Pathology & Oncology Research (2019) 25:653–658
https://doi.org/10.1007/s12253-018-0525-2

ORIGINAL ARTICLE

Triiodothyronine Promotes Cell Proliferation of Breast Cancer via Modulating miR-204/Amphiregulin

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Received: 20 September 2018 / Accepted: 25 October 2018 / Published online: 8 November 2018

Abstract

Breast cancer (BC) severely threatens women's life, and Triiodothyronine (T3) shows a positive role on BC cell proliferation, while the potential mechanism underlying it is still unclear. T3 was used to stimulate BC cell lines MCF-7 and T47-D. Real-time PCR was performed to determine the expression of miRNAs, while western blot was used to measure protein expression of Amphiregulin (AREG), AKT and p-AKT. The interaction between miR-204 and AREG was determined using luciferase reporter assay. MTT was performed to detect cell viability. The expression of miR-204 was decreased, while AREG and p-AKT was increased in T3 stimulated BC cell lines. T3 stimulation promoted cell viability. miR-204 targets AREG to regulate its expression. T3 promoted expression of AREG and p-AKT, while miR-204 overexpression reversed the effect of T3, however, pcDNA-AREG transfection abolished the effect of miR-204 mimic. T3 promoted cell viability of BC cells via modulating the AKT signaling pathway. The detailed mechanism was that the down-regulated miR-204 that induced by T3 stimulation promoted the expression of AREG, the up-regulated AREG activated AKT signaling pathway, while the activated AKT signaling promoted cell proliferation.

Keywords Breast cancer · T3 · Cell viability · miR-204 · AREG

Introduction

Breast cancer (BC) is a death-related disease that severely threatens women's life worldwide. Even though great advancement has been achieved on its therapy, the annually mortality remains high. Currently, it has been reported that the standard prognostic factors of breast cancer have been established, such as tumor size, pathological grade, clinical stage, the presence of lymph node metastases, and hormone receptor status. All these parameters have been widely applied in determine the biological characteristics of BC, however, due to that both genetic and environmental factors contribute to the complex etiology of BC, the underlying pathogenesis remains unclear. Therefore, it is essential and urgent that researchers investigate the mechanisms underlying BC pathologies.

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Thyroid hormones (THs), Triiodothyronine (T3) and T4 are involved in all mammalian tissues and regulated the physiological functions in the body. The activated deiodinase (D1, D2 and D3) can locally alter the THs signaling. T3 is generated from T4, and affects many biological effects of THs. In addition, T3 could bind with the thyroid receptor [1], and modulate gene expression in cellular through ligand-dependent transcription factors of TRs [2]. Preliminary studies have declared that THs affected the cell differentiation and BC cell proliferation [3–5]. The T3 promoted cell proliferation of MCF-7 and T47-D (BC cell lines) in a dose-dependent mode [6]. While the potential mechanism of T3 on BC was still unclear.

MicroRNA (miRNA) is a set of non-coding RNA with the length of ~22 nucleotides, and regulates genes expression at post-transcriptional level by binding the 3'UTR. It has been reported that miRNA is involved in various diseases, and modulates the onset and progression of mounting human diseases. MiRNAs played an important role on BC, as they were reported to mediate the tumor progression. For example, miR-530 served as an suppressor and inhibited cell proliferation of BC [7]. miR-125a suppressed tumor progression of BC by downregulating BAP1 [8]. In addition, miR-155, miR-373 and miR-520c were increased in BC, and served as the

oncogenes [9–11]. MiR-205, miR-200c and miR-30a were decreased, and acted as the tumor suppressor genes in BC [12–15]. It has been reported that miR-204a was decreased in BC tumor tissues than that in the adjacent tissues [16], and overexpressed miR-204 dramatically suppressed cell proliferation and migration, but promoted cell apoptosis of BC [17]. While the potential mechanism of miR-204 on BC was still unclear.

Amphiregulin (AREG) belongs to the epidermal growth factor (EGF) family. AREF was discovered in enriched conditioned medium of MCR-7 cells [18]. It has been reported that the expression of AREG was increased in T3 stimulated MCF-7 cell lines [19, 20]. In addition. The BC cells inducted by AREG could significantly increased cell proliferation [21], indicating the potential role of AREG on BC.

