miR-7-5p suppresses cell proliferation and induces apoptosis of breast cancer cells mainly by targeting REGγ

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Original Articles

Proteasome activator subunit 3 (REGγ) has a key role in breast cancer by promoting protein proteolysis, but methods to block REGγ expression remain elusive. In this study, we found that the expression of REGγ is significantly upregulated in breast cancer, and that the knockdown of REGγ expression suppresses cell proliferation and induces apoptosis in vitro. Furthermore, REGγ was identified as a direct downstream target of miR-7-5p, and there was an inverse correlation between the expression of REGγ and miR-7-5p. The overexpression of miR-7-5p inhibited cell proliferation and induced apoptosis by mainly targeting REGγ in vitro and in vivo. Our data indicate that miR-7-5p has a critical function through blocking REGγ in breast cancer cells.

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Materials and methods

Clinical tissue samples

Primary breast cancer tissues and matched adjacent non-cancerous tissues were obtained from the Department of Endocrine Surgery in the First Affiliated Hospital of Chongqing Medical University, P.R. China, from September 2012 to April 2013. Tissues were obtained from the routine therapeutic surgery of patients who did not receive any anti-tumor treatment and all specimens were confirmed by pathology. Written informed consent was obtained from each patient before the operation and this research was approved through the Institute Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Cell culture and transfection

A panel of eight human breast cancer cell lines was used: BT549, MDA-MB-231, MDA-MB-468, MCF7, SK-BR-3, T47D, HBL100, and MCF-10A. Cells were cultured in RPMI 1640 (Gibco-BRL, Karlsruhe, Germany), supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL) and 100 U/ml of penicillin and streptomycin (Gibco-BRL), and maintained at 37 °C with 5% CO2. All transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions to analyze the incorporation of EdU during DNA synthesis. pcDNA3.1 have been reported previously [3,11] pcDNA3.1-REGγ and its negative control pcDNA3.1 have been reported previously [3,11]. pG2V68-mir-7-5p and pgV268 were purchased from GeneChem (Shanghai, China). The constructs were transfected into cells at a concentration of 4 μg, and selected by neomycin at 48 h after transfection.

Reverse transcription, semi-quantitative PCR and quantitative PCR

Total RNA was extracted from tissue samples and cell lines using TRIzol reagent (Invitrogen). The reverse transcription procedure and semi-quantitative RT-PCR of REGγ (β-actin as a control) using Go-Taq (Promega, Madison, WI, USA) was performed as previously described [12]. Quantitative RT-PCR was performed through the SYBR green assay (Invitrogen) with the Applied Biosystems 7500. U6 and β-actin were used as controls for mir-7-5p and REGγ, respectively. The reverse transcription primers and quantitative PCR primers of mir-7-5p and U6 were purchased from RIBOBIO (Guangzhou, China). mir-7-5p and REGγ relative expression was evaluated by using the 2−ΔΔCt method. All PCR assays were performed in triplicate.

Cell proliferation assay

MDA-MB-231 and MCF7 cells were cultured at a density of 20,000 cells/well in six-well plates and transfected after 24 hours of culture. At 24 h, 48 h and 72 h after transfection, cell proliferation was measured using the Cell Counting Kit (CCK-8) (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer’s instructions. The data were obtained from three independent cultures and experiments were repeated three times.

EdU assay

Cell proliferation was measured using EdU kits (RIBOBIO) according to the manufacturer’s instructions to analyze the incorporation of EdU during DNA synthesis. Assays were performed in triplicate.

Soft agar assay

The soft agar assay was performed, as previously described, to evaluate anchorage-independent growth [13]. Briefly, 0.7% soft agar was placed into a six-well plate until the lower 0.7% soft agar solidification. Ten thousand transfected cells were plated in complete medium at a concentration of 0.35% soft agar and the cells were cultured for 2–3 weeks. The data were obtained from three independent assays.

Flow cytometry analysis

Flow cytometry was used as previously described to analyze cell cycle distribution and apoptosis [14]. Briefly, transfected cells after 48 hours were trypsinized and washed with PBS twice, fixed with ice-cold 75% ethanol, and then stained with propidium iodide (PI) or PBS and annexin V FITC/PI double-staining to analyze cell cycle and apoptosis, respectively. All data were evaluated using Cell Quest (BD Biosciences, San Jose, CA) in triplicate.

