3'-UTR reporter plasmid containing core complementary sequence 4T1 Cells; mutant control group: co-transfected the control miR with the 3'-UTR reporter plasmid containing the mutated core complement of the 4T1 cell; mutant experimental group: the miR-382 mimics and the 3'-UTR containing the mutated core complement The reporter plasmid was co-transfected into 4T1 cells. Each set of experiments was repeated 3 times.

Biological characteristics of 4T1 cells

Control miR pCDNA3.1 group: control miR pCDNA3.1 co-transfected into 4T1 cells; control miR pCDNA-PGC-1α group: control miR pCDNA-PGC-1α co-transfected into 4T1 cells; miR-382+pCDNA3. Group 1: miR-382 mimics and pCDNA3.1 were co-transfected into 4T1 cells; miR-382+pCDNA-PGC-1α. Group 2: miR-382 mimics and pCDNA-PGC-1α were co-transfected into 4T1 cells. Each set of experiments was repeated 3 times.

qRT-PCR

Each group of cells was collected, RNA was extracted using TRIzol, and then cDNA was synthesized using reverse transcription kit and then subjected to qRT-PCR detection. Mouse PGC-1α mRNA PCR primer: sense strand: 5'-AACCACACCCACAGGATCAGA-3'; antisense strand: 5 '-TCTTCGCTTTATTGCTCCATGA-3'. The reaction conditions and relative expression levels of RNA were calculated in the same study as previous studies [6].

Western blot

The BCA protein was quantified after each group of cells was collected, and the protein concentration was determined. The specific method is reported in the literature [6].

Dual luciferase assay

Target Scan and PicTar software were used to predict miR-382 target genes to screen for target genes with consistent predictions. Using the Target Scan and NCBI databases, we designed the PGC-1 3'-UTR PCR primer using Primer5 software: (sense strand: 5'-GCAAGCTTCGTGTTCCCAG-GCTGAGGAATG-3' antisense strand: 5'-CGGAGCT-CGTCTGCTCCTCAGAAGGAGCCA-3'. Using DNA as a template PCR amplification, synthesis of 3'-UTR sequence fragment amplification products identified by 2% agarose gel electrophoresis, PCR recovery of PCR products, selection of HindIII/SacI restriction enzymes added to the 5' end of the primer, plus base GC The plasmid was incubated overnight at 37 °C. The plasmid and the desired fragment were purified,

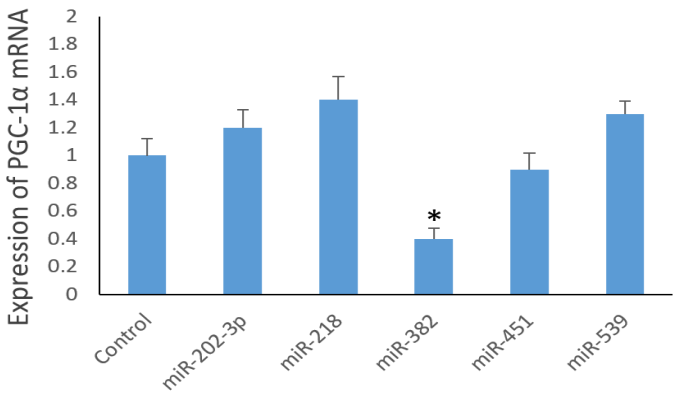

Figure 1. Effect of potential miRNA on the expression of PGC-1α mRNA by online prediction. * P < 0.05, compared to the control.

