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LncRNA BCAR4 up-regulates EGFR and thus promotes human thyrocyte proliferation

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This study establishes that BCAR4 has pro-proliferative effects on normal thyroid cells. BCAR4 up-regulation promotes proliferation and suppresses apoptosis of NTHY-ORI 3-1 cells and human primary thyrocytes, and EGFR is highly expressed in BCAR4 over-expressing-cells. In contrast, BCAR4 up-regulation did not modulate cell viability or cell cycle progression when EGFR was knocked-down. Further, the phosphorylated forms of PI3K, AKT, mTOR and p70S6K were up-regulated by BCAR4 up-regulation, and the alteration in these kinases induced by BCAR4 up-regulation was abolished when EGFR was knocked-down. This study therefore revealed the pro-proliferative role of lncRNA BCAR4 in NTHY-ORI 3-1 cells and human primary thyrocytes; where BCAR4 accelerated thyroid cells proliferation by EGFR up-regulation and the modulation of PI3K/AKT/mTOR signaling. These findings indicate that targeting BCAR4 has potential in hyperthyroidism treatment.

Key words: lncRNA, Breast Cancer Anti-estrogen Resistance 4 (BCAR4), human thyroid cell, hyperthyroidism, EGFR, proliferation

Hyperthyroidism is a condition that occurs due to excessive production of thyroid hormone by the thyroid gland [1]. It is one of the most common endocrine disorders, with a prevalence of 1.3% in the United States and 0.7% in Brazil [2]. Signs and symptoms vary in patients, and these can include palpitation, fatigue, heat intolerance, hyperhidrosis, bulimia, weight loss, emotional sensitivity, exophthalmos and thyroid enlargement. There are many therapeutic modalities of hyperthyroidism, including pharmaceutical, ^{131}I radiotherapy and surgical intervention and selection of these modalities depends upon the age, cause, prevailing medical condition and patient preference [3].

Human thyroid follicular epithelial cells are responsible for synthesis and secretion of thyroid hormone. Therefore, suppressing their proliferation is a feasible novel therapeutic strategy for hyperthyroidism.

Long non-coding RNAs (lncRNA) have more than 200 nucleotides with crucial function in translation, RNA splicing,and gene regulation [4, 5]. Recent studies have indicated that at least 75% of the human genome is transcribed into RNAs and most of these are lncRNAs [6]. LncRNAs have many biological actions, including transcriptional and post-transcriptional regulation, organization of protein complexes, cell-cell signaling, allosteric regulation of protein and chromatin structure modulation [7, 8]. Further, literature reports increasingly implicate lncRNAs in cell proliferation, apoptosis, cell-cycle progression, invasion and migration, and indicate that they have potential in the treatment of a wide variety of diseases, including cancer [9–11].

Breast Cancer Anti-estrogen Resistance 4 (BCAR4) is an lncRNA up-regulated in breast cancer and it contributes to metastasis by directing the downstream chemokine signal cooperation of epigenetic regulation and activating the non-canonical Hedgehog/GLI2 transcription program [12]. Current research also records that BCAR4 is increased in non-small cell lung cancer (NSCLC) and osteosarcoma tissues, and it also correlates with patient distant metastasis and clinical stage [13, 14]. Moreover, BCAR4 has important roles in the progression of breast cancer, NSCLC and osteosarcoma by controling tumor cell proliferation, invasion and migration [13, 15, 16]. Although, these previous studies have identified BCAR4 as a mediator of tumor cell proliferation, BCAR4 function in non-cancer cells remains unclear.

Herein, we investigate the role of BCAR4 in the NTHY-ORI 3-1 human thyroid cell line and primary thyrocytes in order to determine BCAR4 effect on thyroid growth.

Our results showed that BCAR4 is critical for human thyrocytes proliferation, and its up-regulation promotes these cells' proliferation by up-regulating epidermal growth factor receptor (EGFR). This study therefore establishes BCAR4 as a potential therapeutic target in hyperthyroidism.

Materials and methods

Cell culture. Human thyroid follicular epithelial cell line NTHY-ORI 3-1 derived from adult thyroid tissue was purchased from ECACC (Wiltshire, UK). The cells were cultured in RPMI 1640 medium (Gibco, US) containing 2 mM Glutamine and 10% heat-inactivated fetal calf serum (HI-FCS) (Gibco). The cells were routinely cultured in 25-cm^2 plastic flasks (Costar, US) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Culture of primary human thyrocytes was performed as previously described [17]. Human thyroid samples were collected from the contra-lateral disease-free lobe of five patients who underwent thyroidectomy. Specimens were washed twice with cold PBS, and cells isolated within 4 hours of surgery. The tissue sample was minced on ice and then incubated with 3 mg/ml Collagenase solution (Gibco) for 30 min, at 37 °C on a shaker. This process was repeated three times. The monolayer cells were obtained by centrifugation at 150×g for 5 min at 4 °C, and then cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% HI-FCS (Gibco) at 37 °C, and humidified atmosphere with 5% CO_2. This study was approved by the Ethics Committee of Liaocheng People's Hospital, and was performed in accordance with the ethical standards. Written informed consent was obtained from each individual.