In the present study, we investigate the role of miR-204a and AREG on the T3 stimulated BC cell lines, and explore the potential mechanism of BC, which will provide great help for further BC therapy.

Materials and Methods

Cell Culture

BC cell lines MCF-7 and T47-D were all purchased from American Type Culture Collection (ATCC). All cells were cultured in RPMI1640 medium containing 10% fetal bovine serum in a humidified incubator with 5% CO2 at 37 °C.

Triiodo-l-thyronine sodium salt (T3, Sigma) was dissolved in NaOH at 1 mg/ml and diluted in dH_2O at 200 μM. Then the sterilized T3 were stored at −20 °C. For cell treatment, T3 solutions were diluted using dH_2O at 100 μM. 2 μl of T3 solution were added to 200 μl cell culture.

Real-Time PCR

Total RNA was isolated from cells using RNA Extract Kit (Qiagen, CA) according to the manufacture's instruction. The quantified RNA was used as the template to obtain cDNA. Real-time PCR was performed using SYBR green kit (BioRad, CA) following manufacturer's instruction. The mRNA relative expression was calculated using $2^{-\Delta\Delta ct}$.

Western Blot

Cells were pretreated using lysis buffer and the proteins were extracted after centrifugation. The protein extracts were separated using 10% SDS-PAGE, and the separated protein was transferred onto PVDF membrane, which was incubated with the primary antibodies (Abcam) of AREG, p-AKT and AKT at 4 °C for 24 h. Then the membrane was washed using PBS, and incubated with the secondary antibody (Beyotime, Shanghai, China) at room temperature for 1 h. The protein bands were visualized using BeyoECL Plus Kit (Beyotime, Shanghai, China).

Methyl Thiazolyl Tetrazolium (MTT) Assay

Cells was plated in 96-well plates at a concentration of 5×10^4 cell/mL and incubated at 37 °C in 5% CO_2 for 24 to 48 h until the cultures were sub-confluent. After incubation with 20 μL of 5 mg/mL 3-[4, 5-dimethylthiazol-2-yl] - 2, 5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) for 4 h at 37 °C, the MTT solution was discarded. The formazan precipitates were resuspended in 100 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich), and the absorbance was measured on a plate reader at a wavelength of 570 nm.

Cell Transfection

Cells were grown in a 24-well plate until the cultures were sub-confluent. The inhibitors and mimics were all purchased from Shanghai GenePharma Co., Ltd. (China). The plasmid were transfected to the cells using Lipofectamine2000 reagent (Invitrogen). After 24 h, the transfection efficiency was determined using real-time PCR.

Luciferase Reporter Assay

The AREG 3'UTR was amplified and cloned into the vector to construct luciferase reporter plasmid. When cells grown into 70% confluence, the mimic/inhibitor and the vector were co-transfected into the cells using Lipofectamine2000 reagent (Invitrogen). The relative luciferase reporter activity was determined using Dual-Luciferase Reporter Assay (Promega, USA).

Statistical Analysis

All data were presented as means±SD. SPSS18.0 software were used to process data. Statistical difference were analyzed using one-way analysis of variance (ANOVA) or t-test when appropriate. $*P < 0.05$ considered as statistically significant difference.

Results

T3 Stimulation Induced the Reduction of miR-204

To determine the dysregulated miRNAs in BC cells under the stimulation of T3, miRNAs that extracted from cells were

