microRNA-335 inhibits proliferation, cell-cycle progression, colony formation, and invasion via targeting PAX6 in breast cancer cells

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Abstract. microRNAs (miRNAs) have been demonstrated to play crucial roles in tumorigenesis. However, the molecular mechanism underlying the roles of miRNAs in breast cancer remains largely unknown. In this study, we showed that miR-335 is downregulated in a number of breast cancer tissues and cell lines. Luciferase reporter assays identified the paired box 6 gene (PAX6) as a novel target of miR-335. Further investigation revealed that miR-335 negatively regulates the expression of PAX6 in human breast cancer MCF-7 cells. Our results further suggested that overexpression of miR-335 inhibits MCF-7 cell proliferation by inducing cell-cycle arrest at the G1 phase via targeting PAX6. Western blot analysis showed that overexpression of miR-335 promotes p27 protein expression but inhibits cyclin D1 expression in MCF-7 cells; however, overexpression of PAX6 decreased the p27 protein level but increased the cyclin D1 protein level in MCF-7 cells. Furthermore, miR-335 overexpression reduced colony formation and cellular invasion in MCF-7 cells, an effect that was reversed by PAX6 overexpression. In conclusion, this study provides novel insights into the in vitro regulatory patterns of miRNA-335 and PAX6 in breast cancer, and indicates that miRNA-335 may constitute a promising candidate for the treatment of breast cancer.

Introduction

Breast cancer is the most frequently diagnosed cancer among women, accounting for 23% of the total cancer cases (1). Over the past two decades, the death rate due to breast cancer has decreased by <30% due to the improvement of therapeutic strategies. However, breast cancer remains the leading cause of cancer-related death in women worldwide (1,2). Furthermore, breast cancer accounted for 29% of all new cancer cases among women in 2013 (2). Therefore, in vitro investigations on the molecular mechanisms underlying breast cancer may provide important data for the development of therapeutic strategies for breast cancer.

miRNAs (miRNAs) are a type of endogenous non-coding RNAs. They act as gene expression suppressors via binding to 3’ untranslated region (3’ UTR) of their target mRNAs, and further leading to either translational repression or mRNA degradation (3). Recently, accumulating evidence has demonstrated that downregulation of miR-335 is involved in tumorigenesis. Xiong et al (4) showed that miR-335 is downregulated in prostate cancer tissues and cells, and that its low expression is significantly associated with a high Gleason score, advanced clinical stage, and metastasis in prostate cancer patients. Moreover, miR-335 also plays a role in the regulation of cancer progression. For instance, miR-335 was recently found to suppress osteosarcoma cell migration and invasion by targeting ROCK1 (5). In addition, miR-335 was reported to inhibit small cell lung cancer bone metastases (6). It has been also demonstrated that miR-335 acts as a tumor suppressor in breast cancer. Heyn et al (7) showed that overexpression of miR-335 leads to decreased cell viability and an increase in apoptosis in breast cancer cells. Moreover, miR-335 has been suggested to suppress breast cancer metastasis (8,9). However, the underlying molecular mechanism by which miR-335 affects these biological processes, especially cell-cycle progression, in breast cancer cells remains largely unknown.

Paired box 6 (PAX6), a highly conserved transcription factor, plays a crucial role in the development of the eyes, central nervous system, and pancreas (10,11). The role of PAX6 in malignant tumors has been elucidated in recent studies. PAX6 was demonstrated to act as an oncogene in...
various cancers, including breast cancer (12-14). Therefore, PAX6 may constitute a promising therapeutic target for breast cancer. However, whether miR-335 regulates breast cancer by targeting the PAX6 gene has not been studied to date.

The present study mainly aimed to investigate the molecular mechanism by which miR-335 regulates breast cancer in vitro. Our findings suggest that miR-335 inhibits cellular proliferation, cell-cycle progression, colony formation and invasion, via directly targeting PAX6 in breast cancer cells.

