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Research Article

Bone Marrow Mesenchymal Stem Cells (BMSC)-upregulated miR-139 Inhibited the Migration and Invasion of Breast Cancer Cells *In Vitro* by Inhibiting PXN Expression and EMT

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Abstract

Short non-coding RNAs (microRNAs) is reported to exerted a crucial impact in tumor biology. While, the biological effect of miR-139 in breast cancer cells remain unclear yet. Here we intend to clarify the effect and mechanism of miR-139 derived from Bone marrow mesenchymal stem cells (BMSCs) on the biological behavior of gastric cancer cells. Breast cancer cells were divided into BMSC group (mixed culture of BMSC and breast cancer cells 1:1). Cells in the miR-139 mimics group, si-PXN group and control group were not treated. miR-139 abundance is evalued through Real time PCR, cell activity is analysed by MTT assay.Finally, the targeted binding of miR-139 to PXN was verified by double luciferase reporter genes. In relative to control, miR-139 abundance was notably declined in gastric cancer cells, while PXN abundance was higher, and the higher expression of PXN was linked to the prognosis of patients. miR-139 can be up-regulated by BMSCs or miR-139 mimics, thereby regulating EMT process through targeted inhibition of PCN, and ultimately inhibiting *in vitro* activity of breast cancer cells. BMSC co-culture can inhibit PCN by up-regulating miR-139, thus regulating EMT process and inhibiting the progression of gastric cancer. The results of this study revealed the mechanism of breast cancer progression to a certain extent, and suggested that miR-139 and PXN could be used as therapeutic targets for metastatic breast cancer, and BMSC could be of great value as a novel biologic therapy.

Keywords: Gastric Cancer; MiR – 139; Cell Proliferation; Cell Invasion

Introduction

Breast cancer is a phenomenon of malignant proliferation of breast epithelial cells under the action of a variety of carcinogens. Breast cancer is often called "pink killer", and its incidence rate is the first place in female malignant tumors. In the late stage, cancer cells may have distant metastasis and multiple organ lesions, which directly threaten the life of patients [1]. It is crucial to clarify the biological behavior of tumor to improve the curative effect and improve the quality of life of patients.

Short non-coding RNAs, which do not have protein-coding capabilities, are involved in many pathological and physiological processes and are key regulators of multiple biological processes, such as breast cancer [2]. Mesenchymal stem cells (MSCs) have received a lot of attention as a new regenerative therapy in the past

few years. BMSC exosomal-derived miRNAs have been shown to be effective in controlling a variety of cancers [2-5]. Exosomes are extracellular vesicles with diameters ranging from 30 to 100nm and are widely involved in paracrine processes [6]. Exosomes contain microRNAs (miRNAs) that can be delivered to target cells that activate genetic and epigenetic changes to perform biological functions. Altered microRNA expression can act as oncogenes or tumor suppressors to regulate cancer initiation and progression [7]. For example, BMSC exosomal-derived microRNA-136 inhibits bladder cancer cell proliferation and the epithelial-mesenchymal transformation process by regulating RAB1A [8]. In nasopharyngeal carcinoma, BMSC-derived miR-1528 inhibits cell invasion and migration through competitive binding with ANXA2.Among these miRNAs, miR-139, located in chromosome 1p22 region, has attracted much attention due to its abnormal down-regulation, and linked to tumor inhibition in a variety of tumors [9]. For example, the study say [10,11], miR-139 is down-regulated in breast cancer and can inhibit the migration and invasion potential of breast cancer cells. PXN is a local adhesion associated protein that regulates the process of cell movement and migration by forming structural connections between the extracellular matrix and the actin cytoskeleton. According to the report [12,13], PXN is highly expressed during the development of cancer. In gastric cancer, PXN promotes the *in vitro* activity of GC cells by up-regulating GAS6 level. While the effect of PXN and miR139 in breast cancer is unknown yet.

Materials and Methods

Tissue specimens

Taking jilin university first hospital patients with breast cancer, breast cancer tissue and tissue adjacent to carcinoma, a total of 60 for tissue samples (age 52 +/- 6.8 years, 42 patients with III period, 18 patients with IV period) were harvested.

Cells and culture

Breast cancer cell lines, human BMSC cell lines and MCF10A are obtained from the American Typical Culture Preservation Center (USA) at 37°C, 5%CO_2The logarithmic growth phase cells were harvested.