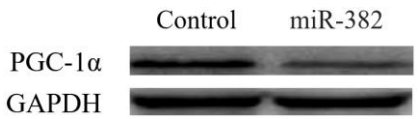

Figure 2. Effect of overexpression of miR-382 on the expression of PGC-1α protein. The expression of PGC-1α protein is increased after treatment with miR-382.

and T4 ligase was added to react at 4 °C overnight, and then the ligation product was transformed into competent cells. Then, single colonies were picked for culture, and plasmid extraction reagent was used. The plasmid was extracted and verified by sequencing. The sequencing was performed by Shanghai Jikai Co. After sequencing, the plasmid was extracted, and 293T cells were cultured to logarithmic growth phase, and seeded in 24-well plates. The number of cells per well was 5×10^4, transfected with Mirus. Reagent transfection, according to the experimental group requirements, take control, normal or mutant plasmid, diluted with opti-MEM 50 μL, and gently mixed with miR382; opti-MEM 50 μL added Mirus 2 μL. Incubated for 5 min at room temperature; mix the above two steps of the solution and incubate at room temperature for 20 min; add the mixture to the 24-well plate once and mix well; after incubating for 5-6 h in the incubator, replace with normal medium before a luciferase assay was performed.

Transwell assay

MiR-382 or control miR was co-transfected into 4T1 cells with pCDNA3.1 or pCDNA-PGC1α, respectively, and the cells were in logarithmic growth phase, washed twice with D-Hanks solution, and then serum-free RPMI1640 medium was added. Cells starved for 12 h. The cell suspension was prepared by adding 0.25% trypsin and adding serum-free RPMI1640 medium to adjust the cell density to 1.33×10^6 /mL. 500 μL of complete medium containing 20% FBS was added to the Transwell plate and the chamber was placed in the plate. 200 μL of the cell suspension was added to the Transwell chamber, and the Transwell plate was cultured in a CO_2 incubator at 37 °C for 24 h. The chamber was taken out, the medium was washed away with PBS, and the crystal violet was stained for 10 min; the surface crystal crystal was washed away by tap water, and the cells on the inoculation side in the upper chamber were wiped clean with a cotton swab, and the non-cell inoculation side was photographed under a microscope. The number of transmembrane cells was observed and counted under a microscope, and 5 fields were randomly taken to obtain the mean value.

CCK-8 assay

MiR-382 or control miR was co-transfected into 4T1 cells with pCDNA3.1 or pCDNA-PGC1α, respectively, and 100 μL (about 10 000 cells) of cell suspension was placed in a 96-well culture plate, with 5 replicates in each group. The plate was preincubated for 48 h in an incubator to allow the cells to adhere. 100 μL of each group of the test substance was added to the culture plate. After the culture plates were intervened in the incubator for 6, 12, 24, and 48 h, 10 μL of CCK-8 solution was added to each well, and the plates were further incubated in an incubator. The optical density value D (450) at 450 nm was measured with a microplate reader. The obtained value can be prepared by using the number of cells as the X-axis and the optical density as the Y-axis.

Subcutaneous transplantation tumor

miR-382 or control miR was co-transfected into 4T1 cells with pCDNA3.1 or pCDNA-PGC-1α, respectively, and 5×10^6/100 μL cell suspension was inoculated into the right groin of the mice, each injection of 0.2 mL (The number of cells was 1×10^7/well), and 0.2 mL of normal saline was injected as a negative control under the left inguinal region. The tumor growth at the injection site was observed every day. The mouse tumor volume was measured and calculated using a vernier caliper.

Statistical analysis

Statistical processing was performed using SPSS 20.0 software. The data were expressed as x±s, and the variance analysis and t-test were compared between groups.

Results

Effect of overexpression of miR-382 on the expression level of PGC-1α

The bioinformatics online website (www.microrna.org/) found that there are multiple potential miR binding sites in the 3'-UTR of mouse PGC-1α gene: mmu-miR-382, mmu-miR-

