

Original Article

Luteolin suppresses tumor progression through IncRNA BANCR and its downstream TSHR/CCND1 signaling in thyroid carcinoma

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Abstract: The flavonoid luteolin is a natural antioxidant that usually occurs in its glycosylated form in many green vegetables, and has shown anticancer effects against various cancers. However, the potential tumor-suppressive role of luteolin in thyroid carcinoma and its underlying mechanism remain largely unknown. In current study, SBR assay, clone formation assay were employed to evaluate the effects of luteolin on thyroid cancer. We found that luteolin significantly inhibits thyroid cancer growth. The further mechanisms of its anticancer activity were analyzed by flow cytometry, quantitative real-time PCR, and Western blotting. We found that luteolin decreased the expression of BRAF-activated long noncoding RNA (BANCR), which further led to downregulation of TSHR and downstream oncogenic signaling. Moreover, overexpression of BANCR/TSHR signaling can largely abolish the anti-tumor effects of luteolin on thyroid carcinoma in vitro and in vivo. In conclusion, luteolin may serve as a potential important anti-cancer agent for thyroid carcinoma by blocking the BANCR/TSHR signaling.

Keywords: Luteolin, BANCR, TSHR, thyroid cancer

Introduction

Thyroid carcinoma is the most common malignant tumor of the endocrine organs, and has shown steadily increasing morbidity in recent decades [1]. There are three classifications for thyroid cancer based on the pathological characteristics, including papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), and anaplastic thyroid carcinoma (ATC) [2]. PTC is the main form of nonmedullary thyroid cancer, accounting for about 80% of all thyroid cancers, while ATC is the most aggressive solid and its 5-year survival rate is 3 to 5 months after diagnosis [3]. Previous study related to radiation, chemotherapy, and surgery are the common treatment methods for thyroid cancer, but they all produce poor satisfactory [4, 5].

Flavonoids, which are commonly found in the vegetables and fruits, have been suggested to possess anticancer and chemopreventive potential. The flavonoid luteolin, 3',4',5,7-tetrahy-

droxyflavone, is a natural antioxidant that usually occurs in its glycosylated form in many green vegetables such as artichoke, broccoli, cabbage, celery, cauliflower, green pepper, and spinach [6, 7]. Luteolin has shown anticancer effects against lung cancer, head and neck cancer, prostate, breast, colon, liver, cervical, and skin cancer. It triggers apoptotic cell death by activating apoptosis pathways and suppressing cell survival pathways. Luteolin has displayed anticancer effects by inducing cell cycle arrest, senescence, or apoptosis in lung carcinoma cells [8, 9]. There was also evidence to anticancer effects of flavonoids on thyroid cancer [8]. However, the potential tumor-suppressive role of luteolin in thyroid carcinoma and its underlying mechanism need to be fully understood.

Recent study has found that epigenetic alteration may also play an important role in development of variety types of tumors including thyroid carcinoma. Long noncoding RNAs (lncRNAs)

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utilize a variety of mechanisms in the regulation of gene expression including chromatin modification, transcriptional, or post-transcriptional modulation [10, 11]. In thyroid carcinoma, BRAF-activated long noncoding RNA (BANCR), papillary thyroid cancer susceptibility candidate 3 (PTCSC3), and noncoding RNA associated with mitogen-activated protein kinase pathway and growth arrest (NAMA) are identified as crucial lncRNAs. Specifically, the alteration of BANCR has been reported in a variety of cell types of thyroid, and silence of BANCR with siRNA resulted in remarkable suppression of thyroid stimulating hormone receptor (TSHR), thus inhibiting of cell proliferation, and cell cycle arrest at G0/G1 phase [12-15].

In this study, we designed to investigate the role and molecular target of luteolin in thyroid carcinoma. Here, we reported that luteolin exhibited an anti-tumor effect on three thyroid carcinoma cell lines as demonstrated by SBR assay, clone formation assay, and cell cycle analysis. In addition, for the first time, BANCR was identified as target of luteolin and BANCR/TSHR/CCND1 signaling largely mediated the tumor-suppressive role of luteolin in thyroid carcinoma.

