MiR-200c inhibits metastasis of breast tumor via the downregulation of Foxf2

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ABSTRACT. The forkhead box F2 (Foxf2) gene suppresses epithelial-mesenchymal transition via the modulation of transcription of zinc finger E-box-binding homeobox 1 (Zeb1) and epithelial (E)-cadherin, thereby inhibiting tumor metastasis. Additionally, the specific binding of microRNA (miR)-200c to Foxf2 mRNA impedes metastatic pulmonary cancer. However, the role of miR-200c in breast cancer is still unknown. Therefore, in this study, miR-200c mimics were transfected into the highly metastatic breast cancer cell line MDA-MB-231. Their invasion and migration abilities were observed by scratch and transwell migration assays. Real-time PCR was used to detect mRNA levels of Foxf2, Zeb1, and E-cadherin, whereas Foxf2 protein level was determined by western blot analysis. Our results showed that, compared to the control group, miR-200c inhibited the migration or invasion of MDA-MB-231 cells. Real-time PCR and western blot analysis exhibited significant decreases in Foxf2 expression in the presence of miR-200c, along with a decrease in Zeb1 and increase in E-cadherin mRNA expressions. Thus, our preliminary data demonstrated that miR-200c could inhibit the metastasis of breast
cancer cells by downregulating Foxf2 expression, providing leads for the discovery of a novel breast cancer treatment.

**Key words:** Breast cancer cell; Epithelial-mesenchymal transition; miRNA-200c; Foxf2; E-cadherin; Zeb1

**INTRODUCTION**

Breast cancer is the third most popular malignant tumor worldwide and is the most common cancer in women, affecting about 12% women worldwide, with rapidly increasing incidence and mortality rates (Berrada et al., 2010). It has been estimated that in 2015, around 268,600 new cancer cases and 72,000 cancer-related deaths occurred in China and 2.8 million women were affected by breast cancer (McGuire et al., 2015). According to the subtyping guidelines by St. Gallen International Breast Cancer Congress (Goldhirsch et al., 2011), basal-like type (triple-negative breast cancer type mainly) is more invasive due to the lack of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 expressions and overexpression of basal-like molecular markers in breast cancer cells, making them insensitive to either molecular targeting or endocrine treatment (Goldhirsch et al., 2011). Recent studies have shown that epithelial-mesenchymal transition (EMT) plays a critical role in the invasion-metastasis cascade (Kumar et al., 2016). EMT represses cell-cell adhesion through inhibition of epithelial (E)-cadherin, zonula occludens-1, occludin, claudin-1, and claudin-7, and facilitates multiple immunosuppression, drug resistance, and evasion of apoptosis, thus promoting cancer progression and metastasis (Chaffer and Weinberg, 2011). Studies have revealed that a multitude of factors, such as forkhead box M1 (FoxM1)-caveolin-1, tumor growth factor β, nuclear factor-kappa B, Notch-1, and hypoxia trigger EMT, resulting in metastasis (Ellenrieder et al., 2011; Huang et al., 2012; Shimojo et al., 2013). Interestingly, Foxf2 can stimulate EMT, further accelerating tumor metastasis (Kundu et al., 2016). Foxf2 gene belongs to the forkhead/winged helix transcription factor family, and its product can specifically recognize and bind the binding regions on DNA sequences in its monomeric form; thus, it recruits co-activating molecules to initiate transcription, playing a crucial role during embryonic development and tissue differentiation (Nik et al., 2013). During such processes, the relationship between Foxf2 and EMT is the key to illustrating the tumor metastasis mechanism. Some studies have indicated that EMT is significantly impeded by Foxf2 deficiency, and that Foxf2 exerts an inhibitory effect on EMT by regulating the transcription of TWIST1 in basal-like breast cancer cells (Marschall and Beuers, 2013; van den Brink and Rubin, 2013; Wang et al., 2015). Another study on gene expression profiling demonstrated that FOXF2 was highly co-expressed with basal- and metastasis-related genes in breast cancer, but was frequently silenced in luminal-type and human epidermal growth factor receptor 2-positive breast cancers, indicating that it plays dual roles depending on the breast tumor subtype (Lo et al., 2016).