Materials and methods

Tissue collection. All protocols in this study were approved by the Ethics Committees of the Guangxi Medical University and the People's Hospital of Guangxi Zhuang Autonomous Region. Each patient in this study signed an informed consent. The breast cancer and the adjacent healthy tissues were collected from 24 patients from the Department of General Surgery, the First Affiliated Hospital of Guangxi Medical University, and the Department of Hepatobiliary and Endocrine Surgery, the People's Hospital of Guangxi Zhuang Autonomous Region, between March 2012 and September 2012. All samples were stored in liquid nitrogen until further use.

Cell culture. Three human breast cancer cell lines, MCF-7, Bcap-37 and CWR22-RV1, and the mammary epithelial cell line MCF-10A were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Life Technologies, Grand Island, NY, USA) at 37°C in a humidified incubator containing 5% CO₂.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues or cells using the Invitrogen™ TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The RevertAid First Strand cDNA Synthesis kit (#K1621; Thermo Fisher Scientific) was used to reverse transcribe the RNA into cDNA, which was further used as the PCR template. Expression of the PAX6 mRNA was detected using a SYBR-Green qPCR assay kit (Bio-Rad, Hercules, CA, USA). The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as an endogenous control. The specific primers for amplification were the following: PAX6 forward (F), 5'-AAGGATAAATCCGAGAAGCTACGCTGTTT-3'; reverse (R), 5'-GGTCTGCGTTCAACAGACCATGCTCCC-3'; GAPDH F, 5'-ACAACTTTTCAAACTGAGTCTGAC-3', and reverse (R), 5'-GCCATCTACGCGCACTTGTTCT-3'. The cycling conditions were as follows: 95°C for 15 sec, then 40 cycles at the following conditions: 94°C for 15 sec, 60°C for 30 sec, then 72°C for 30 sec, then 72°C for 5 min. The PCR product was ligated into a psiCHECK™-2 vector (Promega, Madison, WI, USA) downstream of the luciferase gene sequence. A psiCHECK-2 construct containing the mutant 3' UTR of PAX6, that contains mutant binding sites to miR-335, was synthesized using the Mut-PAX6 primers (FulenGen Co., Ltd., Guangzhou, China). MCF-7 cells were plated in 96-well plates, and the wild-type (Wt)-PAX6-3' UTR-psiCHECK-2 or the Mut-PAX6-3' UTR-psiCHECK-2 vector was co-transfected with the miR-335 mimic, the miR-335 inhibitor (FulenGen Co., Ltd.), the negative control (NC) miRNA mimic, or the NC miRNA inhibitor. Untransfected cells were used as the control.

Luciferase reporter assays. A fragment of the 3' UTR of PAX6 containing the putative miR-335 binding site was amplified by PCR using the Takara LA PCR Amplification kit (Takara Biotechnology Co., Ltd., Dalian, China) and the following primers: F, ATACGGGCTTTCAACAGACCATGCTCCC, and R, GCACTAGTGTTTCACATGCGACGTAG.

Cell proliferation assay. A cell proliferation assay was performed to determine the effects of miR-335 and PAX6 on MCF-7 cell proliferation. Five thousand cells in each group were plated into a 96-well plate. Following incubation for 12, 24, 48 and 72 h, 20 µl of MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) were added to each well. The cells were then incubated at 37°C for 4 h, the reaction was terminated by adding 150 µl of dimethyl sulfoxide, and the cells were left for 10 min at room temperature. Formazan production was detected using the Nuclear-Cytosol Extraction kit (Applygen Technologies, Inc., Beijing, China), separated by 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked in 5% nonfat dried milk in phosphate-buffered saline (PBS) with Tween-20 for 4 h, and next incubated overnight at 4°C with mouse anti-PAX6, -p27, -cyclin D1 or -GAPDH primary antibodies (Abcam, Cambridge, UK). Following incubation with the rabbit anti-mouse secondary antibody (Abcam), the proteins were visualized using an enhanced chemiluminescence (ECL) substrate (Millipore, Billerica, MA, USA). GAPDH was used as an endogenous control for normalization.