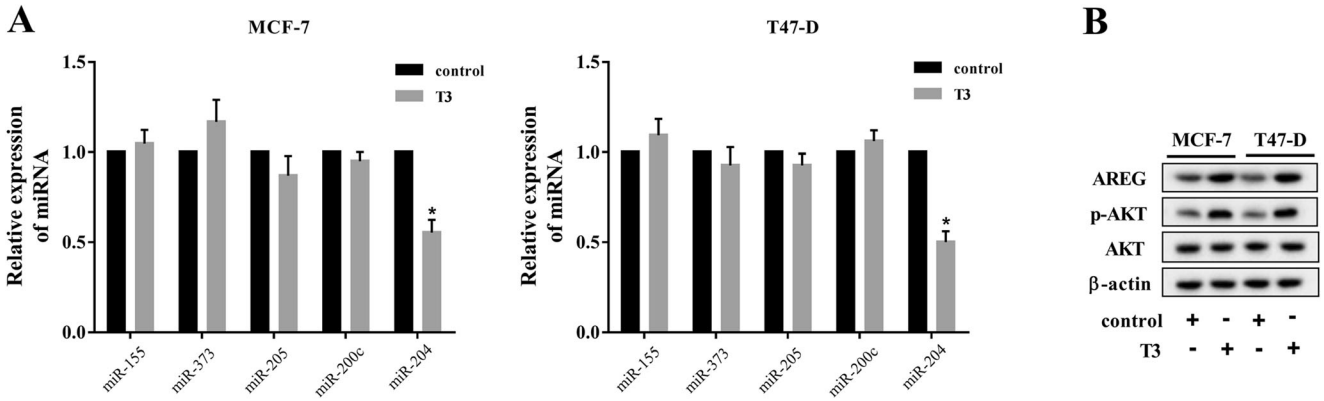

Fig. 1 The dysregulated miRNA and proteins in T3 stimulated BC cell lines MCF-7 and T47-D. a the expression of miRNAs was determined using real-tiem PCR. **b** the expression of AREG, AKT and p-AKT were determined using western blot. *$p < 0.05$ vs control

analyzed using real-time PCR. As is presented in Fig. 1a, the expression of miR-155, miR-373, miR-205 and miR-200c showed no significant difference between control and T3 stimulated cells, while miR-204 was significantly decreased during the T3 stimulation. In addition, the expression of AREG and p-AKT was increased in T3 stimulated BC cells (Fig. 1b).

T3 Stimulation Induced Cell Viability Via Regulating miR-204

To determine the role of T3 on cell viability, BC cell lines MCF-7 and T47-D were transfected with miR-204 mimic. Real-time PCR revealed that T3 stimulation significantly decreased the expression of miR-204, while miR-204 mimic transfection significantly increased its expression (Fig. 2a). Additionally, T3 stimulation significantly increased cell viability, while miR-204 mimic reversed the effect of T3 (Fig. 2b).

MiR-204 Targets AREG to Regulate its Expression

An online prediction revealed that miR-204 could bind with AREG 3'UTR. MCF-7 and T47-D cells transfected with miR-204 mimic significantly decreased relative luciferase activity, while miR-204 inhibitor significantly increased relative luciferase activity (Fig. 3b) in cells transfected with the recombinant plasmid of AREG 3'UTR. In addition, miR-204 mimic decreased the expression of ARGE, while miR-204 inhibitor significantly promoted the expression of ARGE (Fig. 3c).

T3 Stimulation Activated AKT by miR-204/AREG

To determine the mechanism of T3 on AKT activation, MCF-7 and T47-D cells were stimulated using T3. As is presented in Fig. 4a, T3 stimulation significantly promoted the expression of AREG, and miR-204 mimic transfection significantly reversed the effect of T3, while pcDNA-AREG co-tranfection significantly abolished the effect of miR-204 mimic.

T3 Stimulation Promoted Cell Viability through Regulating miR-204/AREG

To determine the mechanism of T3 on cell viability, MCF-7 and T47-D cells were pretreated with T3, as is presented in Fig. 5a, b, T3 stimulation significantly