MicroRNAs (miRNAs) are a group of highly conserved RNA molecules of 22-24 nt length and are important in the endogenous regulatory pathway for protein expression (Bartel, 2009; Tekirdag et al., 2013; Zhang et al., 2016). Animal miRNAs are usually partially complementary to the 3’-untranslated region of the target gene, leading to the less efficient translation of the mRNA into proteins (Bartel, 2009). A previous study on metastatic pulmonary cancer has shown that miR-200c specifically binds the Foxf2 mRNA to inhibit the translation
of Foxf2, which binds zinc finger E-box-binding homeobox 1 (Zeb1) (Ahmad et al., 2011). This elevates E-cadherin expression and retards EMT, arresting cell migration. Moreover, the miR-200 family of miRNAs is downregulated in basal-like breast cancer cell line MDA-MB-231, with a more significant change in their expression levels than other family members (Castilla et al., 2012). However, the correlation between miR-200c and Foxf2 in breast cancer cells is still unknown. Thus, the purpose of this study was to show that miR-200c has a similar function in breast cancer cells as it does in pulmonary cancer, which may provide profound evidence for the development of novel breast cancer treatments.

MATERIAL AND METHODS

Cell line and reagents

Human breast cancer cell line MDA-MB-231 was purchased from American Type Culture Collection (ATCC, USA). MiR-200c mimics were designed and synthesized by Gimma (Gimma, China). Gibco Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Life Technologies (Thermofisher Scientific, USA). Fetal bovine serum was obtained from Gibco (Thermofisher Scientific). Lipofectamine 3000 and mRNA primers were purchased from Invitrogen (Thermofisher Scientific). RNA extraction kit was used for RNA extraction (TaKaRa, Japan), ReverTra Ace kit for reverse transcription, and rTaq DNA Polymerase for PCR were obtained from Toyoabo (Japan). Transwell chamber (6.5 mm transwell, with 8.0 mm pore size polycarbonate membrane insert) was a product of Corning (USA). Foxf2 polyclonal antibody was purchased from Abcam (USA).

Cell culture

Transient transfection of miRNA with Lipofectamine 3000 was performed based on manufacturer instructions. One day before transfection, cells were seeded on a 6-well plate (2 x 10^5 cells per well) in antibiotic-free high-glucose DMEM medium. Cells were divided into control (no treatment), miR-NC (blank transfection), and miR-200c transfection groups. After the cells reached 60-70% confluence, 3.75 µL Lipofectamine 3000 was diluted in 125 µL DMEM, whereas equal volume of DMEM was used to dilute 30 nmol miR-200c mimics or miR-NC. These two solutions were mixed and incubated for 5 min at 25°C to form the transfection mixture, which was then added to culture plates (1.5 mL each well). After incubating it for 36-48 h, further experiments were performed.

Scratch assay

Forty-eight hours after transfection, a scratch was made in the center of the 6-well plate using pipette tips. D-hanks’ solution was used to wash the cells and culture medium was added. Experiments were carried out in triplicates. Images were captured at 0 and 24 h, to assess the wound closure rate.

Transwell cell migration assay

Forty-eight hours after transfection, 1.1 mL high-glucose DMEM medium was added
to a 24-well plate and a transwell chamber. Cells were counted and adjusted to a concentration of $10^5$ per mL. Serum-free cell suspension (0.2 mL) containing 0.4% bovine serum albumin was then added to the chamber, and 1.1 mL high-glucose DMEM medium was added to the lower chamber. The transwell was incubated for 24 h and was fixed in methanol. After staining with 5 g/L hematoxylin, images were taken to count cell numbers in six randomly selected fields, with a microscope (magnification 200X; Olympus CX41, Japan). Experiments were carried out in triplicates.