Cell-cycle progression assay. For each group, 10⁶ cells were collected in 1X PBS, resuspended in 70% ethanol, and allowed to
fix overnight at -20°C. Cells were pelleted at 1,000 x g for 5 min, washed in 1X PBS, and then pelleted at 1,000 x g for 5 min. Following resuspension in 300 µl of propidium iodide staining buffer, the cells were incubated for 30 min at room temperature, and the DNA content was analyzed using a FACSCalibur flow cytometer (Beckman Coulter, Brea, CA, USA).

**Colony formation assay.** For each group, 4 ml of complete medium (DMEM + 10% FBS) containing 200 cells were added to a 60-mm dish. Following a 14-day cell culture at 37°C with 5% CO₂, the supernatant was discarded, and cells were washed with PBS 3 times. Cells were then fixed with 4% paraformaldehyde for 15 min, and stained with Giemsa (Solarbio Science & Technology, Co., Ltd., Beijing, China) for 20 min. Colonies were counted under an inverted microscope (Nikon, Tokyo, Japan). This assay was repeated 3 times.

**Cell invasion assay.** For the cell invasion assay, 24-well Transwell chambers (Chemicon International, Temecula, CA, USA) with a layer of matrix gel were used. For each group, a cell suspension (5x10⁵ cells/ml) was prepared in serum-free DMEM, and 500 µl of DMEM with 10% FBS were added into the lower chamber, while 300 µl of the cell suspension were added into the upper chamber. Following incubation at 37°C with 5% CO₂ for 24 h, the non-invading cells and the matrix gel were removed, and cells that had invaded through the membrane were stained for 20 min with 0.1% of crystal violet (Nlunbio, Changsha, China). The cells were next rinsed with water, and dried in the air. The number of stained cells was counted in six random fields under an inverted microscope (ECLIPSE TE2000-S; Nikon). This assay was repeated 3 times.

**Statistical analysis.** Statistical analysis was performed using the statistical software SPSS 19.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as mean values ± standard deviation (SD) of triplicate experiments, and all experiments were repeated at least 3 times. The data were analyzed by one-way analysis of variance (ANOVA) and Student’s t-tests. P-values <0.05 were considered to indicate statistically significant differences.

**Results**

**miR-335 is downregulated in breast cancer tissues and cell lines.** We first examined the expression of miR-335 in breast cancer and adjacent tissues using RT-qPCR. As shown in Fig. 1A, the expression level of miR-335 in breast cancer tissues was markedly reduced compared to adjacent healthy (normal) tissues. We further examined the expression of miR-335 in three breast cancer cell lines, MCF-7, Bcap-37 and CWR22-RV1. The normal human mammary epithelial cell line MCF-10A was used as a control. miR-335 expression was significantly reduced in breast cancer cell lines compared to MCF-10A cells (Fig. 1B). These findings suggest that miR-335 may be involved in breast cancer.

**miR-335 negatively regulates PAX6 by binding to its 3’ UTR in MCF-7 cells.** As predicted by the online software miRWalk (15), the PAX6 gene is a putative target of miR-335. To determine whether PAX6 is a direct target of miR-335, a luciferase reporter assay was further performed. In MCF-7 cells co-transfected with miR-335 and the 3’ UTR of PAX6, the Renilla/Firefly value was significantly reduced, when compared to the control group (P<0.01), and this effect was reversed by the miR-335 inhibitor (Fig. 2A). However, in MCF-7 cells co-transfected with miR-335 and the mutant 3’ UTR of PAX6, the Renilla/Firefly value did not decrease, indicating that miR-335 can not bind to the mutant 3’ UTR of PAX6. In addition, the NC miRNA and the NC inhibitor also had no effect on luciferase activity. Based on these findings, we suggest that PAX6 is a novel target of miR-335.
Since miRNAs generally inhibit the expression of their target genes at the post-transcriptional level, we next investigated the effects of miR-335 over- or underexpression at the PAX6 protein level. Following transfection of MCF-7 cells with the miR-335 mimic or inhibitor, we first determined the efficiency of transfection, which was satisfactory (Fig. 2B). Next, western blot analysis was performed to examine the protein level of PAX6 in each group. The protein level of PAX6 was reduced when miR-335 was overexpressed, but increased when miR-335 expression was inhibited, suggesting that miR-335 negatively regulates the protein expression of PAX6 (Fig. 2C).