Cell transfection. Small interfering RNA (siRNA) specific for BCAR4 (si-BCAR4) and EGFR (si-EGFR) and their correspondingly non-targeting control siRNA (si-NC) were synthesized by Ribobio (Guangzhou, China). BCAR4 and EGFR expression vectors (pc-BCAR4 and pc-EGFR) were constructed by sub-cloning the full-length wild-type BCAR4 and EGFR coding sequence into pcDNA3.1 (Sangon Biotech, China). The empty construct pcDNA3.1 plasma was transfected as the negative control. All transfections were performed by Lipofectamine 3000 reagent (Invitrogen, USA) according to the manufacturer's instructions. The final concentration of siRNA and vectors was 100 nM and 2 μg/ml. The stably transfected cells were selected from culture medium containing 0.5 mg/ml G418 (Sigma-Aldrich). G418-resistant cell clones were established after approximately 4 weeks.

qRT-PCR. Total cellular RNA was extracted by Trizol reagent (Invitrogen) according to the manufacturer's instructions. Five micrograms of total RNA from each sample was subjected to reverse transcription using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). FastStart Universal SYBR Green Master (ROX) (Roche) was used in real-time PCR and each real-time PCR was carried out in triplicate for a total of 20 μl reaction mixtures on ABI PRISM 7500 Real-time PCR System (Applied Biosystems, US). The GAPDH used for normalizing fold changes in each sample and data was analyzed according to $2^{-\Delta\Delta Ct}$ [18].

Cell viability assay. The transfected cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After 48 hour incubation at 37 °C, the culture medium was removed and 10 μl CCK-8 solution was added (Dojindo Molecular Technologies, US). The plates were incubated at 37 °C for another 4 hours and absorbance of each well at 450nm was detected by microplate reader iMark (Bio-Rad, US).

Apoptosis quantification. Cell apoptosis was detected post-transfection by Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, China). In brief, the transfected cells were seeded in 6-well plates with a density of 5×10^5 cells/well. After 48 hour incubation at 37 °C, the cells were collected and re-suspended in 200 μl binding buffer containing 10 μl Annexin V-FITC and 5 μl PI. After incubation in the dark at room temperature, 300 μl PBS was added to each sample and apoptotic cells were distinguished by FACS can (Annexin-V positive – Beckman Coulter, US). Data was analyzed by FlowJo software (TreeStar, US).

Brdu assay. 5×10^4 transfected NTHY-ORI 3-1 cells were seeded in 24-well plates and a sterilized coverslip was placed on each well. When the cells achieved 70~80% confluence, 10 μM Brdu solution was added and cultured at 37 °C for another 4 hours (Sigma-Aldrich). The culture medium was removed, the cells were washed twice with PBS and then fixed with 4% cold paraformaldehyde for 20 minutes. After incubation with 0.2% Triton X-100 (Sigma-Aldrich) for 10 minutes, the cells were probed by monoclonal anti-BrdU antibody (1:1000, Sigma-Aldrich) at 4 °C overnight. The cell nucleus was stained with 0.5 μg/ml DAPI (Sigma-Aldrich) and directly counted under a fluorescence microscope (BX61, Olympus, Japan).

Cell cycle assay. Cell cycle distribution was detected post-transfection by the classical PI staining method and flow-cytometry analysis [19]. The transfected cells were seeded in 6-well plates with a density of 5×10^5 cells/well. After 48 h of incubation at 37 °C, the cells were starved for 24 h in non-serum culture medium to synchronize them. The cells were then collected and suspended in chilled 70% ethanol at 4 °C overnight and incubated with a solution containing 50 μg/ml PI (BD Biosciences, US), 20 μg/ml RNase A (Sigma-Aldrich), and 0.1% Triton X-100 (Sigma-Aldrich). This was for 30 min at 37 °C in the dark. Subsequently, 3,000 events per sample were recorded by FACS can (Beckman Coulter, US) and the proportion of cells in G1/S, S and G2/M phases were analyzed by ModFit software (Verity Software House, US).

Western blot analysis. Total cellular proteins were extracted by RIA lysis buffer (Beyotime) and quantified using the BCA™ Protein Assay Kit (Pierce, US). The protein (0.1 mg) was resolved over 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were

blocked in 5% non-fat dry milk for 1 h at room temperature, and then incubated with primary antibodies at 4 °C overnight for Bcl-2 detection (1:2000, ab182858), Bax (1:1000, ab182734), caspase-3 (1:250, ab13586), caspase-9 (1:1000, ab184786), CyclinD1 (1:10000, ab134175), CyclinE (1:1000, ab33911), CDK4 (1:500, ab137675), p21 (1:1000, ab109520), p27 (1:500, ab62364), EGFR (1:1000, ab52894), PI3K (1:1000, ab86714), p-PI3K (1:500, ab182651), AKT (1:500, ab8805), p-AKT (1:500, ab38449), mTOR (1:2000, ab2732), p-mTOR (1:1000, ab109268), p70S6K (1:500, ab47504), p-p70S6K (1:500, ab59208), GAPDH (1:2500, ab9485) (all from Abcam, US). After rinsing, the membranes were incubated with anti-mouse (1:2000, ab6789) and anti-rabbit (1:2000, ab6721) (Abcam) secondary antibodies for 1 hour at room temperature. Positive signals were visualized by enhanced chemi-luminescence.