Cell transfection

The mimics targeting miR-139 were synthesized by Genepharma, and the PXN-specific small molecule interfering RNA and si-NC were synthesized using non-specific mimics as negative control (NC).All plasmids were transfected with Liposome 3000(InvitGen) and the transfection efficiency was verified by PCR 48h later.

The cells were divided into BMSC group (mixed culture of BMSC cells and breast cancer cells 1:1).Cells in the miR-139 mimics group and the si-PXN group were not treated in the control group.

qRT-PCR

Cells were collected and washed 3 times with precooled PBS and added appropriate amount of solution. After 20 min of ice bath, they were transferred to 1.5 mL EP tube for the next step or put at -80°C preservation reserve.Proper amount of tissue was fully shredded and put into 2 mL thick wall EP tube and added beads and 1 mL trizol solution. After centrifugation with chloroform at low temperature, the upper aqueous phase was collected to harvest RNA precipitate, and RT reaction solution was configured on the ice after collecting the total RNA (1 μg). 1 μg RNA was reverse transcribed in IDTE buffer (pH=8.0, Integrated DNA Technologies, USA). Diluted to 10 ng/μL, and qPCR was actualized following per reagent vendor instructions. Set reaction conditions: 90°C for 60s, predenaturation at 95°C for 90 s;95°C for 60s; denaturation at 95°C n for 45 s and 60°C for 45s, total of 40 cycles. The value of each gene CT was determined after the reaction, that is, the initial cycle number of the gene when it entered linear amplification. The gapdh expression was taken as the internal parameter and the expression of each sample and related genes was evaluated by $2^{-\Delta\Delta CT}$ method.

Western blotting

After the extraction of total protein with BCA protein analysis the kits protein concentration, transfer film after sds-page protein separation, after closed 2 h at room temperature in 5% skim milk, with a resistance (E cadherin, Vimentin, q, PXN, Bax, Bcl2 PXN GAPDH), 4°C for the night, and then clean with TBST solution membrane, and the corresponding secondary antibody incubation at room temperature and 2 h, and then use the enhanced chemiluminescence reagent kit (GE Healthcare, the United States) for development.

MTT assay

Logarithmic phase cells were collected, the concentration of cell suspension was adjusted, 100 μ l was added into each well, and the density of cells to be tested was adjusted to 1000-10000 wells (the edge wells were filled with sterile PBS). 5% CO2, incubation at 37 °C, until the cell monolayer is covered with the bottom of the hole (96 hole flat plate), add the drug of concentration gradient. In principle, the drug can be added after the cell adheres to the wall, or two hours, or half a day, but we often lay the plate in the afternoon of the previous day and add the drug in the morning of the next day. Generally, there are 5-7 gradients, each hole is 100 uL, and 3-5 multiple holes are set. It is suggested to set five, otherwise it is difficult to reflect the real situation. 5% CO_2, incubated at 37 °C for 16-48 hours, observed under inverted microscope. MTT was added to each well. If the drug can react with MTT, the culture medium

can be discarded after centrifugation, washed with PBS carefully for 2-3 times, and then the culture medium containing MTT can be added. Stop the culture and carefully suck out the culture medium in the well.

MTT assay is the method to detect cell survival and growth. MTT is a yellow compound, which can accept hydrogen ions and act on the respiratory chain of mitochondria in living cells. Under the action of succinate dehydrogenase and cytochrome c, exogenous MT is reduced to water-insoluble blue purple crystal formazan and deposited in cells. Dead cells have no such function. Dimethylsulfoxide (DMSO) can dissolve methyl Zan in cells. The light absorption value measured by enzyme-linked immunosorbent assay at 570nm (reference wavelength 630m) can indirectly reflect the number of living cells. Within a certain range of cell number, the amount of MT crystal formation is directly proportional to the number of living cells.

Flow cytometry analysis

Wash harvested MSC cells (5×106) twice with PBS, and stained with V-FITC and PI (Xian Ding Biotechnology Co., Ltd., China) and dyes with fluorescein isothiocyanate (FITC), China) for dyeing, with mouse immunoglobulin labeled with FITC or PE (Nantong Ishi Biotechnology Co., Ltd., staining cells as a negative control, the analysis was carried out in WINMDI software after 30 minutes of room temperature incubation.