Overexpression of miR-335 inhibits MCF-7 cell proliferation by targeting PAX6. Cell proliferation was examined in breast cancer MCF-7 cells following transfection with the miR-335 mimic or the miR-335 inhibitor, or co-transfection with the miR-335 inhibitor and the PAX6 small interfering RNA (siRNA). Control, non-transfected MCF-7 cells.
miR-335 upregulation induces cell-cycle arrest at the G1 phase via PAX6 targeting in MCF-7 cells. Cell-cycle distribution was examined following transfection of MCF-7 cells with the miR-335 mimic or inhibitor, or co-transfection with the miR-335 inhibitor and the PAX6 small interfering RNA (siRNA). miR-335 overexpression induced cell-cycle arrest at the G1 phase; however, inhibition of miR-335 expression significantly promoted cell-cycle progression, an effect that was reversed by PAX6 silencing (Fig. 4). These data indicate that miR-335 inhibits cell-cycle progression of human cancer MCF-7 cells by inducing cell-cycle arrest at the G1 phase via PAX6.

We further investigated the underlying molecular mechanism, and found that miR-335 overexpression upregulates the protein p27, while it downregulates cyclin D1. However, PAX6 overexpression decreased the protein level of p27 but increased the protein level of cyclin D1. These findings suggest that miR-335 can induce cell-cycle arrest at the G1 phase at least partially via targeting PAX6, which is in accordance with the upregulation of p27 and the downregulation of cyclin D1.

Overexpression of miR-335 reduces the ability of MCF-7 cells to form colonies by inhibiting PAX6. We further examined the roles of miR-335 and PAX6 in the regulation of colony formation in breast cancer MCF-7 cells. Overexpression of miR-335 significantly inhibited colony formation in MCF-7 cells (Fig. 5). However, the inhibitory effect of miR-335 on MCF-7 cell colony formation was markedly attenuated by overexpression of PAX6. These findings suggest that the PAX6 protein may be involved in the miR-335-induced inhibition of colony formation in breast cancer MCF-7 cells.

miR-335 overexpression reduces MCF-7 cell invasion by inhibiting PAX6. Since tumor cell invasion is a key index of tumor malignancy, we further determined the effects of miR-335 and PAX6 on MCF-7 cell invasion by performing a Transwell assay. Overexpression of miR-335 significantly reduced MCF-7 cell invasion (Fig. 6). However, the inhibitory effect of miR-335 on MCF-7 cell invasion was markedly attenuated by PAX6 overexpression. These data suggest that miR-335 inhibits MCF-7 cell invasion at least partially via targeting PAX6.

Discussion

Recently, accumulating evidence has suggested that miRNAs play a vital role in human cancer via directly targeting oncogenes or tumor suppressors (16). Among miRNAs, miR-335...
MENG et al: miR-335 INHIBITS BREAST CANCER in vitro VIA TARGETING PAX6

miR-335 has been demonstrated to be involved in various human malignancies, including ovarian, small cell lung, prostate and gastric cancer, meningioma, osteosarcoma, and hepatocellular carcinoma (4-6,17-22). Furthermore, miR-335 was shown to suppress cell viability and induce cell apoptosis in breast cancer cells (7), while it may also play a role in breast cancer metastasis (8,9). However, the effect of miR-335 on breast cancer cell proliferation, as well as the underlying molecular mechanism remain largely unknown. In this study, we showed that miR-335 can inhibit cellular proliferation by inducing cell-cycle arrest at the G1 phase in breast cancer cells.