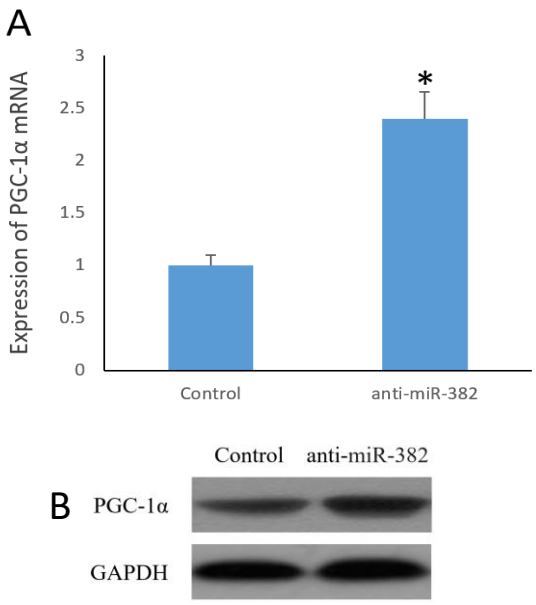

Figure 3. Effect of inhibition of miR-382 on the expression of PGC-1α. A: QT-PCR detectionof PGC-1α mRNA. *P < 0.05, compared with the control group; B: Western blot results shows the PGC-1α protein is increased in cells treated with anti-miR-382.

539, mmu-miR-218, mmu-miR-202-3p, mmu-miR-451, mmu-miR-320, mmu-miR-375, mmu-miR-129-5p, mmu-miR-335-5p, mmu-miR-362-3p, mmu-miR-543. The miRs of the top 5 predicted scores were transfected into mouse breast cancer cells 4T1. As shown in Fig. 1, miR-382 had the most significant inhibitory effect on the expression level of PGC-1α mRNA (0.25±0.04). The protein was collected for 48 h and detected by Western blot. As shown in Fig. 1, compared with the control group (0.33±0.05), miR-382 significantly inhibited the level of PGC-1α protein [(0.16±0.03), P < 0.05].

Inhibition of miR-382 expression on the expression of PGC-1α

The inhibitory probe anti-miR-382 or control miR was transfected into 4T1 cells, and Real-time PCR and Western blot were performed 48 hours later. As shown in Figure 3, anti-miR-382 significantly up-regulated PGC-1α mRNA levels (0.53 ± 0.02) and protein levels (P < 0.05) compared to the control group (0.30 ± 0.04).

Luciferase assay to confirm the interaction between miR-382 and PGC-1α

The sequence of mouse miR-382 (mmu-miR-382) is highly complementary to the mouse wild-type PGC-1α 3'-UTR, and the site of action is located at PGC-1α 3'-UTR 565-582. The complementary sequence is ACAACUU. The plasmid with the core complement was mutated into UAAAUCU, and

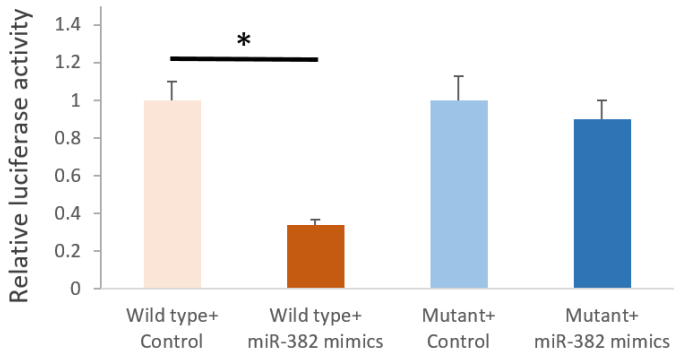

Figure 4. Luciferase assay confirms the interaction between miR-382 and PGC-1α and found that the PGC1αis the direct target of miR-382. *P < 0.05, compared to the Wild type+Control.

the luciferase reporter gene was transfected into 293T cells. The results in Fig. 4 showed that miR-382 significantly inhibited wild-type compared to the control [relative luciferase activity (1.02 ± 0.11)]. The activity of the reporter plasmid [relative luciferase activity was (0.28 ± 0.04), P < 0.05]; whereas in the mutant reporter plasmid group, miR-382 had no significant inhibitory effect.