Statistical analysis. All experiments were repeated three times. Statistical analysis was by SPSS 19.0 statistical software (SPSS Inc., US) and data is presented as mean ± standard derivation. A p-value was calculated using one-way analysis of variance (ANOVA); with $p<0.05$ statistically significant.

Results

BCAR4 up-regulation promotes human thyrocyte viability and inhibits apoptosis. The expression levels of BCAR4 in NTHY-ORI 3-1 cells and primary throcytes were first altered by transfection with the specific targeted siRNA (si-BCAR4) and pc-BCAR4 expression vector. The qRT-PCR analytical results showed that cells transfected with pc-BCAR4 had significant over-expression compared to controls, while transfection with si-BCAR4 resulted in significant expression decrease ($p<0.01$ or $p<0.001$, Figures 1A–D). This indicates transfection success.

The effect of BCAR4 de-regulation on NTHY-ORI 3-1 cells and primary thyrocytes viability and apoptosis was then assessed. Figures 2A–D shows that the cell viability in the pc-BCAR4 transfected group was much higher than in pcDNA3.1 transfected group ($p<0.05$), and cells transfected with si-BCAR4 had less cell viability than siNC cells ($p<0.05$). Figures 2E–H highlights that the apoptotic cell rate was unaffected by pc-BCAR4 transfection ($p<0.05$) but significantly increased by si-BCAR4 transfection ($p<0.01$ or $p<0.001$). Similarly, Figure 2I illustrates that the BrdU-positive cell rate was significantly increased by pc-BCAR4 but decreased by si-BCAR4 transfection, compared to controls (both $p<0.05$). Finally, Western blot analysis established that Bcl-2 was down-regulated, Bax was up-regulated and caspase-3 and caspase-9 were activated in si-BCAR4 transfected cells (Figures 2K–J).

BCAR4 up-regulation arrests more human thyrocytes in the S phase. We evaluated the effects of BCAR4 de-regulation on cell cycle progression of NTHY-ORI 3-1 cells and primary thyrocytes, and Figures 3A–D illustrate that the ratio of cells in the S phase was significantly increased by pc-BCAR4, and the G0/G1 phase was accordingly decreased (both $p<0.05$). si-BCAR4 then reversed these effects ($p<0.05$). Western blot analysis determined that CyclinD1, CyclinE and CDK4 were up-regulated and p21 and p27 down-regulated by pc-BCAR4 (Figures 3E–F); and again si-BCAR4 reversed these effects.

BRCA4 upregulates EGFR in human thyrocytes. A previous study recorded that breast cancer cell growth driven by BRCA4 is counteracted by EGFR inhibition [15]. Herein, we investigated the EGFR effect on BRCA4-mediated proliferation in normal human thyrocytes; and Western blot and qRT-PCR established that EGFR mRNA and protein levels were up-regulated in cells transfected with pc-BCAR4 ($p<0.05$ or $p<0.01$) (Figures 4A–D). This confirms that EGFR is positively regulated by BCAR4 over-expression. We then silenced EGFR expression in NTHY-ORI 3-1 cells and primary thyrocytes to reveal the functional effects of EGFR on BCAR4-modulated cell proliferation. qRT-PCR and western blot analytical results showed that EGFR expression levels were significantly down-regulated by transfection with EGFR targeted siRNA (si-EGFR) ($p<0.01$) (Figures 4E–H); thus indicating transfection success.

BCAR4 promotes human thyrocyte viability and S-phase arrest by EGFR up-regulation. NTHY-ORI 3-1 cells and primary thyrocytes were co-transfected with pc-BCAR4 and si-EGFR, and cell viability and cell cycle distributions were then re-assessed. Figures 5A–D highlight that BCAR4

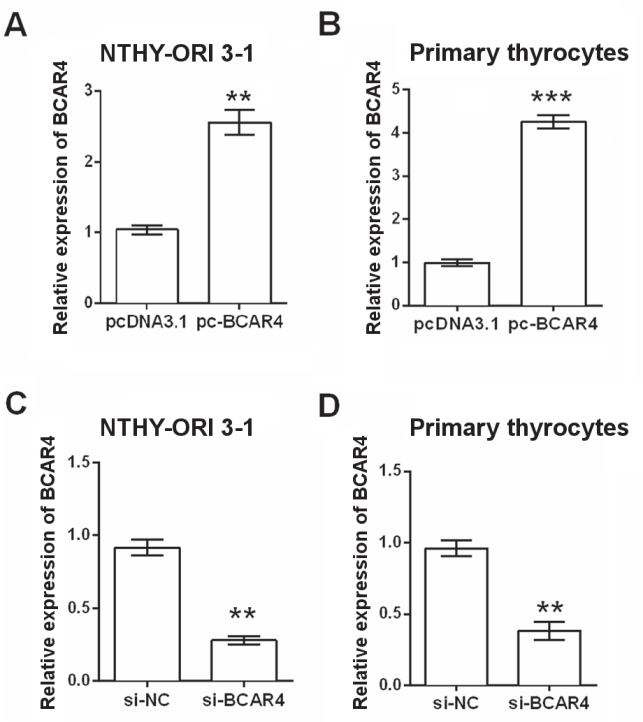

Figure 1. The expression levels of BCAR4 in NTHY-ORI 3-1 cells and human primary thyrocytes were over-expressed or suppressed by transfection with (A–B) pc-BCAR4 (a BCAR4 expressing vector) or (C–D) si-BCAR4 (BCAR4 targeted siRNA). ** $p<0.01$, *** $p<0.001$.