To further investigate the molecular mechanism by which miR-335 exerts its effects on breast cancer cells, we focused on its target genes, and identified, for the first time to the best of our knowledge, PAX6 as a novel target of miR-335. PAX6, a member of the PAX gene family, encodes a transcription factor highly conserved in both flies and mammals, which has been demonstrated to play a role in the development of the eyes, central nervous system, and pancreas (11,23,24). More recently, PAX6 was shown to act as a key regulator in various cancers, including breast cancer. Zong et al (14) suggested a potential role of PAX6 in promoting breast cancer in vitro and in vivo. They found that knockdown of PAX6 leads to decreased cell viability, DNA synthesis and colony formation, as well as significantly reduced tumorigenesis in xenograft nude mice. In the present study, we found that miR-335 negatively regulates PAX6 protein expression by directly binding to the 3' UTR of the PAX6 gene in breast cancer cells. Moreover, we showed that miR-335 inhibits breast cancer cell proliferation and induces cell-cycle arrest by targeting PAX6. PAX6 has been shown to be regulated
by additional miRNAs in cancer. For instance, Huang et al (25) showed that PAX6 is involved in miR-223-mediated glioblastoma cell growth and invasion, while Wang et al (12) reported that miR-365b-3p regulates cell-cycle progression and apoptosis in human retinoblastoma cells by targeting PAX6.

Several molecules have been shown to play vital roles in the regulation of cell-cycle progression, including p27 and cyclin D1. p27, a key protein for the G0/G1 checkpoint, can block the transition from the G1 to the S phase. Cyclin D1 is essential for the progression through the cell cycle, and inhibition of cyclin D1 can lead to cell-cycle arrest at the G1 phase. Accordingly, we further investigated the underlying molecular mechanism by which miR-335 regulates cell-cycle progression in breast cancer cells, and found that overexpression of miR-335 causes an increase in p27 and a decrease in cyclin D1 protein levels. These changes in response to miR-335 can explain the cell-cycle arrest observed upon overexpression of miR-335. The PAX6 protein has also been demonstrated to participate in the regulation of cell-cycle progression. Wang et al (12) reported that PAX6 is involved in the miR-365b-3p-induced cell-cycle arrest via affecting the protein expression of p21, p27, Cdc2 and cyclin D1. Moreover, Zong et al (14) showed that knockdown of PAX6 induces cell-cycle arrest at the G0/G1 phase in breast cancer cells. Here, we showed that the effects of miR-335 overexpression on p27 and cyclin D1 are abolished by restoring PAX6 expression, which further supports the hypothesis that miR-335 regulates cell-cycle progression via targeting PAX6.

PAX6 silencing was shown to significantly inhibit colony formation in breast cancer cells (14). Since we demonstrated that miR-335 negatively regulates the protein expression of PAX6, we hypothesized that miR-335 may have an inhibitory effect on colony formation in MCF-7 cells. The colony formation assay confirmed that overexpression of miR-335 inhibits colony formation at least partially via directly targeting PAX6 in MCF-7 cells. The effect of miR-335 on colony formation has been reported in several types of cancer. For instance, Gong et al (6) reported that overexpression of miR-335 suppresses colony formation in small cell lung cancer SBC-5 cells. Martin et al (26) reported reduced colony survival following irradiation in HeLa cells overexpressing miR-335.

Furthermore, miR-335 has been associated with cancer cell invasion in several types of cancer. Wang et al (27) found that miR-335 overexpression significantly inhibits cellular invasion in non-small cell lung cancer A549 and H1299 cells. Xu et al (28) found that overexpression of miR-335 suppresses gastric cancer cell invasion. Moreover, miR-35 was suggested to act as a suppressor of breast cancer metastasis (8). In this study, miR-335 overexpression significantly reduced MCF-7 cell invasion, an effect that was attenuated by overexpression of PAX6. Our findings suggest that miR-335 inhibits breast cancer cell invasion by directly downregulating PAX6.

In conclusion, this study provides novel insights into the molecular mechanism by which miRNA-335 and PAX6 regulate cellular proliferation, cell-cycle progression, colony formation and cellular invasion in breast cancer in vitro, and suggests that miRNA-335 may constitute a promising candidate for the treatment of breast cancer.

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