Materials and methods

Cell culture

The human thyroid cancer cell lines IHH-4, FTC-133, and 8505C were purchased from American Type Culture Collection (ATCC). All those cells were maintained under Roswell Park Memorial Institute (RPMI)-1640 containing 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 1% penicillin-streptomycin (Sigma, USA) in the humidified incubator with 5% CO₂ at 37°C.

RNA isolation and quantitative real-time PCR

Total RNA was purified from thyroid cancer cells using Trizol Reagent (Takara) and RNA (1 µg) was reverse transcribed using PrimerScript RT-PCR kit (Takara) according to the manufacturer's protocol. Quantitative real time PCR was performed using SYBR green Supermix (Tiangen, China) in ABI 7300 (Applied Biosystems Inc., USA). GAPDH was used as a reference gene. Primers using in this study were described as follows: TSHR forward, 5'-GATATCAACGC-

ATCCCCAG-3'; TSHR reverse, 5'-TTCTGGAAAT-ATTGGGCAGATT-3'; BANCR forward, 5'-ACAGG-ACTCCATGGCAAACG-3'; BANCR reverse, 5'-ATGAAGAAAGCCTGGTGCAGT-3'; GAPDH forward, 5'-ACAACCTTTGGTATCGTGAAGG-3'; GAPDH reverse, 5'-GCCATCACGCCACAGTTTC-3'.

Western blotting

Total protein extracted from patient tissues and cell lines using RIPA lysis buffer (Beyotime, China) followed the protocol. A total of 10 µg protein was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride membrane (PVDF). The membrane was blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 hour. The membrane was then incubated with the primary antibodies (1:1,000 dilution) at 4°C overnight. The following antibodies were used in this study: TSHR, p-CREB, PCNA, and CCND1 were all purchased from Abcam (Shanghai, China). GAPDH was bought from ProteinTech (Shanghai, China). Bound antibodies were visualized with the ECL kit (Beyotime, China).

Generation of stable cell lines

To over-express BANCR and TSHR, Vectors containing full length of BANCR and TSHR were purchased from GeneCopoeia (Shanghai, China). Thyroid cancer cells were transfected using lipofectamine 2000 (Invitrogen) with those vectors following the manufacturer's protocols. The supernatant media containing virus was collected by centrifugation to remove cellular contaminant. The resulting viruses were used to infect indicated cells, and then integrated cells were selected by 2 µg/ml puromycin for 2 weeks.

Cytotoxic assay

Sulforhodamine B (SRB, obtained from Sigma, USA), was used to measure the effect of luteolin (obtained from Sigma, USA) on the viability of thyroid cancer cell lines. Thyroid cancer cells were cultured in a 96-well plate for 24 h. After exposure of cultured cells to luteolin at various concentrations for 24 h or 48 h, cells were fixed with ice-cold trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid. Excess dye was removed by rinsing several times with