Transwell method

Matrix rubber plank: BD Matrigel's 1:8 (according to the amount of MMP produced by cells) is diluted. Preparation of cell suspension: before the preparation of cell suspension, the cells could be starved for 12-24 hours to further remove the influence of serum. culture cells: conventional culture for 12-48 hours (mainly depending on the invasion ability of cancer cells). In addition to considering the invasiveness of cells, the effect of treatment factors on cell number should not be ignored. results statistics: direct counting method, "adherent" cell counting, here the so-called "adherent" means that after passing through the membrane, the cells can adhere to the inferior chamber side of the membrane without falling into the inferior chamber. By staining the cells, the cells can be counted under the microscope.

Analysis of luciferase report

We constructed the plasmid expressing both mirage luciferase and firefly luciferase. Mirage luciferase was an internal reference, and the firefly luciferase gene was linked wild type (WT)- PXN 3'-UTR sequence or mutant(MUT)- PXN 3'-UTR sequences. HEK-293T cell was integrated with bifluorescent WT/MUT- PXN3-1'-UTR plasmid, then treated with miR-NC or MiR-139 Mimics. Reduced activity of the luciferase activity of firefly could indicate that MiR-139 binded to a certain sequence of WT PXN3 3'- UTR.

Statistical analysis

Data was analysed in Graphpad prism 6.0 and expressed as the mean ±SEM. With the double-tailed t test analysis adopted, $P<0.05$ means a significantly statistical differences.

Results and Discussion

PXN is highly expressed in breast cancer

PXN mRNA notably increased in tumor tissues in relative to the paracancerous (Figure 1A), and tumor cell lines showed elevated PXN abundance than normal cells (Figure 1B). Furthermore, elevated PXN links to poor prognosis in breast cancer patients (Figure 1C).

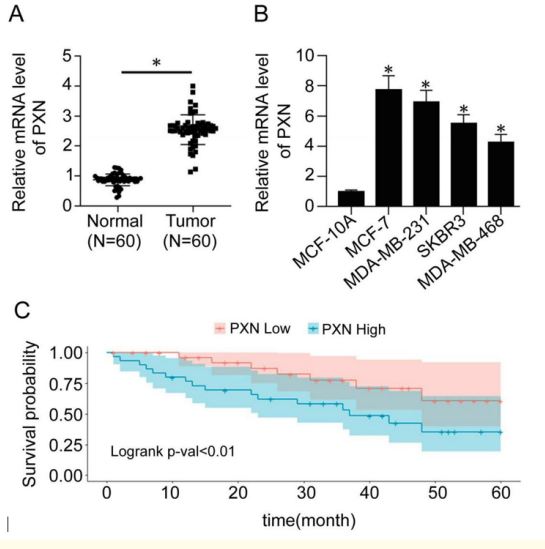

Figure 1: PXN expression is upregulated in breast cancer. (A) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the expression of PXN in breast cancer tissues and adjacent normal tissues. (B) The expression of PXN in human breast cancer cell lines MCF-7, MDA-MB-231, SKBR-3 and MDA-MB-468 and normal breast epithelial cell line MCF-10A were detected by qRT-PCR. (C) Kaplan-Meier analysis of the relationship between PXN expression and patients' overall survival. * $P < 0.05$. Xueqiang Gao*., et al.* Figure 1.

PXN gene knockout can inhibit cell proliferation

Rt-pcr analysis confirmed that after transfection sh - PXN# 1/2 PXN in (Figure 2A), function experiments have shown that cells can significantly reduce PXN knockout and cells proliferation ability (Figure 2B), C - myc protein involved in proliferation and apoptosis resistance protein expression of Bcl - 2 significantly decreased (Figure 3B), flow cytometry analysis showed that PXN silence cells apoptosis rate increased significantly (Figure 2C). The expression of apoptotic protein CCS3 (cleaved Casepase-3) was increased (Figure 3B).