**Total RNA extraction, reverse transcription, and real-time PCR**

After 48 h of transfection, total RNA was extracted from cells using RNA extraction kit (TaKaRa), following manufacturer protocol. After determining RNA concentration, *in vitro* reverse transcription was performed the kit (Promega, USA) containing 4 µL 25 mM MgCl$_2$, 2 µL 10X reverse transcription buffer, 2 µL 10 mM dNTP mixture, 0.5 µL ribonuclease inhibitor, 15 U AMV reverse transcriptase, 0.5 mg random primers, 1 mg total RNA, and nuclease-free water to a final volume of 20 µL, at 42°C for 15 min and 85°C for denaturation Real-time PCR was then performed using SYBR Premix Ex Taq GC kit (TaKaRa) with 7.5 µL SYBR Premix, 10 mM forward and reverse primers, and distilled water to a final volume of 15 µL. The following PCR conditions were used: denaturation at 94°C for 30 s, followed by 40 cycles of denaturation at 94°C for 5 s and annealing at 60°C for 30 s, with LightCycler 480 system (Roche Diagnostics, USA). Primer sequences and amplicon lengths are listed in Table 1. Glyceraldehyde 3-phosphate (GAPDH) was selected as internal reference. Relative gene expression was semi-quantitatively analyzed by the $2^{-\Delta\Delta Ct}$ method, where $2^{-\Delta\Delta Ct} = \frac{\text{gene copy number in the test group}}{\text{gene copy number in the control}}$. Experiments were carried out in triplicates.

**Table 1. PCR primer sequences and product lengths.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer position</th>
<th>Primer sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foxf2</td>
<td>Upstream</td>
<td>5'-TGCAATCCAGCATGTCCTCCTA-3'</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>5'-CGCTAGCTGAGGGATGGAAAG-3'</td>
</tr>
<tr>
<td>Zeb1</td>
<td>Upstream</td>
<td>5'-GCACAACCAAGTGCAGAAGA-3'</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>5'-CATTGCCAGATGGAGGAG-3'</td>
</tr>
<tr>
<td>E-cadherin (CDH1)</td>
<td>Upstream</td>
<td>5'-GGACGATGATGTGAACACC-3'</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>5'-TTGGCTGTGCCTAAACCC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Upstream</td>
<td>5'-AGGAAGCTGGGAGGCTATT-3'</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>5'-AGGGGCGATACGACTTCTC-3'</td>
</tr>
</tbody>
</table>

**Western blot analysis**

After transfection, $10^6$ cells were lysed in RIPA buffer (Thermo Fisher Scientific, USA) and were centrifuged at 12,000 g for 5 min. Supernatants were extracted to purify total proteins, which were quantified using the bicinchoninic acid assay. Protein samples were diluted to equal amounts and were separated by SDS-PAGE at 120 V for 2 h. The separated proteins were then transferred to a polyvinylidene membrane (Bio-rad, USA) at 15 V for 30 min and were blocked with 10% defatted milk powder at 37°C for 2 h. Rabbit anti-human Foxf2 polyclonal antibody (1:1000) or GAPDH (1:1000) at 20 µL final volume was incubated at 4°C overnight. After this, the membrane was rinsed three times (10 min each) with Tris-buffered...
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saline-Tween-20 at 25°C. Goat anti-rabbit secondary antibody, at 20 µL final volume, was then added for 1-hour incubation, followed by the enhanced chemiluminescence chromogenic substrate. Quantity One software (Bio-rad, USA) was employed to analyze the blotting bands, in which the ratio of intensity of target protein to that of GAPDH bands was calculated to reflect the relative protein level.

**Statistical analysis**

SPSS 17.0 software (IBM, USA) was used to process the data collected, which are reported as means ± SD. All data were analyzed by ANOVA. A statistical significance was defined at P < 0.05.

**RESULTS**

**MiR-200c inhibited the migration of breast cancer cells MDA-MB-231**

To evaluate the effect of miR-200c on the invasion and metastasis capacity of tumor cells, we implemented a scratch assay to test the cell movement. Typically, we found that cells showed a budding-pattern growth from the edge of the scratch to the center, in 0 to 24 h. Compared to the control and miR-NC groups, the migration of cells significantly decreased after transfection with miR-200c mimics (Figure 1A). The result also showed that the wounds closed (~75%) on miR-220c overexpression. In contrast, control or miR-NC cells showed ~55% wound closure (Figure 1B).