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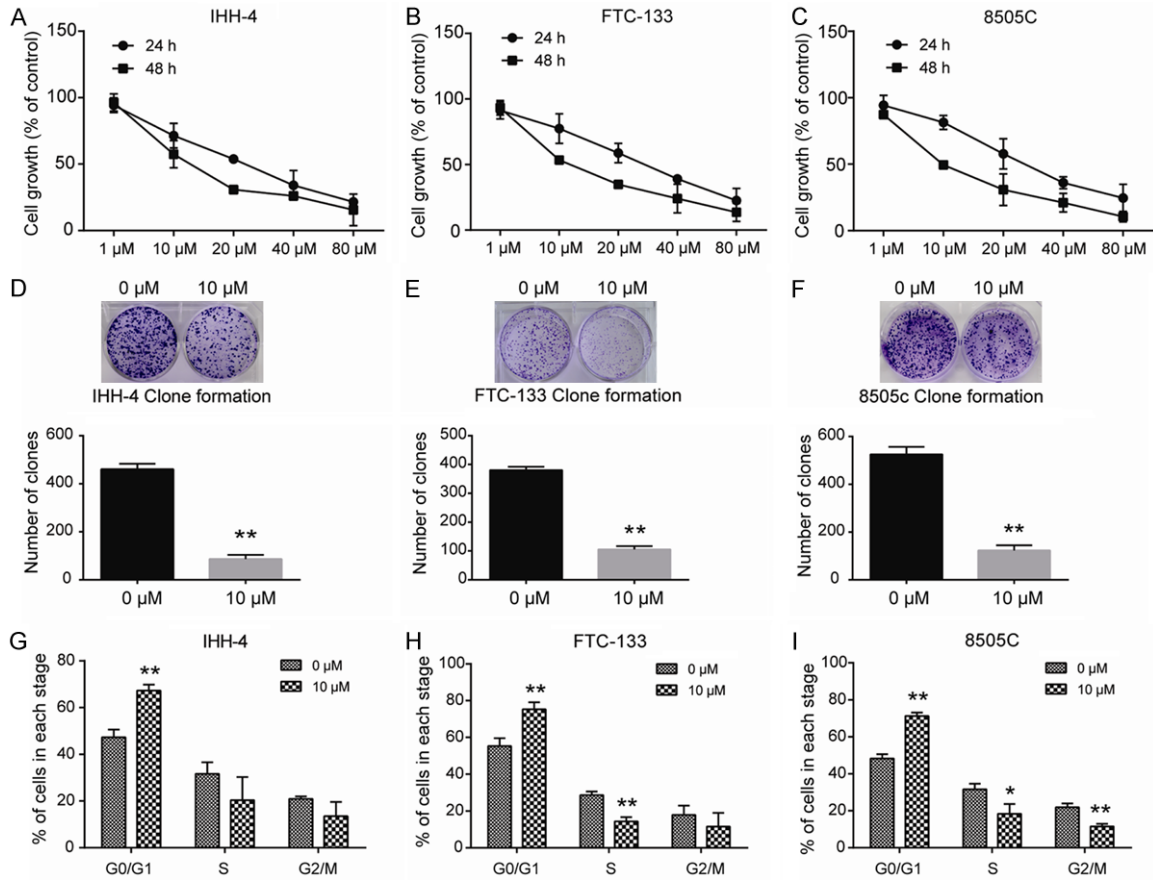


Figure 1. Luteolin exhibits an anti-tumor effect on thyroid cancer cells in vitro. A-C. Thyroid cancer cells, IHH-4, FTC-133 and 8505C were treated with various concentration of luteolin (1, 10, 20, 40 and 10 μ M) for 24 or 48 h. The cell numbers were determined by the SRB assay, and the results are presented as percentage of control. D-F. The clone formation assay was performed in IHH-4, FTC-133, and 8505C cell treated by 10 μ M luteolin, and compared with 0 μ M luteolin. G-I. Roles of Luteolin cell cycle distribution on thyroid cancer cells were analyzed by Flow cytometry. * $P < 0.05$; ** $P < 0.01$. All the *in vitro* assays were triplicate.

acetic acid, and protein-bound dye was dissolved with 10 mM Tris base solution for determination of absorbance with a microplate reader with a filter wavelength of 570 nm. Cell growth inhibition was expressed in terms of percentage of untreated control absorbance following subtraction of mean background absorbance.

Cell viability assay

Cells were seeded into a 96-well plate at 5×10^3 cells per well with 100 μ l cultured medium cultured at 37°C, 5% CO₂. The cell viability was quantified by addition 10 μ l of cell counting kit-8 (CCK8, Dojindo, Japan). After 1.5 hours incubation, the plates were monitored by Power Wave XS microplate reader (BIO-TEK) at an

absorbance 450 nm. Results are presented of three independent experiments.