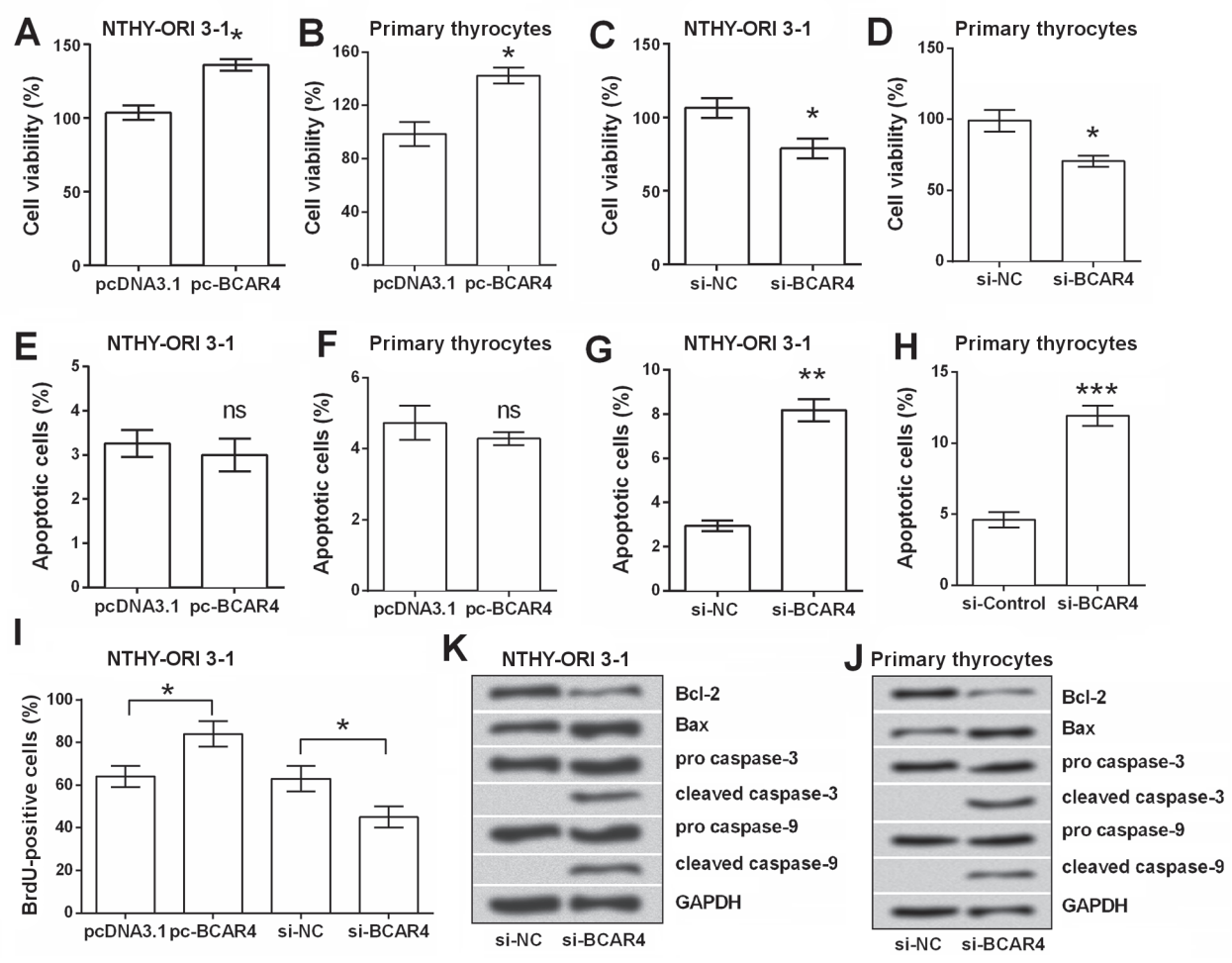

Figure 2. BCAR4 up-regulation promotes cell viability but inhibited apoptosis in human thyrocytes. NTHY-ORI 3-1 cells and human primary thyrocytes were transfected with pc-BCAR4 or si-BCAR4, and then (A–D) cell viability, (E–H) apoptotic cell rate, (I) BrdU-positive cell rate and (K–J) expression changes of apoptosis-related proteins were respectively assessed. ns, no significance, * p<0.05, ** p<0.01, *** p<0.001.

over-expression increases cell viability and S-phase population, and it decreases cells in the G1/S-phase. These were all reversed by si-EGFR ($p<0.05$ or $p<0.01$). Further, Western blot determined that BCAR4 over-expression up-regulates CyclinD1, CyclinE, CDK4 and down-regulates p21 and p27; again all reversed by si-EGFR (Figures 5E–F).

BCAR4 activates PI3K/AKT and mTOR signaling pathways by EGFR up-regulation. Here, we focused on PI3K/AKT and mTOR signaling pathways to find the molecular mechanisms of BCAR4 modulated NTHY-ORI 3-1 cells and primary thyrocytes proliferation, because these are important pathways in modulating cell survival [20, 21] and are also downstream EGFR effectors [22, 23]. We found that pc-BCAR4 remarkably up-regulated p-PI3K, p-AKT, p-mTOR and p-p70S6K protein expression; and these were all reversed by si-EGFR (Figures 6A–D). Further, pc-BCAR4, pc-BCAR4 and si-EGFR had no remarkable effect on total PI3K, AKT, mTOR and p70S6K expression.