Immunoblotting

Total cell lysate, extracted by RIPA lysate buffer (Beyotime Institute of Biotechnology), was degenerated in a 5× sodium dodecyl sulfate (SDS) buffer, and the concentration determined using the bicinchoninic acid (BCA) standard curve. Approximately 40 μg of protein was separated using 12% SDS–polyacrylamide gel electrophoresis, then transferred onto polyvinylidene fluoride (PVDF) membranes.

The membranes were incubated overnight with primary antibodies against REGγ, p21, p27, cleaved-caspase 3, GAPDH and β-actin (1:1000 dilution, Cell Signaling Technology), incubated with relevant secondary antibodies (1:5000 dilution, Cell Signaling Technology), and then visualized using ECL (Invitrogen). All experiments were performed in triplicate. The intensity of the bands was measured using Image Pro Plus (IPP, version 6.0, Media Cybernatics, Silver Spring, MD, USA).

Luciferase reporter assay

REGγ 3′UTR wild-type (WT) and mutant (Mut) were synthesized and cloned into the Sptel/HindIII site of pMIR-REPORT vector, and the resulting constructs designated pMIR-REPORT-REGγ-3′UTR-WT and pMIR-REPORT-REGγ-3′UTR-Mut, respectively. HER-293T cells were transfected using Lipofectamine 2000 reagent with pMIR-report-REGγ-3′UTR-WT or pMIR-report-REGγ-3′UTR-Mut, mir-7-5p mimics or control, and a renilla luciferase pRL-TK vector. Luminescence was detected 48 h later using the Dual-Luciferase Reporter assay system (Promega) following the manufacturer’s instructions. Results were normalized to the Renilla luminescence from the same vector. All assays were performed in triplicate.

In vivo tumor model

Stable cells overexpressing mir-7-5p, and control cells, were subcutaneously injected into nude mice (n = 6/6). The tumor volume was measured once per week, and the tumor weight measured after animal sacrifice. The protocol for the in vivo tumor model was approved through the Institute Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Immunohistochemistry

The two-step immunohistochemistry process was performed as previously described [15]. Antibodies against Ki-67 and REGγ were used (1:50 dilution). All photographs were measured using Image Pro Plus (IPP, version 6.0, Media Cybernatics, Silver Spring, MD, USA).

TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining kit (Roche CA) was used to analyze the cell apoptosis, by following the manufacturer’s instructions.

Statistical analysis

SPSS 17.0 software was used for statistical analysis. Data were represented as mean ± standard deviation (SD). The correlation between the expression of REGγ and mir-7-5p was analyzed by Pearson’s correlation. The two-tailed Student t-test was used to determine the p-value, and a p < 0.05 was considered significant.

Results

REGγ is dysregulated in breast cancer cell lines and breast cancer

We previously demonstrated that REGγ is significantly upregulated in primary breast cancer tissues, especially in the metastatic lymph gland [3]. In this study, we first detected REGγ mRNA levels by RT-PCR in a panel of breast cancer cell lines. The expression of REGγ in tumorigenic BrCa cell lines (BT549, MDA-MB-231, MDA-MB-468, MCF7, SK-BR-3, T47D) was greatly upregulated compared with non-tumorigenic breast cancer cell lines (HBL100 and MCF-10A) (Fig. 1A). In addition, the protein levels of REGγ in tumorigenic BrCa cell lines were investigated by immunoblotting. Notably, BT549, MDA-MB-231, MDA-MB-468 are triple-negative (ER-negative, PR-negative, HER2-negative) BrCa cell lines, MCF7 and T47D are luminal BrCa cell lines, and SK-BR-3 is a HER2-enriched BrCa cell line. Interestingly, MDA-MB-231, a triple-negative BrCa cell line, displayed the highest level of REGγ (Fig. 1A).

Further, the protein level of REGγ was examined in paired BrCa tissues from four cases by immunoblotting and immunohistochemistry. As shown in Fig. 1B, the protein level of REGγ significantly increased in BrCa tissues relative to matched adjacent tissues. Furthermore, the expression of REGγ was evaluated by searching the Oncomine open cancer microarray database (https://www.oncomine.org/). Results showed that the expression of REGγ was significantly increased in DC (ductal carcinoma) in situ, IDC (invasive ductal carcinoma), and ILC (invasive lobule carcinoma).
compared with normal breast tissue without gene amplification (Fig. 1C).