Clonogenic assay

The indicated cells (1×10^3 /well) were plated in a 6-well plate and cultured for 14 days. Subsequently, formed colonies were then stained with 0.5% crystal violet, and the number of colonies under a microscope and cell number in each colony was at least 20 cells.

Cell cycle analysis

The indicated cells (20×10^4 /well) were seeded in the 6-well plates. Then these cells were collected at 24 h, 48 h and 72 h. Cells were washed twice with $1 \times$ PBS, then resuspended and fixed in 2 ml 70% ethanol at -20°C. Cells

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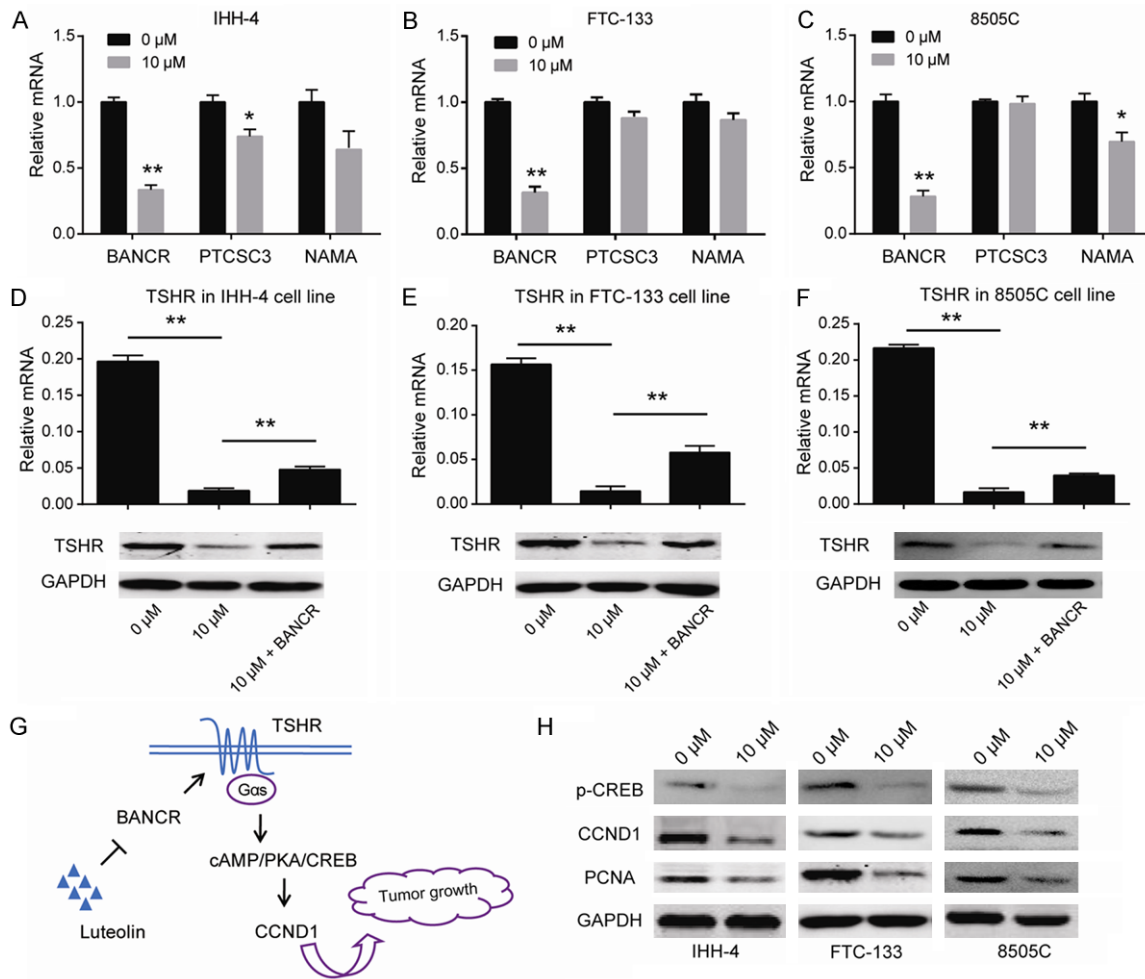