Discussion

Although thyroid gland function is mainly under pituitary TSH control [24], other factors such as miRNAs have significant roles in this process [25]. In contrast to miRNA, much less is known about lncRNA function in human cells, especially thyroid cells. However, herein we highlight the functional effects of lncRNA BCAR4 on NTHY-ORI 3-1 cells and human primary thyrocytes, and show that BCAR4 promotes their proliferation through EGFR up-regulation.

BCAR4 is well conserved in primates, and while distant BCAR4 orthologues exist in other placental species, these are not noted in mice and rats [26].

Several recent studies have focused on the functional roles of BCAR4 in tumor cells. For example, Li and his colleagues demonstrated that BCAR4 knockdown inhibited non-small cell lung cancer (NSCLC) cell invasion, metastasis and proliferation, induced cell cycle arrest and increased cell apoptosis

[13]; thus indicating the pro-proliferative and pro-metastasis roles of BCAR4 in NSCLC cells. A further *in vitro* investigation revealed that BCAR4 promoted chondrosarcoma cell proliferation and migration by activating the mTOR signaling pathway [16]. While these studies identified BCAR4 as a critical modulator in tumor cell proliferation, little is known about its role in normal cells.

In this research, we detected the functional effects of BCAR4 de-regulation on the normal NTHY-ORI 3-1 human thyroid follicular epithelial cell line and primary human thyrocytes. We found that BCAR4 exerted pro-proliferative functions in human thyrocytes; where BCAR4 up-regulation increased cell viability, down-regulated, Bcl-2, caspase-3 and caspase-9 pro-apoptotic proteins and up-regulated Bax anti-apoptotic protein. BCAR4 up-regulation also arrested more thyrocytes in the S phase, increased CyclinD1, CyclinE and CDK4 expression levels and decreased p21 and p27 expression. Moreover, it is established that CyclinD1 and CyclinE are two key proteins in the cyclin family and thatCDK4 is a key protein in the cyclin-dependent kinase (CDK) family;