From the above, we speculate that REGγ dysregulation in primary breast cancers may promote breast tumorigenesis.

**REGγ knockdown inhibits cell proliferation and induces apoptosis**

To validate the hypothesis above, shREGγ was used to knock down the expression of REGγ and evaluate its function in the MDA-MB-231 cell line. Quantitative RT-PCR and semi-quantitative RT-PCR were used to determine the expression of REGγ. CCK-8 assay was used to evaluate proliferative ability. Flow cytometry was used to measure cell cycle distribution and apoptosis. The expression of REGγ was downregulated by shREGγ transfection (Fig. 2A). Further, REGγ knockdown suppressed cell viability (Fig. 2B). In addition, REGγ knockdown induced G0/G1 phase arrest by accumulating p21, but not p27, and apoptosis by activating caspase3 cleavage (Fig. 2C-E). These data suggest that therapeutic REGγ knockdown may favor the prognosis of breast cancer patients.

**REGγ is a direct downstream target of miR-7-5p**

A previous study showed that glucosamine induces tumor cell growth inhibition by downregulating REGγ [16]. Therefore, we explored how REGγ blockage may specifically provide a novel approach for breast cancer treatment. Overall, miRNAs play a vital role in cancers as negative post-transcriptional regulators, so we performed bioinformatic analyses to search for miRNAs that target REGγ. At least three prediction algorithms (Targetscan, PicTar, microwalk) were used to identify its upstream miRNAs. We found that miR-7-5p can bind the 3′UTR of REGγ at the base from 1413 to 1419 (Fig. 3A).
Fig. 2. REGγ regulates cell proliferation and apoptosis in vitro. (A and B) Cells were transfected with shN.C. and shREGγ, and 48h later, the expression of REGγ was determined by quantitative RT-PCR and semi-quantitative RT-PCR (A); cell proliferation was evaluated by CCK-8 assay at 24h, 48h and 72h (B). Mean ± SD, n = 3, **p < 0.01. (C–E) The effect of REGγ on cell cycle (C), apoptosis (D) and associated molecular change (E) were determined by flow cytometry analysis and immunoblot, respectively. Mean ± SD, n = 3, **p < 0.01.
Fig. 3. REGγ is a direct downstream target gene of miR-7-5p. (A) A schematic graph representation of REGγ mRNA 3′UTR sites targeted by miR-7-5p. (B) The expression of miR-7-5p in a panel of tumorigenic breast cancer cell lines. Mean ± SD, n = 3, **p < 0.01. (C) The activity of the reporter gene was measured by dual-luciferase reporter assay, and the firefly luciferase activity was normalized to renilla luciferase activity; the 3′UTRm of REGγ contains three base mutations in the miR-7-5p binding site. Mean ± SD, n = 3, **p < 0.01. (D) Cells were transfected with N.C. mimics or miR-7-5p mimics, and 48 h later, the expression of REGγ determined by quantitative RT-PCR and immunoblot, U6 and β-actin were used as controls. Mean ± SD, n = 3, **p < 0.01. (E) Cells were transfected with N.C. mimics or miR-7-5p mimics, N.C. inhibitor or miR-7-5p inhibitor in MDA-MB-231 cells; N.C. inhibitor or miR-7-5p inhibitor in T47D cells. And 48 h later, the protein level of REGγ was measured by immunoblot (β-actin as a control). Mean ± SD, n = 3, **p < 0.01.
miR-7-5p suppress cell proliferation and induce apoptosis mainly by targeting REGγ in vitro

To illustrate the function of miR-7-5p through targeting REGγ in breast cancers, miR-7-5p was overexpressed in MDA-MB-231 and MCF7 cell lines by transfecting miR-7-5p mimics, followed by quantitative RT-PCR (Fig. 4A). Further, cell proliferation was measured by the CCK-8 (Fig. 4B) and EdU assay (Fig. 4C), and miR-7-5p significantly suppressed cell proliferation in MDA-MB-231 and MCF7 cell lines. Moreover, relative to the N.C. group, colony formation was inhibited by miR-7-5p in MDA-MB-231 cells in soft agar assay (Fig. 4D). In addition, flow cytometry indicated similar cell cycle distribution and apoptosis in cells with knockdown of REGγ through induction of G0/G1 phase arrest and accumulation of p21. Additionally, p27 was upregulated, and apoptosis induced via activating caspase3 cleavage (Fig. 4E and F).