Figure 2. Luteolin modulates the BANCR/TSHR/CCND1 signaling in thyroid carcinoma. A-C. RT-PCR analysis showed that luteolin suppression significantly decreased mRNA expression of BANCR in IHH-4, FTC-133 and 8505C cells but performed no significant effects on lncRNA PTCS3 and NAMA. D-F. The expressions of TSHR in IHH-4, FTC-133 and 8505C cells were determined by qRT-PCR and Western blotting when treated with 10 μ M luteolin and overexpression of BANCR. G. Hypothesis of the mechanism by which luteolin inhibits tumor growth of thyroid carcinoma. H. The potential downstream factors of classical TSHR signaling pathway, p-CREB, CCND1 as well as the proliferation index PCNA in thyroid cancer cells were detected by Western blotting, when cells treated with 10 μ M luteolin. ** $P < 0.01$.

were then stained with PI (BD) and followed as the manufacture's protocol.

Animal study

Athymic nude mice (5-week-old) were obtained from the Animal Facility of Shandong University. They were cultured in pathogen-free sterile conditions with continuous access to sterile food and water. Approximately 5×10^6 cells were injected subcutaneously into the right flank of each nude mouse. After tumors grew to ~ 100 mm³, the mice were treated with 50 mg/kg luteolin. Tumor growth was observed every

three day. Tumor volume was calculated as follows: Volume = (Length) \times (Width)²/2.

Statistical analysis

Statistical analyses were performed using the SPSS 16.0 (SPSS, Chicago, IL, USA) or Prism 5.0 (GraphPad, La Jolla, CA, USA). All Data were presented as mean \pm SD. Paired data were analyzed by Student's t test, and group data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey correction, as appropriate. The p value < 0.05 was considered to be statistically significant.

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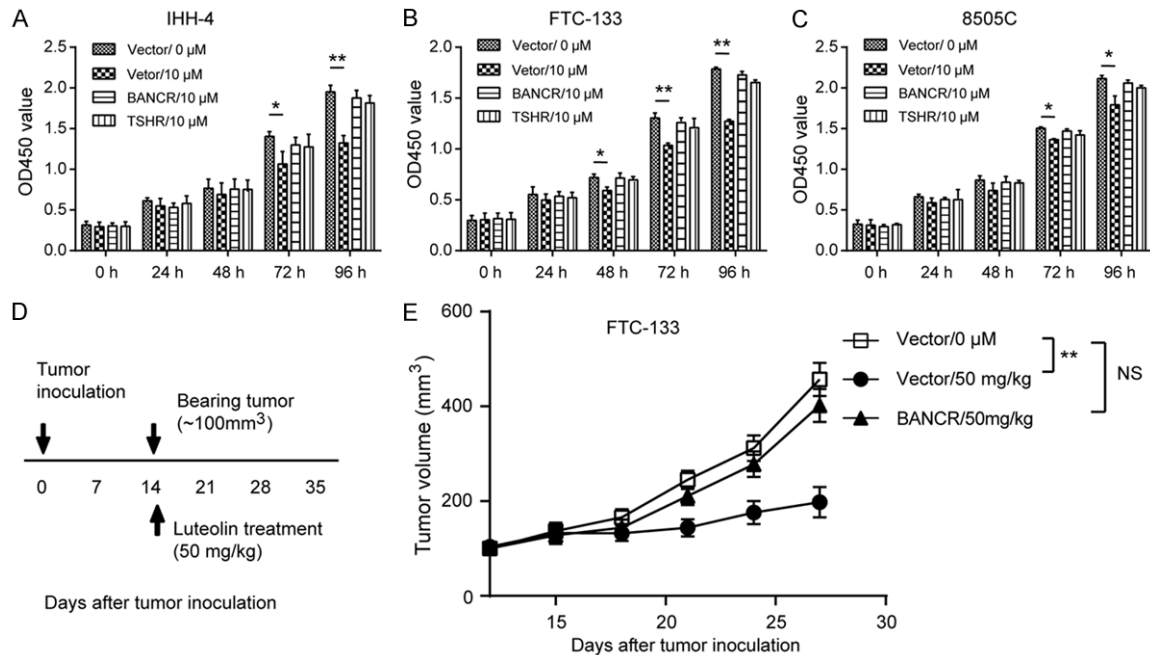