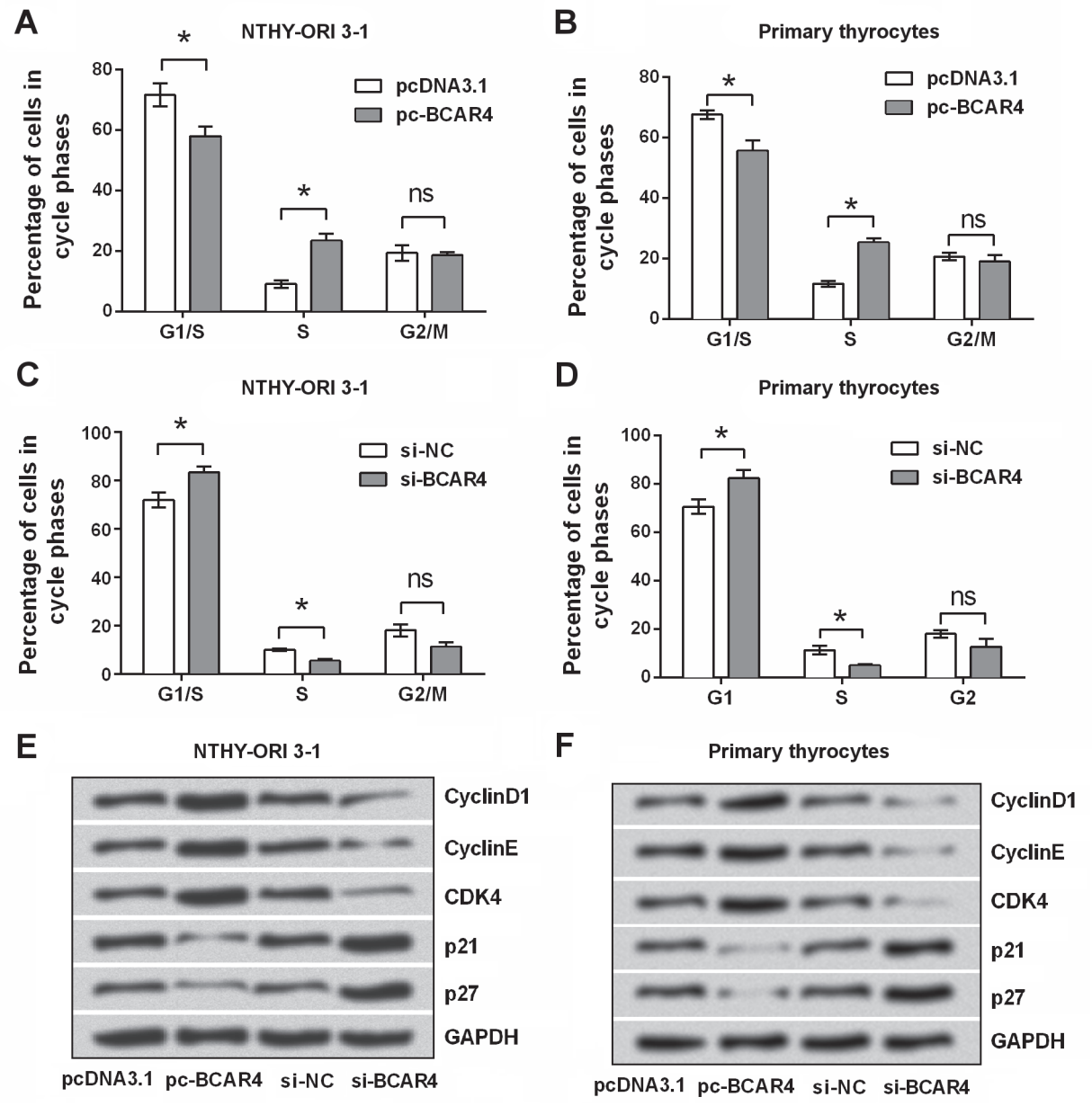

Figure 3. BCAR4 up-regulation arrests more human thyrocytes in S phase. NTHY-ORI 3-1 cells and human primary thyrocytes were transfected with pc-BCAR4 or si-BCAR4, and then (A–D) the percentage of cells in each cycle phases, and (E–F) expression changes of cell-cycle related proteins were detected. ns, no significance, * p<0.05.

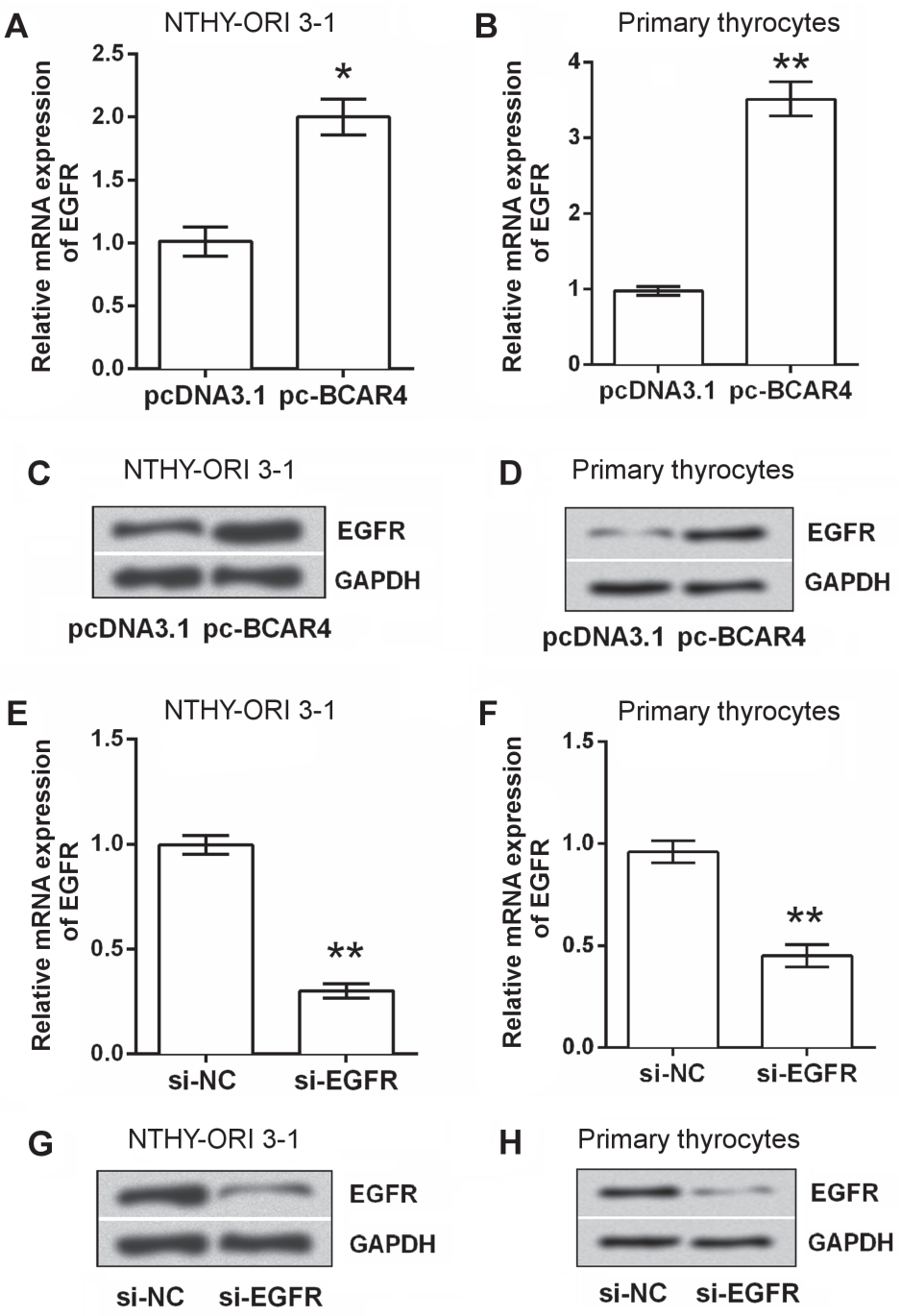

Figure 4. BRCA4 up-regulates EGFR in human thyrocytes. The (A–B) mRNA and (C–D) protein expression levels of EGFR in pc-BCAR4 transfected cells were measured. The (E–F) mRNA and (G–H) protein expression levels of EGFR in cells which were transfected with si-EGFR (EGFR targeted siRNA) were measured. * $p<0.05$, ** $p<0.01$.

and that all of these have pivotal roles in cell cycle progression by accelerating G1/S transition [27, 28].