Effect of miR-382 on the proliferation of 4T1 cells

The miR-382 mimics or the control miR were co-transfected into 4T1 cells with pCDNA3.1 or pCDNA-PGC1α, respectively, and the cell proliferation activity was detected by CCK-8 method, as shown in Fig. 5. At 48 hour, when compared with the control + pCDNA3.1 group (1.16±0.08), miR-382 significantly inhibited the proliferation of 4T1 cells in vitro [(0.65±0.05), P < 0.05]. However, the control +pCDNA-PGC1a (1.61±0.17) increased the proliferation of 4T1 cells in vitro [(1.61±0.17), P < 0.05] which suggest that the overexpression of PGC-1α could significantly promoted cell proliferation and counteract the inhibitory effect of miR-382 on PGC-1α.

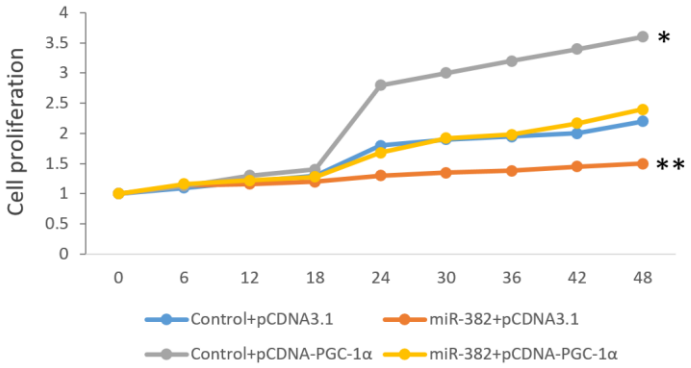

Figure 5. Effect of C miR-382/PGC-1α signaling pathway on proliferation of 4T1 cells in vitro. * P < 0.05, **P < 0.05, compared to the Control+pCDNA3.1.

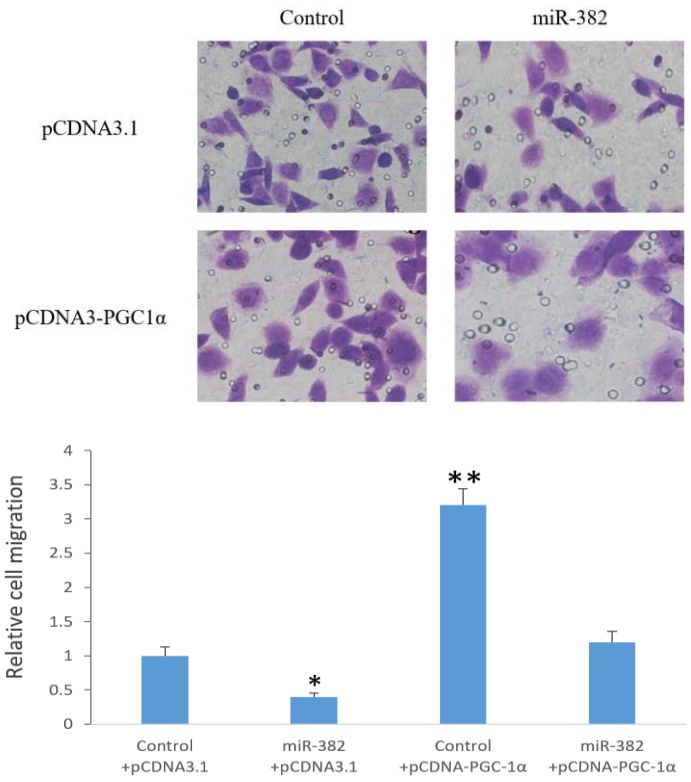

Figure 6. Effect of miR-382/PGC-1α signaling pathway on migration of 4T1 cells by transwell assay. * P < 0.05, **P < 0.05, compared to the Control+pCDNA3.1.