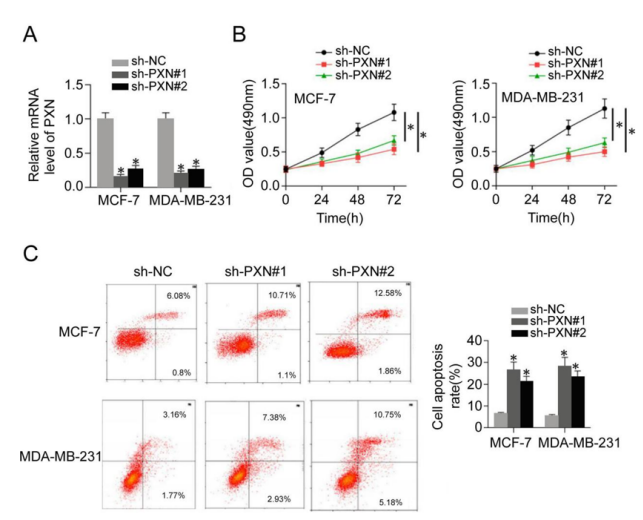

Figure 2: PXN gene knockout can inhibit cell proliferation and promote cell apoptosis.

(A) qRT-PCR was used to detect the knockout efficiency of PXN gene in breast cancer cells. (B) Proliferation of MCF-7 and MDA-MB-231 cells transfected with SH-PXN #1 and #2 was detected by MTT assay. (C) Cell apoptosis was detected by flow cytometry. Xueqiang Gao., *et al*. Figure 2.

PXN gene knockout can inhibit cell migration and invasion, and inhibit the process of EMT

PXN silentation significantly inhibited the migration and invasion of tumor cells (Figure 3A). Ki67, PCNA and Bcl-2 were decreased, while Bax were increased in breast cancer cells. In addition, knockout of PXN gene inhibited the levels of E-cadherin, Vimentin and Snail (Figure 3B), suggesting that knockout of PXN gene inhibited the process of EMT.These results suggested that PXN gene knockout inhibited the malignant behavior of tumor cell and inhibited the process of EMT.

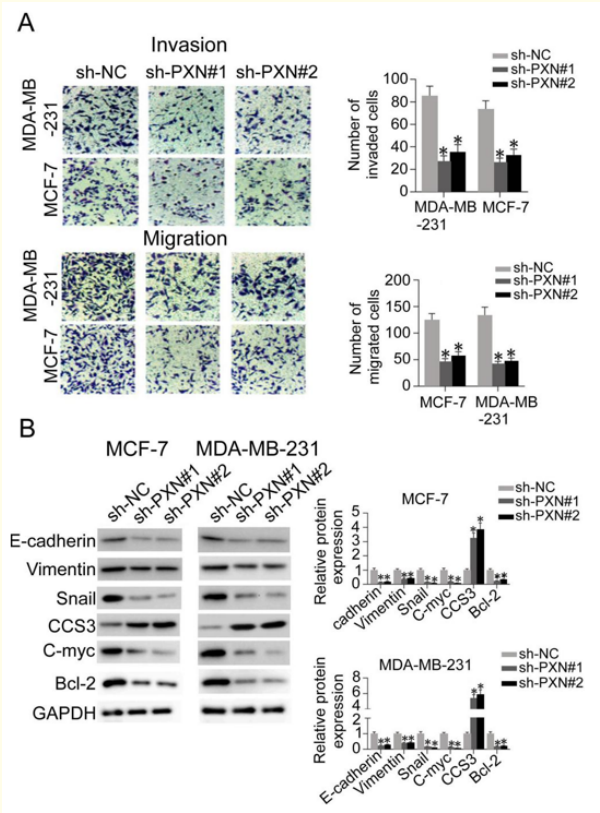

Figure 3: PXN gene knockout inhibited cell migration and invasion, and inhibited EMT process Transwell assay was used to detect.

(A) Cell migration and invasion ability, original image X100, and (B) Western blotting was used to detect the expression levels of proteins associated with cell proliferation and apoptosis (CCS3, c-myc and Bcl-2) and EMT-related proteins. * P < 0.05. Xueqiang Gao., *et al.* Figure 3.

PXN binds to miR-139

Use Starbase forecasts the combination of miR - 139 and PXN (Figure 4B), and miR - 139 analog effectively raised the expression of miR - 139 in cells (Figure 4A), through the luciferase report ex-

periment further verifies the interaction between them, the results show that compared with the simulation of NC group, miR - 139 simulation group pmirGLO PXN - Wt report molecular luciferase activity decreased significantly, However, the luciferase activity of the pmiRGLO-PXN-MUT reporter molecule was not significantly changed (Figure 4C), and the overexpression of miR-139 inhibited PXN abundance in (Figure 4D), while the declined expression of miR-139 is noticed in cell lines (Figure 4E).