In contrast, p27 and p21 are two important CDK inhibitors (CDKIs) which prevent cell cycle progression from the G1 to the S phase [29]. These combined findings provide the first *in vitro* evidence that BCAR4 promotes human thyrocytes proliferation by arresting more cells in the S phase.

The EGFR member of the ErbB (HER) receptor tyrosine kinase family is recognized for its essential roles in regulating numerous biological processes, including cell proliferation,

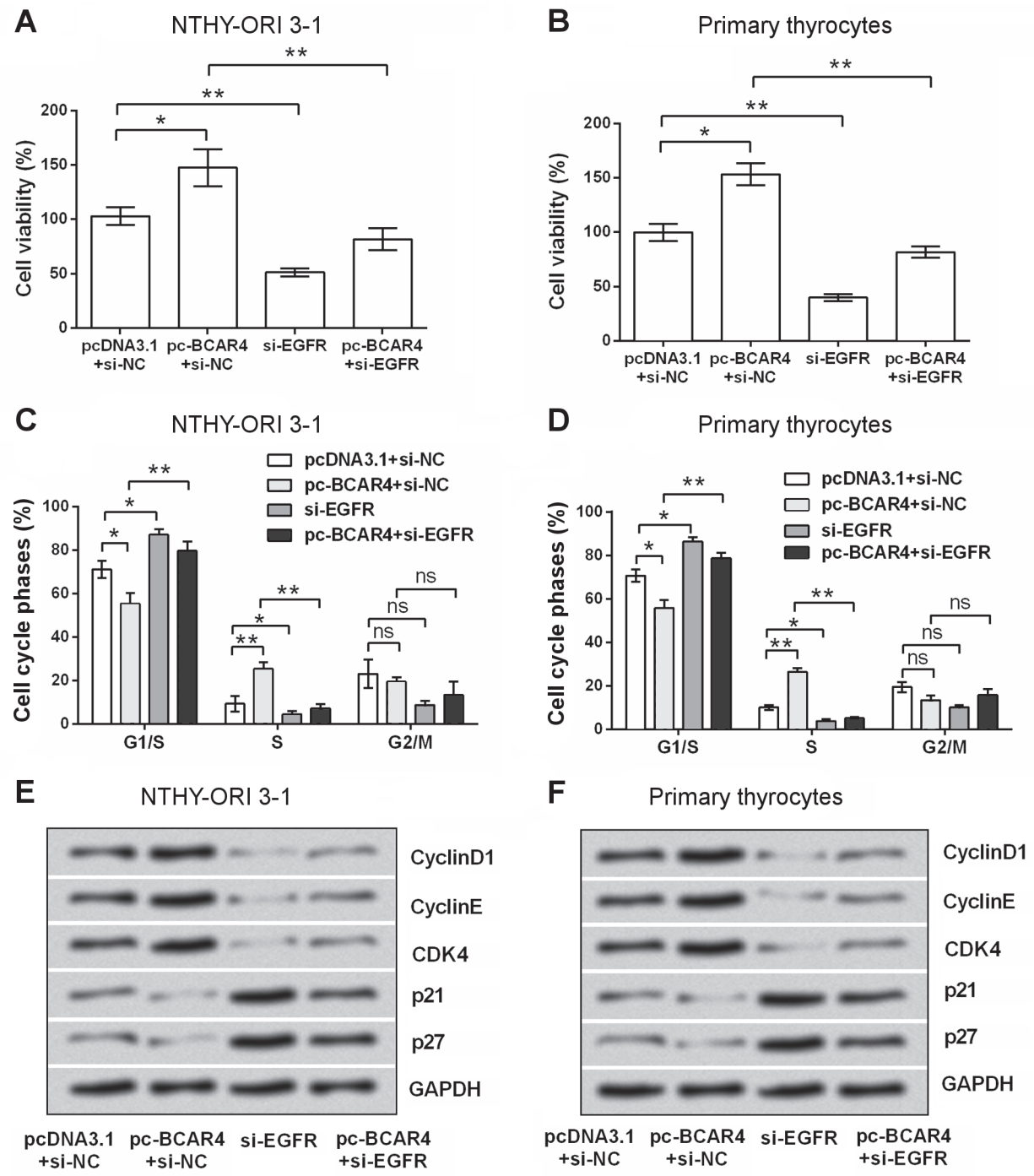

Figure 5. BCAR4 promotes viability and S-phase arrest of human thyrocytes via up-regulation of EGFR. NTHY-ORI 3-1 cells and human primary thyrocytes were co-transfected with pc-BCAR4 and si-EGFR, (A–B) cell viability, (C–D) cell cycle distribution, and (E–F) expression of cell-cycle associated proteins were then assessed. ns, no significance, * p<0.05, ** p<0.01.

differentiation, metabolism and apoptosis [30, 31]. Cells transduced with BCAR4 result in multi-fold signal to noise ratio of EGFR phosphorylated on Tyr1173 [32], and herein, qRT-PCR and Western blot analysis established that EGFR is positively regulated by BACR4 in both NTHY-ORI 3-1 cells and human primary thyrocytes. Most interestingly, BACR4 up-regulation did not promote cell viability and S-phase arrest when EGFR was knocked down, and therefore we infer that BCAR4 promotes human thyrocyte proliferation by EGFR up-regulation.

