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MiR-130a-3p inhibits migration and invasion by regulating RAB5B in human breast cancer stem cell-like cells

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ABSTRACT

Breast cancer stem cells (BCSCs) constitute a subpopulation of tumor cells that express stem cell-associated markers and have a high capacity for tumor generation in vivo. MicroRNAs (miRNAs) are involved in tumorigenesis by regulating specific oncogenes and tumor suppressor genes, and their roles in BCSCs are becoming more apparent. We try to reveal the mechanism by which specific miRNA plays its function in BCSCs. Herein, we show that miR-130a-3p is down-regulated in human breast cancer tissues and exosomes from circulating blood. Overexpression of miR-130a-3p in BCSCs inhibited cellular proliferation, migration, and invasion, and silencing of miR-130a-3p had the opposite effects. We also confirmed that RAB5B is directly down-regulated by miR-130a-3p. Knockdown of RAB5B also inhibited cell proliferation, migration and invasion. Furthermore, we found that lower levels of exosome-derived miR-130a-3p are associated with lymph node metastasis and advanced TNM stage. Taken together, our results demonstrate that miR-130a-3p may act as a disease progression monitoring indicator and therapeutic target in breast cancer.

1. Introduction

Breast cancer is one of the most common malignant tumors in females [1]. Cancer stem cells (CSCs), a sub-population of cancer cells, possess characteristics similar to normal stem cells, such as self-renewal and differentiation. Breast cancer stem cells (BCSCs) characterized by the CSC markers $CD44^{+}CD24^{-}$/low are responsible for the formation, progression, metastasis, therapy resistance and recurrence of malignant tumors [2–4].

As a class of small non-coding RNAs, microRNAs (miRNAs) are approximately 21 nucleotides in length and negatively regulate gene expression through binding to the 3′untranslated regions of target mRNAs, which leads to translational inhibition and/or mRNA degradation [5]. Meanwhile, miRNAs have been shown to play roles in several biological processes, including cellular proliferation, differentiation, apoptosis, and metastasis [6,7]. More importantly, aberrant deregulation of specific miRNAs and their targets are associated with the formation and progression of various cancers, including breast cancer [8–10].

Exosomes are extracellular vesicles produced by various living cells. Exosomes contain a variety of selectively enriched miRNAs, mRNAs and proteins that reflect the physiological and pathological conditions of the cells from which they originated. The outer membrane of exosomes provides comprehensive protection of miRNAs from the extracellular environment which readily digests it. Therefore, it's more practical to study the exosome-derived miRNAs from circulating blood as a noninvasive molecular marker in breast cancer.

Recently, many studies have focused on the relationship between miRNAs and CSCs. Aberrant expression of miRNAs was shown to be involved in the proliferation, self-renewal, differentiation and tumor formation of BCSCs [11–14]. In this study, we investigated the function of miR-130a-3p in $CD44^{+}CD24^{-/low}$ BCSCs derived from the MCF-7 cell line. We found that miR-130a-3p is down-regulated in human breast cancer tissues and exosomes from circulating blood. In addition, miR-130a-3p decreased cellular proliferation, migration, and invasion by directly targeting RAB5B, a member of the RAB subfamily of small GTPases. Similar biological efficacy was obtained when we blocked the expression of RAB5B with siRNA. To our knowledge, this is the first study to comprehensively examine the expression and function of miR-130a-3p in BCSCs, and highlight its potential as a biomarker for breast cancer.

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2. Materials and methods

2.1. Specimens

40 pairs of breast cancer and adjacent normal tissues, 40 pairs of blood samples from patients with breast cancer and healthy controls were all obtained from Shanghai tenth people's hospital following informed consent and ethic approval (time: 2015.1–2016.2). All the samples were confirmed as invasive ductal breast cancer, and no patient had received any chemotherapy or radiotherapy ahead of surgery.

2.2. Cell culture and transfection

Breast cancer MCF-7 and MDA-MB-231 cells, mammary epithelial MCF-10A cells were maintained in high-glucose Dulbecco's modified eagle medium (DMEM) (Gibco, GrandIsland, NY), containing 10% fetal bovine serum (FBS) (Gibco) and incubated in a humidified chamber with 5% CO2 at 37 °C. BCSC isolation and culture were performed following the methods previously described [15,16]. Suspended MCF-7 cells were cultured in BCSC isolation medium which consists of serum-free DMEM/F12 (Gibco),2% B27 (Invitrogen, Carlsbad, CA), 20 ng/mL EGF (PeproTech, London, UK), 20 ng/mL bFGF (PeproTech), 0.4% bovine serum albumin (BSA) (Sigma, St.Louis, MO), and 5 μg/mL insulin.