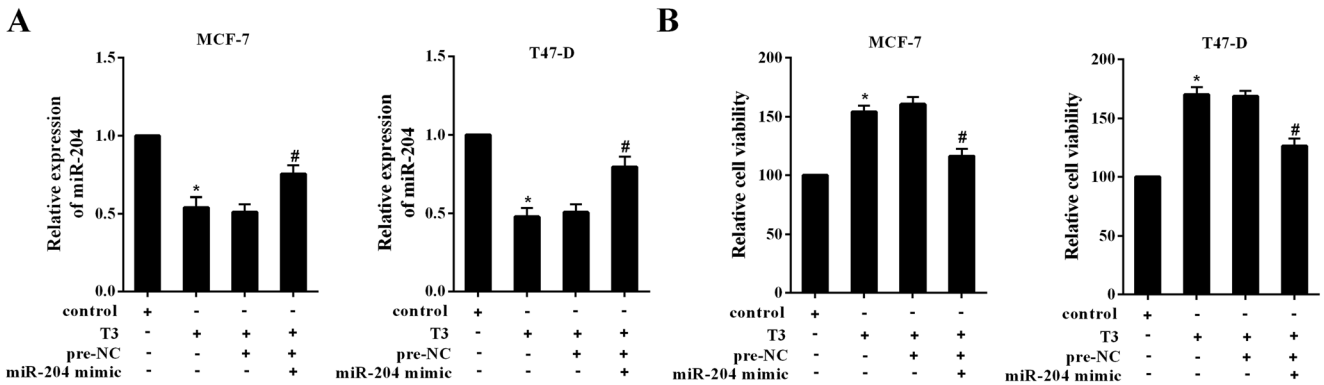

Fig. 2 Role of miR-204 on T3 stimulated cell proliferation. Both MCF-7 and T47-D cell lines were divided into: control, T3, T3 + pre-NC, T3 + miR-204 mimic. A: real-time PCR was performed to determine the expression of miR-204; B: MTT assay was used to determine cell viability. *$p < 0.05$ vs control; #$p < 0.05$ vs T3 + pre-NC