miR-7-5p inhibitors were further used in T47D cells for validating the function of miR-7-5p. The results showed that miR-7-5p is downregulated (Fig. 4G) by specific inhibitors, along with cell viability increase (Fig. 4H) and cell cycle progression (Fig. 4I). Finally, we co-transfected miR-7-5p mimics and the REGγ plasmid to determine whether overexpression of REGγ blocks the function of miR-7-5p. However, REGγ can reverse but not completely inhibit miR-7-5p function (Fig. 5A and B). The data collectively indicated that miR-7-5p inhibits cell proliferation and induces apoptosis mainly by targeting REGγ, and that miR-7-5p may act as a “tumor suppressor” in breast cancers.

miR-7-5p inhibits breast cancer progression in vivo

Further, to validate the function of miR-7-5p and the interaction between miR-7-5p and REGγ, the tumor-bearing nude mice model was constructed using MDA-MB-231 cells. Relative to the N.C. group, the tumor volume and tumor weight were inhibited in groups with miR-7-5p (Fig. 6A). Moreover, proliferation-related Ki-67 and REGγ were suppressed and apoptosis induced upon miR-7-5p overexpression (Fig. 6B). Thus, these data indicate that miR-7-5p inhibits cell proliferation and induces apoptosis by targeting REGγ in vivo.

miR-7-5p and REGγ mRNA levels are negatively correlated in primary breast cancers

It has been demonstrated that miR-7-5p can directly bind the REGγ seed sequence, and the expression of REGγ was suppressed by gain function of miR-7-5p. We speculate there may be an inverse correlation between miR-7-5p and REGγ expression in breast cancers. Accordingly, we initially extracted data on expression of miR-7 and REGγ from the “Curtis breast” microarray in the Oncomine database. Pearson’s correlation analysis confirmed a negative correlation between miR-7-5p and REGγ (Fig. 7A). Furthermore, the mRNA levels of miR-7-5p and REGγ were measured in 20 cases of primary breast cancer tissues and matched adjacent non-cancerous tissues by quantitative RT-PCR. Relative to the adjacent tissues, the expression of miR-7-5p was significantly downregulated (Fig. 7B) and the expression of REGγ significantly upregulated (Fig. 7C). There was an inverse relationship between miR-7-5p and REGγ levels in primary breast cancer tissues by Pearson’s correlation analysis (Fig. 7D).

Taken together, these results strongly indicate a role of miR-7-5p in the negative regulation of REGγ expression, thereby acting as a tumor suppressor in breast cancer cells.

Discussion

Proteasome activator subunit 3 (REGγ), a member of the REG family, acts as an oncogene depending on the proteolysis of target molecules such as p21 and p53 [17,18]. The exact physiological effect of REGγ is unclear, but accumulated data have shown the proteolysis of target proteins in a REGγ-dependent manner through activating the 20S proteasome and ATP or in a ubiquitin-independent manner [18,19]. The expression of REGγ is located in the nucleus, and upregulated in multiple cancers, including colorectal, thyroid, and breast cancers. Particularly, our previous data indicated a positive correlation between the high expression of REGγ and the stage of breast cancers and lymph node metastasis [3]. In colon cancers, upregulated levels of REGγ in the serum can act as a biomarker [16]. Our previous study also suggested that REGγ knockdown could suppress cancer cell proliferation, induce apoptosis, and inhibit migration and invasion [4,11]. Moreover, it has been demonstrated that glucosamine can suppress cancer cell proliferation by downregulating REGγ. In this study, REGγ knockdown suppressed cell viability, inhibited colony formation, induced G0/G1 phase arrest and apoptosis. Hence, we inferred that the knockdown of REGγ could provide new options for breast cancer therapy.

miRNAs are a family of regulators that act as tumor promoters or tumor suppressors depending on downstream target genes. For example, in breast cancers, miR-21 may induce tumor cell migration and invasion by targeting the tumor suppressor tropomyosin 1 (TPM1) [20]. In contrast, miR-145 suppresses tumor cell proliferation by targeting the oncogene c-myc [21]. Comprehensive research data have identified a series of miRNAs in breast cancers, including onco-miRNAs (e.g., miR-10 family, miR-21, miR-17-19 cluster) and tumor suppressor miRNAs (e.g., let-7 family, miR-200 family, miR-205, miR-145) [22]. In this study, our data indicate that REGγ is the main downstream target gene of miR-7-5p, and that miR-7-5p can bind to its 3′UTR and subsequently regulate its expression post-transcriptionally. Re-expression REGγ upon miR-7-5p overexpression could reverse but not completely block the effect of miR-7-5p. Thus, miR-7-5p additionally suppresses cell proliferation via interactions with other target genes, although miR-7-5p suppresses cell proliferation and induces G0/G1 cell cycle arrest and apoptosis by mainly targeting REGγ.