Figure 3. Overexpression of BANCR/TSHR signaling attenuates luteolin-induced growth inhibition in thyroid carcinoma. A-C. The effects of luteolin (10 μM) on the cell proliferation of BANCR-overexpressing, TSHR-overexpressing, and vector IHH-4, FTC-133 and 8505C cells were determined by CCK8 assays at 0 h, 24 h, 48 h, 72 h, and 96 h. The mean and SD of triplicate experiments are presented. D. The schema for animal study. E. The tumor volume of the mice in indicated groups was monitored since the start of luteolin treatment (n = 5). *P < 0.05 and **P < 0.01. NS, not significant.

Results

Luteolin exhibits an anti-tumor effect on thyroid cancer cells in vitro

To demonstrate whether luteolin has a growth inhibitory effect on human thyroid cancer cells, three thyroid cancer cell lines (IHH-4, FTC-133, and 8505C) were subjected to treatment with different concentrations of luteolin and SRB assay was used to evaluate the cell viability. As shown in **Figure 1A-C**, the cell growth of three cell lines was strongly inhibited after 24 h and 48 h of treatment with luteolin in a dose-dependent manner. To determine the effect of luteolin on longer term viability and replicative potential of thyroid cancer cells, the clone formation assay were performed. As shown in **Figure 1D-F**, 10 μM luteolin caused a significantly decrease in colony forming ability of thyroid cancer cells when compared with negative control.

To further examine whether luteolin-induced growth inhibitory effect on thyroid cancer was mediated via alterations in cell cycle progres-

sion, flow cytometry was used to analyze the effect of luteolin on the cell cycle phase distribution in IHH-4, FTC-133 and 8505C cells. As a result, luteolin caused a significant increase in the distribution of cells at G1/S phase (**Figure 1G-I**). Collectively, these findings above indicate that luteolin does exhibit an inhibitory effect on the growth of thyroid carcinoma.

Luteolin modulates the BANCR/TSHR/CCND1 signaling in thyroid carcinoma

Previously, luteolin has been reported to inhibit tumor progression by reversing epithelial-mesenchymal transition [16, 17], induction of endoplasmic reticulum stress via reactive oxygen species [18], inhibition of the Nrf2 pathway [19], targeting p90 ribosomal S6 kinase [20, 21], and etc. However, we failed to observe these phenomena in thyroid carcinoma cells (data not shown). Recently, involvement of lncRNAs in regulation of thyroid cancer cell proliferation has been reported [22]. To investigate the molecules affected by luteolin, we focused on lncRNAs in thyroid cancer formation and progression, including BANCR, PTSC3 and

NAMA. The results showed that treatment of IHH-4, FTC-133 and 8505C cells with 10 μ M luteolin resulted in a dramatic decrease in mRNA level of BANCR but no significant alteration of PTSC3 and NAMA, as shown in **Figure 2A-C**. Recently, we have reported that BANCR is enriched by polycomb enhancer of zeste homolog 2 (EZH2), and silencing BANCR lead to decreased chromatin recruitment of EZH2, which results in significantly reduced expression of thyroid stimulating hormone receptor (TSHR) [23]. Indeed, TSHR was significantly decreased by treatment with luteolin at both mRNA and protein level, and this trend could be partially rescued by overexpression of BANCR, especially at protein level (**Figure 2D-F**). Classically, activation of TSHR leads to activation of downstream cAMP/PKA/p-CREB signaling, which ultimately contributes to CCND1 expression (**Figure 2G**) [24]. As shown in **Figure 2H**, we found that downstream factors of TSHR, p-CREB and CCND1, were significantly decreased by luteolin treatment. Collectively, these findings above suggest that luteolin may suppress tumor growth of thyroid carcinoma through inhibition of BANCR/TSHR signaling pathway.