Effect of miR-382 on migration ability of 4T1 cells

As shown in Figure 6, the number of transmembrane cells in the control +pCDNA3.1 group was 780±97 and in the miR-382+pCDNA3.1 group, it was 211±43), which indicate that miR-382 significantly inhibited the migration of 4T1 cells (P < 0.05). However, the number of cells in the control +pCDNA-PGC-1α group was 1579±132, which suggest that overexpression of PGC-1α significantly promoted the migration activity of 4T1 cells and counteracted the inhibition of PGC1α by miR-382.

Effect of miR-382 on the growth of subcutaneous xenografts

As shown in Figure 7, miR-382 mimics or control miR were co-transfected with 4C1 cells with pCDNA3.1 or pCDNA-PGC1α, respectively, and a mouse subcutaneous xenograft model was established. On day 14, was used to show: the volume of transplanted tumor in control + pCDNA3.1 group was (0.74±0.16) cm^3, and that in miR-382+pCDNA3.1 group was (0.29±0.12) cm^3, which miR-382 significantly inhibited the growth of 4T1 xenografts (P < 0.05). Overexpression of PGC1α significantly promoted the growth of transplanted tumors in control+pCDNA-PGC-1α group [(1.89±0.19) cm^3, P < 0.05]. In addition, the inhibitory effect of miR-382 was blocked by overexpression of PGC1α.

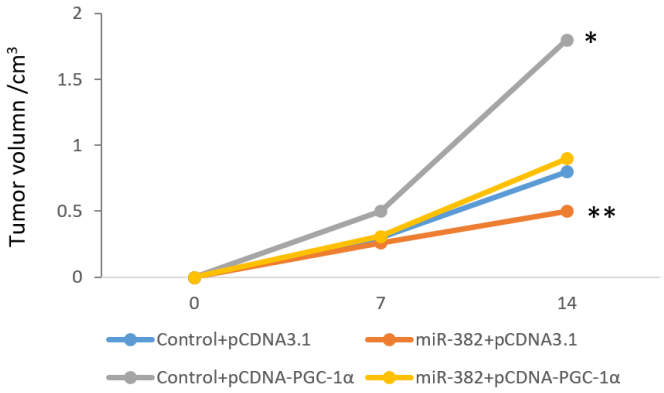

Figure 7. Effect of miR-382 on the growth of subcutaneous xenografts. * P < 0.05, **P < 0.05, compared to the Control+pCDNA3.1.

Discussion

Triple-negative breast cancer is a heterogeneous disease with complex origin. Its occurrence and development are closely related to environmental and genetic factors [8], accounting for 15%-20% of all breast cancers. It has unique clinicopathological features, such as histopathological grades are high, easy to local recurrence, early metastasis, strong invasiveness, poor prognosis, etc. [9-10]. At present, with the continuous improvement of the level of surgery, further regulation of radiation therapy and chemotherapy, the diagnosis and treatment of breast cancer has also made great progress. However, distant metastasis remains a therapeutic bottleneck and is one of the research hotspots of breast cancer.

This PGC-1α is one of the subtypes of the PGC-1 family and is a coactivator of peroxisome proliferator-activated receptor γ (PPARγ) [11]. The human PGC-1 gene is located on chromosome 4p15 and has a molecular weight of 91 kDAa. It was first discovered by PUIGSERVER [12] using yeast two-hybrid technique from a cDNA library constructed from brown adipose tissue of mice, and ST-PIERRE et al [13] found that PGC -1α can effectively inhibit ROS production, promote mitochondrial replication and transcription, increase ATP production, and promote tumor cell metastasis. In breast cancer, patients with high plasma PGC-1α levels have a lower prognosis and survival rate than breast cancer patients with low plasma PGC-1α levels [12]. In invasive breast cancer, PGC-1α promotes distant metastasis of cancer cells by enhancing mitochondrial function [14]. However, the specific mechanism of upstream regulation of PGC-1α has not been clarified.