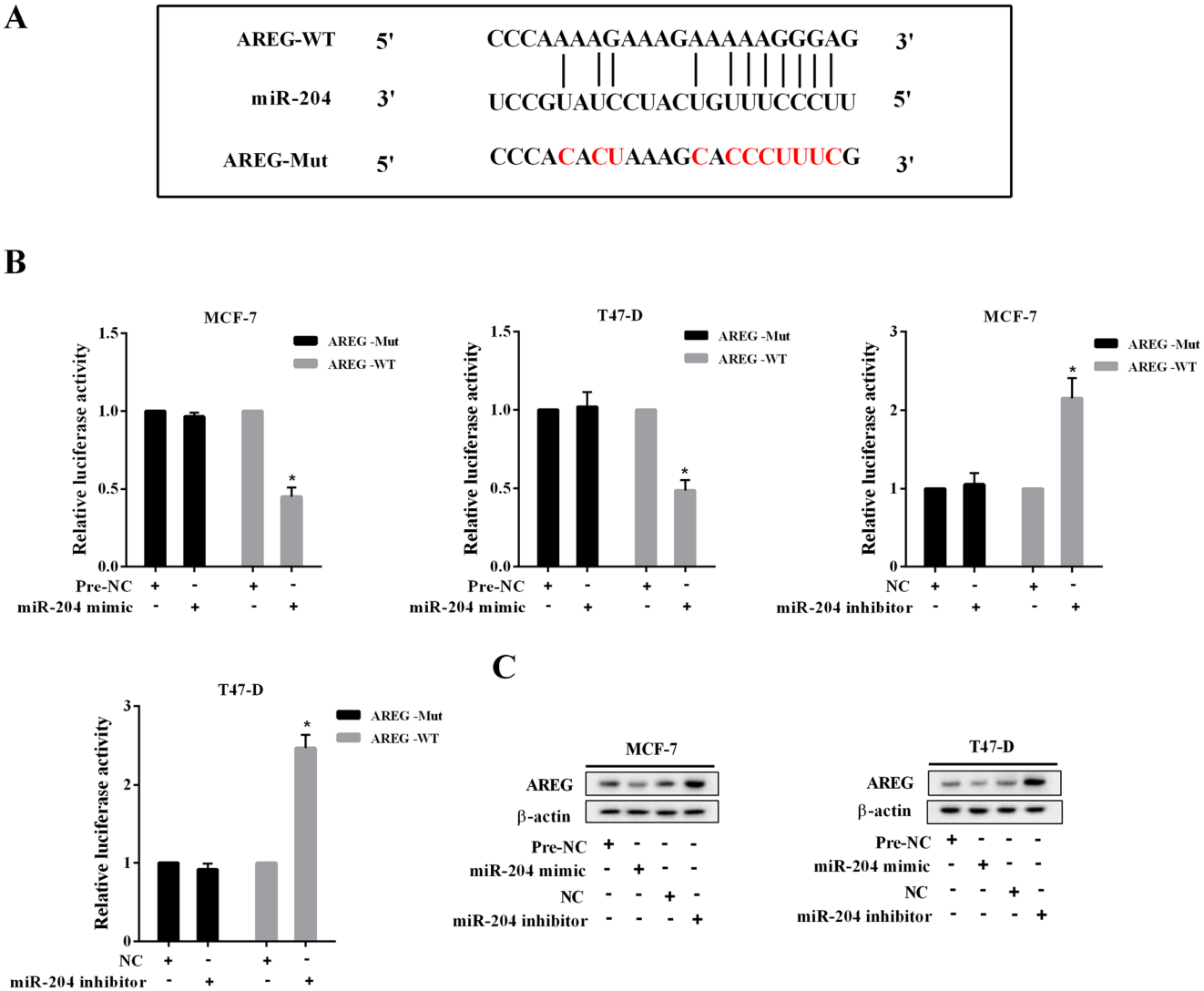

Fig. 3 The interaction between miR-204 and AREG. a online prediction revealed that miR-204 could bind with AREG 3'UTR. **b** luciferase reporter assay was carried out to determine relative luciferase activity under the treatments of miR-204 mimic and miR-204 inhibitor. **c** western blot was used to determine the protein expression of AREG under the treatments of miR-204 mimic and miR-204 inhibitor. *p < 0.05 vs NC-pre-NC

promoted cell viability, while miR-204 mimic transfection decreased cell viability, however, cells co-transfected with miR-204 mimic and pcDNA-AREG significantly abolished the effect of miR-204 mimic.

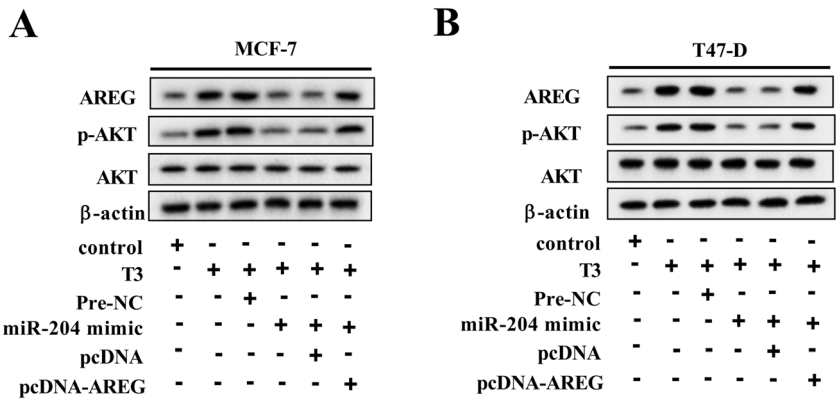

Fig. 4 Effects of miR-204 and AREG on T3 stimulated AKT activation. Both MCF-7 and T47-D cell lines were divided into: control, T3, T3 + pre-NC, T3 + miR-204 mimic, T3 + miR-204 mimic+pcDNA, T3 + miR-204 mimic+pcDNA-AREG. **a** and **b**: western blot was performed to determine the expression of AREG, p-AKT and AKT