**Figure 1.** Analysis of breast cancer cell migration by scratch assay. **A.** Cell migration after 0 and 24 h (magnification 200X). **B.** Quantitative analysis of cell migration assayed in A. 1, Control group; 2, miR-NC group; and 3, miR-200c group. Data are reported as mean ± SD for N = 3. **P** < 0.01, compared to the control group.
Similarly, further in vitro invasion assay with transwell plate revealed a statistically small amount of migrated cells (~50%) in the miR-200c group, compared to those in the control or miR-NC group (~100%), suggesting that cell migration was inhibited after miR-200c transfection (Figure 2).

Figure 2. Analysis of MDA-MB-231 cell migration by transwell assay. Cell migration in control group (A), miR-NC group (B), and miR-200c group (C) (magnification 400X). D. Quantitative analysis of cell migration assayed in A, B, and C. Data are reported as means ± SD for N = 3. *P < 0.01, compared to the control group.

**MiR-200c altered the mRNA levels of Foxf2, Zeb1, and E-cadherin**

We detected the mRNA expressions of Foxf2, Zeb1, and E-cadherin, which play significant roles in tumor metastasis. Our real-time PCR data showed that mRNA levels of Fox2 and Zeb1 were significantly decreased, but mRNA level of E-cadherin in cells transfected with miR-200c was upregulated, compared to that in the control group (Figure 3). These results indicated that the overexpression of miR-200c suppressed the expression of Foxf2 and Zeb1, whereas it induced E-cadherin expression.

**MiR-200c downregulated the expression of Foxf2 protein**

To further assess the effect of miR-200c at protein level, we detected the expression of Foxf2 protein via western blotting. The result demonstrated that the protein level of Foxf2 statistically decreased to about 50% of its basal level in the miR-200c group, compared to
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that in the control or miR-NC group, being consistent with the data on mRNA level (P < 0.05; Figure 4).

**Figure 3.** Effect of miR-200c on mRNA levels of Foxf2, Zeb1, and E-cadherin. **P < 0.01, compared to the control group.** Foxf2, forkhead box f2; Zeb1, zinc finger E-box-binding homeobox 1; E-cadherin, epithelial cadherin.

**Figure 4.** Foxf2 protein expression level after miR-200c transfection. β-actin was used as a loading control. **P < 0.01, compared to the control group.** Foxf2, forkhead box f2.

**DISCUSSION**

The occurrence of breast cancer is regulated by various genetic, hormonal, and environmental factors, including age, height, alcohol consumption, use of estrogen-progesterone contraceptives, X-ray or gamma radiation, tobacco smoking, and shiftwork.
involving circadian disruption (Parkin et al., 2011). Generally, metastasis and invasion of tumor in the host involves multiple steps. For example, integrin, paracrine Wnt-β-catenin signaling plays a significant role in regulating breast cancer progression, and growth factor receptors, apoptosis markers, and signaling cascades change to various degrees, among which EMT is an important marker of the early stages of metastatic tumor (Allen et al., 2014). EMT is crucial for the establishment of multicellular organisms, including newly formed mesenchymal cells, which can exhibit locomotory and invasive phenotypes, suggesting that EMT contributes to the progression of carcinoma (Vincent-Salomon and Thiery, 2003). EMT was originally used to describe certain morphological changes occurring in epithelial cells during embryonic development (Ye et al., 2015). In the process of tumor invasion, metastasis, and progression, the de-differentiation and alteration of polymorphism is a typical feature (Wei et al., 2015). A fibroblast-like morphology in the later stages of the disease was progressively observed during EMT in squamous cell carcinoma of the skin (Oft et al., 2002). Data have revealed that EMT prolongs the survival time of cancer cells during movement from their primary site to distal metastasis site (Iwatsuki et al., 2010). During EMT, many tumor cells from the primary tumor lesion lose their epithelial polarity and cell-to-cell adhesion via the suppression of E-cadherin, zona occludens-1, occludin, claudin-1, and claudin-7, thus acquiring the ability to invade and migrate (McCatt Reed et al., 2016). In addition, recent studies suggest that a multitude of factors are involved in the regulation of EMT. Foxf2, which belongs to the forkhead transcription factor family, can stimulate EMT (Kundu et al., 2016). E-cadherin, a calcium-dependent transmembrane glycoprotein, was thought to be involved in oral carcinogenesis via Wnt pathway dysregulation and as an EMT marker (Chaw et al., 2012). Zeb1, which encodes a zinc finger and homeodomain transcription factor, is positively correlated to EMT (Díaz-López et al., 2015).