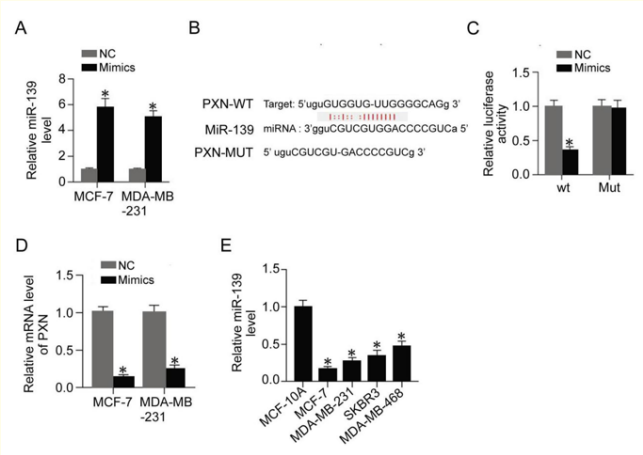

Figure 4: Targeting binding of PXN to miR-139.

(A) qRT-PCR was used to detect the expression of miR-139 in MCF-7 and MDA-MB-231 cells transfected with NC simulants or miR-139 simulants, (B) Tarbase database predicted PXN-bound miR-139, (C) luciferase report assay confirmed the interaction between miR-139 and PXN, (D) qRT-PCR was used to detect the expression of PXN in MCF-7 and MDA-MB-231 cells transfected with NC simulants or miR-139 simulants.(E) Detection of miR-139 expression in human breast cancer cell lines by qRT-PCR.* P < 0.05, P < 0.05. * *. Xueqiang Gao., *et al.* Figure 4.

Co-culture of miR-139 mimics or BMSCs can inhibit PXN level

In relative to control, the use of miR - 139 mimics or BMSC after cocultivation, MCF - 7 and miR - 139 level in cells increased significantly (Figure 5C), and the expression of PXN significantly reduced (Figure 5B), further cell phenotype analysis revealed that cells compared with control group, the use of miR - 139 mimics or BMSC after cocultivation, breast cancer cell proliferation activity, invasion and migration ability significantly reduced (Figure 5-5 d f), cell apoptosis increased significantly (Figure 5e). These results suggest that miR-139 mimics or BMSC co-culture can inhibit the PXN level, thus inhibit the activity of breast cancer cells *in vitro*.

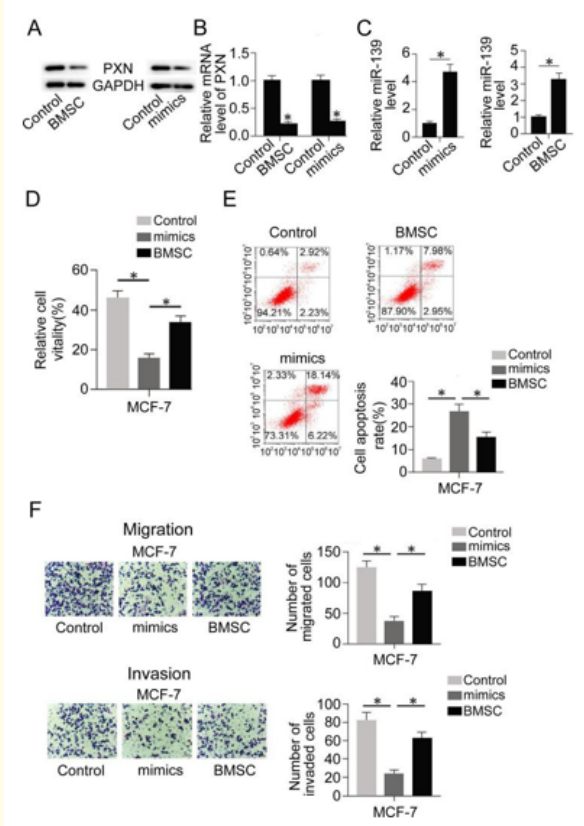

Figure 5: Co-culture of miR-139 mimics or BMSC inhibited the expression of PXN.