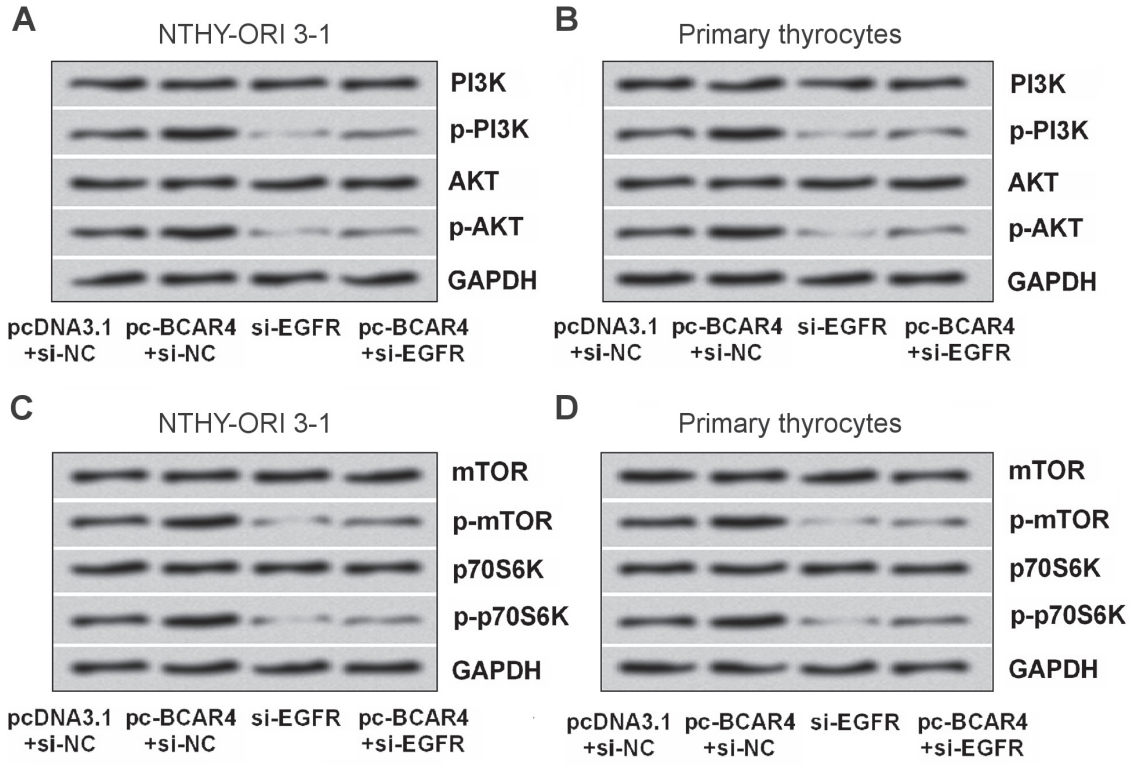

Figure 6. BCAR4 activates PI3K/AKT and mTOR signaling pathways via up-regulation of EGFR. NTHY-ORI 3-1 cells and human primary thyrocytes were co-transfected with pc-BCAR4 and si-EGFR, expression of main proteins in (A–B) PI3K/AKT and (C–D) mTOR signaling pathways were detected.

It is increasingly apparent that PI3K/AKT and mTOR signaling pathways affect most major cellular functions by regulating basic cell proliferation, cycle progression and apoptosis [20, 21]. The PI3K/AKT/mTOR pathway has also been recognized as a promising therapeutic target for thyroid cancer [33, 34], and herein we determined that BCAR4 up-regulation activates the PI3K/AKT and mTOR signaling pathways in NTHY-ORI 3-1 cells and human primary thyrocytes. The phosphorylated forms of PI3K, AKT, mTOR and p70S6K were all up-regulated. However, BCAR4 up-regulation did not activate these four proteins when EGFR was knocked down, and we therefore consider that BCAR4 activates the PI3K/AKT and mTOR signaling pathways in an EGFR-dependent manner.

The combined results suggest that BCAR4 knockdown is a most effective method of inhibiting human thyroid follicular epithelial cell proliferation and reducing thyroid hormone secretion; thereby preventing hyperthyroidism and aiding treatment. We established BCAR4 influence on NTHY-ORI 3-1 cells and confirmed that the pro-proliferative BCAR4 effects can be reproduced in primary thyrocytes. Limitations of this study are lack of thyroid hormone detection which would further confirm our findings, and the underling mechanisms of PI3K/AKT and mTOR activation by BACR4 require clarification.

In conclusion, our research confirmed the pro-proliferative function of lncRNA BCAR4 in the NTHY-ORI 3-1 human thyroid follicular epithelial cell line and human primary thyrocytes. BCAR4 accelerated cell proliferation by up-regulating EGFR and activating PI3K/AKT/mTOR signaling. This study therefore introduces the great potential of targeting BCAR4 in hyperthyroidism treatment.

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