We found multiple potential miR binding sites in the 3'-UTR of the PGC-1α gene by gene online prediction software. miRNAs are the largest group of post-transcriptional regulators. MicroRNA (miRNA) is one of the post-transcriptional regulators [15], and its 5' end binds to the 3'

untranslated region (3'-UTR) of the target gene, thereby degrading gene translation and reducing protein expression [16]. miRNAs are involved in a range of biological processes, including cell cycle, growth, apoptosis, differentiation, and stress response [17]. Multiple miRNAs regulate cell proliferation, angiogenesis, invasion, and metastasis of breast cancer through targeted genes. The first discovered miRNA associated with breast cancer was miR-206. The miR-206 was significantly decreased in breast cancer tissues, and the low miR-206 level was closely related to the late clinical stage and the overall overall survival rate [18]. miR-125 also inhibits ErBb2 by RTNK, and miR-145 also plays a role in breast cancer metastasis [14, 19]. Studies have shown that miR-382 is also closely related to the development of cancer. For example, miR-382 is involved in the development and metastasis of non-small cell lung cancer by inhibiting SETD8 expression [20]. In colorectal cancer, miR-382 regulates NR2F2 to promote the development of cancer [21, 22]. However, whether miR-382 has a targeted regulatory relationship with PGC-1α in breast cancer remains to be determined. Our previous study found that miR-382 inhibits the activation of aerobic oxidation-dependent M2 macrophages in TAMs by inhibiting the expression of PGC-1α, thereby inhibiting cancer cell metastasis [6-7]. But the relationship between the two in breast cancer cells is unclear.

Therefore, this experiment first used the bioinformatics website to find potential binding sites for multiple miRNAs to PGC-1α, namely mmu-miR-382, mmu-miR-539, mmu-miR-218, mmu-miR-202-3p, mmu-miR-451, mmu-miR-320, mmu-miR-375, mmu-miR-129-5p, mmu-miR-335-5p, mmu-miR-362-3p, mmu-miR-543. Among them, miR-382 can significantly inhibit the expression of PGC-1α gene and protein, and the expression of PGC-1α is significantly increased after inhibiting miR-382, indicating that PGC-1α is regulated by miR-382, but the site of action is still unclear. We then constructed a PGC-1α dual luciferase reporter plasmid and a mutant plasmid, and co-transfected 293T cells with miR-382 mimics to detect changes in luciferase activity. The principle of the luciferase reporter gene is that the miRNA acts mainly by acting on the 3'-UTR of the target gene, and the 3'-UTR region of the target gene can be constructed behind the reporter gene Luciferase to construct a luciferase plasmid. Then, the cells are transfected into cells, and after comparing or overexpressing the miRNA, the activity of the luciferase is monitored, and the expression of the reporter gene is detected to quantitatively reflect the inhibitory effect of the miRNA on the target gene. This experiment found that miR-382 can significantly reduce the expression of luciferase activity of PGC-1α, and it is proved that miR-382 has a regulatory effect on PGC-1α, and the site of action is located at PGC-1α 3'-UTR 565-582.

Although we have demonstrated the regulation of PGC-1 α by miR-382 through the above two experiments, the site of action is located in PGC-1α 3'-UTR 565-582, but the regulation of PGR-1α by miR-382 is specifically in the biological characteristics of breast cancer. The role of the aspect is still unclear, so we used pcDNA3.1 and pcDNA3.1-PGC-1α plasmid to change the role of miR-382/PGC-1α signaling pathway in the proliferation, migration, tumorigenesis and lung metastasis of 4T1 cells. It was found that overexpression of miR-382 can significantly reduce the proliferation and migration ability of cells in vitro, and significantly inhibit the tumor formation and lung metastasis of cancer cells. Overexpression of PGC-1α can significantly increase the proliferation and migration ability of cells in vitro, and significantly promote tumor formation and lung metastasis.

In conclusion, miR-382 may inhibit the proliferation and lung metastasis of 4T1 cell line by PGC-1α, and its site of action is located at the PGC-1α 3'-UTR 565-582.

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Conflicting interests

The authors have declared that no conflict of interests exist.

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