WB test (A) after different treatment of MCF - 7 cell PXN expression, (B) qRT - PCR detection after different treatment of MCF - 7 cell PXN expression, (C) qRT - PCR detection after different treatment of MCF - 7 cell in the expression of miR - 139, (D) qRT PCR detection after different treatment of MCF - 7 cell PXN expression, (E) determined by MTT method to detect different processed MCF - 7 cell proliferation and flow cytometry to test different DE MCF - 7 cell apoptosis after processing, (F) Transwell assay was used to detect the migration and invasion ability of MCF-7 cells after different treatments.* P < 0.05. Xueqiang Gao., *et al.* Figure 5.

GENE	Positive	Reverse
GAPDH	ACCCAGAAGACTGTGGATGG	TCAGCTCAGGGAT-GACCTTG
miR-139	TCAGCTCAGGGATGACCTTG	TCAGCTCAGGGAT-GACCTTG

Table 1: Primers for quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Discussion

Breast cancer is a phenomenon of malignant proliferation of breast epithelial cells under the action of a variety of carcinogens. Breast cancer is often called "pink killer", and it is crucial to clarify the biological behavior of tumor to improve the curative effect and improve the quality of life of patients. PXN is highly expressed during the development of cancer.MiR-139 is differentially expressed in several tumors. The results of this study revealed the overexpression of PXN in breast cancer as well as declined miR-139 abundance. MiR-139 overexpression/BMSC co-culture/PXN gene knockout can inhibit the *in vitro* activity of breast cancer cells and promote the apoptosis by inhibiting the expression of PXN. Further, knockdown of PXN can inhibit the malignant behavior of breast cancer cells through the EMT pathway, suggesting the regulatory role of PXN in EMT.

Short non-coding RNAs, which do not have protein-coding capabilities, are involved in many pathological and physiological processes and are key regulators of multiple biological processes, such as breast cancer. Mesenchymal stem cells (MSCs) have received a lot of attention as a new regenerative therapy in the past few years. BMSC exosomal-derived miRNAs have been shown to be effective in controlling a variety of cancers. Exosomes are extracellular vesicles with diameters ranging from 30 to 100nm and are widely involved in paracrine processes. Exosomes contain microRNAs (miRNAs) that can be delivered to target cells that activate genetic and epigenetic changes to perform biological functions. Altered microRNA expression can act as oncogenes or tumor suppressors to regulate cancer initiation and progression [13-20]. microRNAs are involved in tumor [21] development by regulating EMT and multiple signaling pathways. For example, microRNA-129 promotes EMT in colorectal cancer cells through the PI3K/Akt pathway and thus facilitates their migration and invasion.

As a member of the miR-14h family, miR-139 has been widely identified as a tumor suppressor gene. For example, miR-139 suppresses the invasion process of gallbladder cancer cells by down-regulating the expression of TGF-β, and miR-319 suppresses chemotherapy resistance by targeting FOXQ1 by inhibiting dry maintenance of hepatoma cells. In triple-negative breast cancer cells, miR-139 reduced cell proliferation by binding to HDAC8 [22]. In this study, RT-PCR analysis revealed miR-139 abundance was notably reduced in breast cancer cell lines, and functional test revealed miR-139 abundance was significantly increased when treated with miR-139 mimics or when MCF-7 was co-cultured with BMSC, while the malignant phenotype of breast cancer cells was significantly inhibited. Previous studies have shown that [14,23,24], as a tumor suppressor, miR-139 inhibits the migration and invasion of nasopharyngeal carcinoma cells by targeting PXN, and in addition, miR-139 inhibits the EMT process of prostate cancer by interacting with PXN [25]. Consistent with this, bioinformatics analysis in this study predicted that miR-139 and PXN had complementary base pairings, while dual luciferase reporter gene results showed that miR-139 could target PXN expression inhibition.

Conclusion

In short, we confirmed that PXN expressed in breast cancer high expression and miR - 139 low, low expression of miR - 139 can be handled BMSC or miR - 139 mimics the rise, thereby inhibit PCN to adjust EMT process, mediating the proliferation, migration and invasion of breast cancer cells and apoptosis, but also has certain limitation, such as research and save the experiment failed in the body.

Ethical Compliance

Research experiments conducted in this article with animals or humans were approved by Qingdao University Hospital following all guidelines, regulations, legal, and ethical standards as required for humans or animals.

Conflicts of Interest

There are no conflicts to declare.

Acknowledgements

There are no acknowledgements.

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