Accumulated studies have demonstrated that miR-7-5p, downregulated in multiple cancers, exerts outstanding antitumor effects in multiple cancers, except renal cancer. miR-7-5p inhibits cell proliferation and metastasis by targeting PI3K/Akt in hepatocellular carcinoma and glioblastoma [6,23]. Moreover, epidermal growth factor receptor (EGFR) also acts as a downstream target of miR-7-5p in multiple cancers, including gastric cancer and glioblastoma [24,25]. In breast cancer cells, miR-7-5p inhibits cell metastasis by targeting focal adhesion kinase (FAK) or...
miR-7-5p inhibits breast cancer progression in vitro. (A–C) Cells were transfected with N.C. mimics and miR-7-5p mimics, respectively, and 48 h later, the expression of miR-7-5p was determined using quantitative RT-PCR (A); cell proliferation was evaluated by the CCK-8 assay at 24 h, 48 h, and 72 h (B) and EdU assay (C). Mean ± SD, n = 3, *p < 0.05, **p < 0.01. (D) The effect of miR-7-5p on colony formation was evaluated by the soft agar assay. Mean ± SD, n = 3, **p < 0.01. (E and F) The effects of miR-7-5p on cell cycle and apoptosis (E) and the associated molecular changes (F) were determined by flow cytometry analysis and immunoblot, respectively. Mean ± SD, n = 3, **p < 0.01. (G–I) Cells were transfected with N.C. and miR-7-5p inhibitor, respectively, and 48 h later, the expression of miR-7-5p was measured using quantitative RT-PCR (G); cell viability was determined by the CCK-8 assay (H) and cell cycle was evaluated by flow cytometry (I).
Kruppel-like factor 4 (KLF4) [7,8]. Recently, it has been demonstrated that miR-7-5p suppresses cell proliferation by targeting REGγ in lung cancer [9]. Further, miR-7-5p induced cell cycle arrest and apoptosis in multiple cancers, including colorectal, hepatocellular, cervical and breast carcinomas [26–29]. However, in renal cancer, miR-7-5p was identified as an oncogene, although its target genes remain to be identified. The expression of miR-7-5p is downregulated in primary breast cancers, but the detailed mechanisms for this downregulation are still unclear. Thus, we can conclude that miR-7-5p may act as a tumor suppressor in breast cancers.

Moreover, p21 is a central element in regulating the G0/G1–M phase transition [30]. In this study, we found that the miR-7-5p–REGγ–p21 axis plays a vital role in suppressing cell proliferation by inducing G0/G1 phase arrest. In this study, p27, also known to be regulated by miR-7-5p, was not observed to change with REGγ knockdown, indicating that miR-7-5p may have other targets that regulate p27 in breast cancers. Sanchez et al. showed that miR-7-5p induces G0/G1 cell cycle arrest via targeting Skp2 to increase the p27 level in CHO cells [31]. This is in accordance with the principal effect of miRNAs on many target genes.

It is well-known that miR-7-5p exerts anti-metastasis effects in multiple cancers, including breast cancer [7,32]. Moreover, our previous study showed that dysregulated REGγ was associated with lymph node metastasis in breast cancer cells. We speculate that miR-7-5p may suppress tumor metastasis by targeting REGγ and will assess this in the future. On the basis of bioinformatic analysis and the data in this study, we conclude that miR-7-5p is a functional regulator that can serve as a tumor suppressor, and that REGγ is a novel target gene of miR-7-5p in breast cancer cells.

Collectively, the present study provides evidence that the dysregulation of REGγ results from miR-7-5p downregulation in breast cancers and may promote cancer progression. Therefore, this study demonstrates a novel regulator of REGγ and enriches our knowledge on the interactions between miR-7-5p and its targets in breast cancers. Thus, our findings provide new prospects for...
miR-7-5p and REGγ as promising molecular therapies in breast cancer treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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