The proportion of BCSCs was evaluated using the BCSC marker $CD44^{+}CD24^{-/low}$ after mammosphere (MMS) formation. Human CD44 (APC) and CD24 (PE) monoclonal antibodies (BD Bioscience) were added to the cell suspensions and incubated at 4 °C for 30–40 min in the dark. BCSC proportion was analyzed by flow cytometry on FACScalibur (Becton Dickinson). Cells were then cultured in DMEM containing 10% FBS for 24 h. The miR-130a-3p mimics (miR-130a-3p), the miR-130a-3p inhibitor (anti-miR-130a-3p) sequences were synthesized by Integrated Biotech Solutions Company (Shanghai, China), siR-RAB5B was synthesized by Genechem Company (Shanghai, China), and miR-NC and siR-NC were used as negative controls. Transfection was conducted with Lipofectamine 2000 (Invitrogen) following the instructions. Exosome extraction was conducted with ExoQuick Exosomal Extraction Kit (System Biosciences) following the instructions.

2.3. Cell viability assays

BCSCs were seeded in a 96-well plate at 5000 cells per well and incubated for 96 h. Cell viability was determined at 24, 48, 72, and 96 h after transfection using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). The absorbance of each well was measured with a microplate reader set at 450 nM.

2.4. Cell cycle assay

For cell cycle analysis, 72 h after transfection, cells were collected and fixed with 70% ethanol at −20 °C for 18 h, propidium iodide (PI) (BD Bioscience) was then added to the cells. Samples were analyzed by flow cytometry on FACScalibur (Becton Dickinson).

2.5. Scratch wound assay

Cell monolayer was scraped with 1-mL sterile pipette tip at 24 h after transfection. Thereafter, cells were washed with medium and stimulated as indicated. Images tagged with a point of the plates were taken at 0, 36, and 72 h using a phase contrast microscope at 200× magnification (Olympus IX81, Tokyo, Japan).

2.6. Invasion assay

Transwell chambers coated with Matrigel (BD Biosciences) were used to examine invasion capability of the BCSCs in vitro. 5×10^4 cells were seeded into the upper chamber which contains 200 μl DMEM with 1% FBS, and the lower chamber was filled with 500 μl of DMEM with 20% FBS as a chemoattractant. Transwells were then incubated at 37 °C for 48 h. Cells that were adhered to the lower membrane of the inserts were counted as previously described [16]. Images of different fields were taken.

2.7. RNA extraction and qRT-PCR

Total RNA was extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen). The miR-130a-3p level was quantified by qRT-PCR using TaqMan MicroRNA Assay kits (Applied Biosystems, Foster City, USA) with U6 small nuclear RNA (snRNA RNU6B) as an internal normalized reference. Their relative levels were measured in triplicate on a Prism 7300 real-time PCR machine (Applied Biosystems). For the quantification of RAB5B mRNA, extracted total RNA was reverse transcribed to cDNA using an oligo (dT) 12 primer and RevertAid™ Reverse Transcriptase (Fermentas). The RAB5B expression was measured in triplicate on a MyiQ Single -Color RT-PCR Detection System (Bio-Rad, Hercules, USA) with GAPDH and β-actin as internal control.

2.8. Western blot

Cells were lysed at 4 °C for 30 min by RIPA buffer. Total protein was quantified by BCA assay and separated by electrophoresis in SDS–polyacrylamide gels before transferring to nitrocellulose membranes (Bio-Rad). They were then blocked in 5% skim milk in TBST. Immune complexes were formed by incubation of membranes with anti-Rab5B (1:200) (Santa Cruz Biotechnology, USA) and anti-GAPDH (1:1000) (Santa Cruz Biotechnology) antibodies overnight at 4 °C. Blots were washed and incubated for 1 h with anti-rabbit secondary antibody. Immunoreactive protein bands were detected with an Odyssey Scanning system.

2.9. Luciferase reporter assay

The putative targets of miR-130a-3p were predicted using the TargetScan, PicTar, and miRanda algorithms. Only common targets were considered for experimental analyses. The RAB5B 3′-untranslated mRNA region (3′-UTR) containing the predicted miR-130a-3p-binding sites were cloned into the pMIR-REPORT vector (Ambion, Austin, TX) using PCR-generated fragments (WT-UTR). A mutant luciferase vector with miR-130a-3p one pairing site deleted (DEL-UTR) was also constructed. When HEK293T cells reached 60–70% confluence in 24-well plate, 100 ng of Luciferase plasmid was cotransfected with 50 ng of Renilla plasmid (Ambion) and 650 ng of miR-130a-3p mimics or NC using Lipofectamine 2000 as previously described [17]. After 48 h, luciferase activities were measured using a dual-luciferase reporter assay system (Promega, Madison, US) according to the manufacturer's instructions.

2.10. Statistical analysis

All experiments were done in triplicate. Continuous variables were expressed the mean ± standard deviation, $^{*}P < 0.05$, $^{**}P < 0.01$. The two-tailed *t*-test was used to draw a comparison between groups. All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, U.S.).

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