As endogenous non-coding small RNAs, miRNAs can modulate the expression of target proteins via degrading mRNA or inhibiting translation (Elgheznawy et al., 2015). Recent studies have revealed the important role of miR-200 family in maintaining the epithelial status. Alteration in miR-200 family transcription leads to change in expression of EMT-targeted modulatory factors Zeb1, Snail, and transcription-specific factor Sp1 (Díaz-López et al., 2015). Zeb family initiates protein expression via a negative feedback mechanism (Hilmarsdóttir et al., 2015). A study by Hur et al. (2013) revealed that one of the miR-200 family members, miR-200c, downregulates the expression of Zeb family and vimentin proteins and induces the expression of E-cadherin in human colorectal cancer metastasis. Similarly, our data on breast cancer demonstrated that the expression of Zeb1 decreased, whereas that of E-cadherin increased, in the miR-200c group. Additionally, we found that both the mRNA and protein levels of Foxf2 in MDA-MB-231 breast cancer cells were downregulated by miR-200c, which was consistent with a recent study on pulmonary cancer suggesting that Foxf2 was under the direct regulation of miR-200 (Kundu et al., 2016).

FOX proteins are a family of transcription factors that play critical roles in regulating cell growth, proliferation, differentiation, and longevity (Tuteja and Kaestner, 2007). Research shows the vital involvement of FOX family in cancer onset and progression, and miRNAs play a crucial regulatory role in tumors via the modulation of these FOX proteins (Zhang et al., 2015; Wang et al., 2016). Foxf2 gene belongs to the forkhead/winged helix transcription factor family and has been implicated as an oncogene that modulates metastasis of various tumors (Nilsson et al., 2010). Our preliminary data exhibited that migration of breast cancer cell MDA-MB-231 was repressed by miR-200c, and the expression of Foxf2 was downregulated
correspondingly. Coincidentally, recent studies have shown that other members of the Fox family, such as FoxM1 and FoxC1, can induce EMT, thus, activating tumor invasion and metastasis (Sarrió et al., 2008). Therefore, the overexpression of miR-200c impedes tumor progression via the inhibition of Fox-EMT cascade. Other studies have also indicated the role of Foxf2 in inducing EMT and tumor migration in pulmonary carcinoma, possibly by binding to Zeb1 and inhibiting E-cadherin expression at transcription level (Kundu et al., 2016). Our study also validated that in breast cancer cells MDA-MB-231, the transfection of miR-200c significantly reduced Zeb1, whereas it elevated E-cadherin expression. This indicated that the inhibition of Foxf2 expression by miR-200c, to eliminate its binding to Zeb1 and prevent degradation of E-cadherin, leads to the repression of EMT and retardation of tumor invasion. However, our study had some limitations. As another study contends that in human breast cancer samples, the loss of E-cadherin is not commonly observed in EMT and is not necessary (Hollestelle et al., 2013), the precise anti-tumor mechanism of miR-200c, in regulating the cascades involving Foxf2, Zeb1, and E-cadherin-EMT, requires to be further investigated.

In summary, this study further illustrated the critical role of miR-200 family in regulating EMT of breast cancer cells and revealed that miR-200c might affect Zeb1/E-cadherin expression to further eliminate EMT and inhibit tumor metastasis. This offers leads for the discovery of a precise effective target for molecular targeting therapy against breast cancer.

Conflicts of interest

The authors declare no conflict of interest.

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