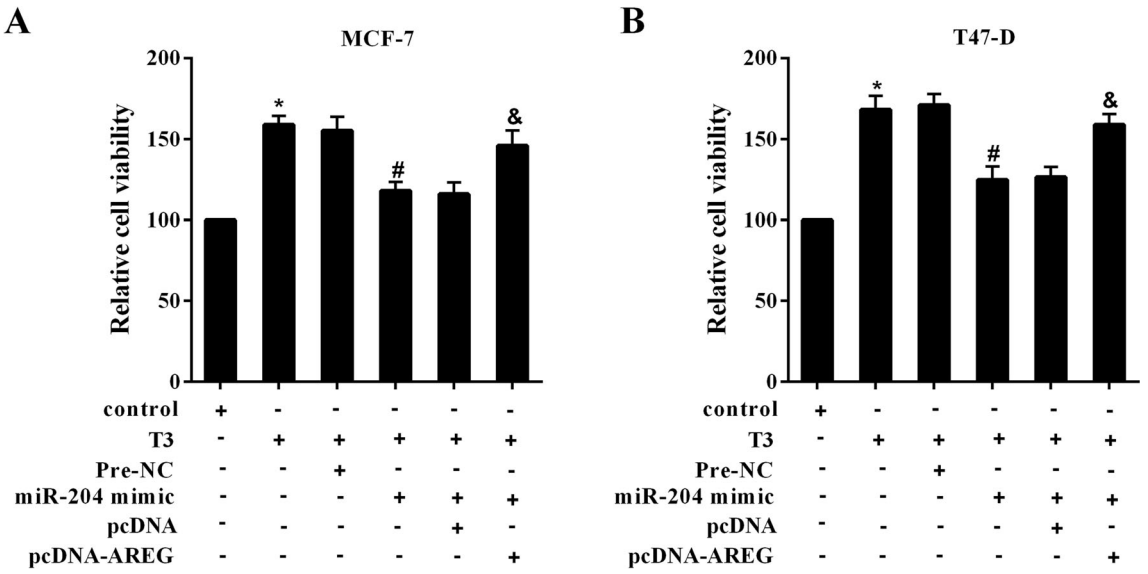

Fig. 5 Role of miR-204 and AREG on T3 stimulated cell proliferation. Both MCF-7 and T47-D cell lines were divided into: control, T3, T3+ pre-NC, T3+ miR-204 mimic, T3+ miR-204 mimic+ pcDNA and T3+ miR-204 mimic+ pcDNA-AREG. **a** and **b**: MTT was performed to determine cell viability. *$P < 0.05$ vs control; #$P < 0.05$ vs T3+ pre-NC; &$P < 0.05$ vs T3+ miR-204 mimic+ pcDNA

Discussion

miRNAs has been reported to be dysregulated in BC via a series of biological processes, such as cell proliferation, apoptosis, cell cycle, invasion and migration. miR-204 was reported to downregulate in various tumors, such as gastric [22], glioma [23], thyroid cancer [24], and non-small cell lung cancer [25], and identified as an anti-oncogene. In the present study, miR-204 was significantly decreased in T3 stimulated BC cell lines, indicating the potential role of miR-204 in T3 induced BC cells proliferation. It has been verified that miRNA regulated gene expression in post-transcriptional level by binding 3'UTR of the mRNA. For example, miR-506 targets NEK6 to regulate cell proliferation of retinoblastoma [26]. miR-139 targets DHFR to promote cell differentiation of bovine skeletal muscle-derived satellite cells [27]. In the present study, miR-204 was identified to target AREG to regulate its expression, indicating that miR-204 and AREG were involved in T3 induced BC cell proliferation.AREG is a differentially expressed gene that could code for glycoprotein. Numerous studies have reported that AREG is associated with estrogen response, for example, AREG is involved in the mammary maturing and oncogenesis [19]. Additional study has identified that AREG was up-regulated in BC and E2 treatment could significantly decrease the expression of AREG [28], and the up-regulated AREG is associated with cell invasion [18]. The increased AREG was observed in BC cells after they were stimulated by T3 [19]. In the present study, T3 stimulation significantly promoted the expression of AREG. The online prediction combined with the luciferase reporter assay identified that miR-204 targets AREG to regulate its expression, indicating that both miR-204 and AREG served as the important molecular in T3 induced BC cell proliferation.

AKT signaling pathway has been identified to involve in the cell proliferation. For example, inhibition of AKT-ERK pathway suppressed cell proliferation in colorectal cancer [29]. The IGF-1R/PI3K/AKT signaling inhibition could arrest cell cycle in human nasopharyngeal carcinoma [30]. Another study has verified that AREG could activate AKT signaling pathway [31]. In our present study, T3 stimulation activated the AKT signaling pathway by regulating miR-204/AREG, and further promoted cell proliferation.

Taken together, in the present study, T3 stimulation significantly promoted BC cell proliferation, among which, the down-regulated miR-204 targets AREG to promote its expression, and the up-regulated AREG further activated AKT signaling pathway to promote cell proliferation. The present study provided great help for clinical therapy of BC. The further study should pay attention to the in vivo experiments to verify the mechanism.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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