Overexpression of BANCR/TSHR signaling attenuates luteolin-induced growth inhibition in thyroid carcinoma

To further prove the role of luteolin on BANCR/TSHR signaling, we overexpressed BANCR and TSHR in IHH-4, FTC-133 and 8505C cells and applied CCK8 assay to examine the cell viabilities at 0 h, 24 h, 48 h, 72 h and 96 h upon luteolin treatment. We found that overexpression of BANCR or TSHR largely compromised the inhibitory effect of luteolin on cell viabilities (**Figure 3A-C**). Furthermore, we validated the anti-tumor effect of luteolin in FTC-133 derived xenograft models (**Figure 3D**). The results showed that growth of FTC-133 tumors was significantly inhibited by administration of luteolin. Meanwhile, overexpression of BANCR also largely abolished the inhibitory effect of luteolin on tumor growth (**Figure 3E**).

Discussion

Although there are numerous articles reported that luteolin has influence on a series of cancer development [25-29], the effects of luteolin in the pathogenesis of thyroid cancer have new

understanding. In the current study, we show that luteolin significantly suppress thyroid cancer growth, suggesting the possible potential natural compound to prevent thyroid cancer. Luteolin can inhibit the proliferation, arrest cell cycle progression and induce apoptosis of KKKU-M156 cells, and also suppresses the viability of TNBC cells and blocks their metastasis to the lungs [30]. Our findings not only similarity with these previous reports that luteolin significantly decrease cancer progression but also extend its molecular mechanism to thyroid carcinoma.

Because carcinogenesis is characterized by unregulated clonal expansion of malignant cells, one of the requisite actions of an anticancer agent is to impede the uncontrollable proliferation and accelerate death of tumor cells. In this study, we found that luteolin has a remarkable effect in the induction of growth arrest in thyroid cancers. The ability of luteolin to inhibit thyroid cancer cell growth seemed to be more potent when compared to other investigators [16, 20, 31]. To examine the mechanism responsible for cell growth inhibition, cell cycle distribution was determined by flow cytometry. The inhibition capacity of thyroid cancer cells treated by luteolin was associated with G1/S phase arrest, which related to downregulation of BANCR and TSHR. This pattern was similarity with the silence of BANCR in our previous study. Only several lncRNAs including PTSC3, NAMA and BANCR has been demonstrated as the key lncRNAs in the development and progression of thyroid cancer, and TSHR expression is often silenced in thyroid cancer associated with absent TSH-promoted iodine uptake and has been found mainly on FTC cells [13-15]. In the current study, BANCR is dramatically decreased in thyroid cancer cells when treated with luteolin, but no significant change of NAMA and PTSC3. The downregulation of BANCR may result in significant decline of the TSHR mRNA and protein level because of luteolin. The potential downstream molecular of TSHR in the different thyroid cancer cells was also down-regulated when treated with luteolin, especially CCND1, which is responsible for cell cycle progression [32]. Therefore we conclude that luteolin can suppress thyroid cancer cell growth through decreasing BANCR and TSHR as evidenced by both in vitro and in vivo studies (**Figure 3**). However, the clear link between lute-

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olin and BANCR, in other words, how luteolin inhibits BANCR expression in thyroid cancer warrants further investigation.

In summary, for the first time, the potential anti-cancer activity against thyroid cancer and the underlying mechanisms of luteolin were investigated in this study. Luteolin exhibits effective cytotoxicity against thyroid cancer cells *in vitro* and *in vivo*. Cell cycle arrest could be attributed in part to its proliferation inhibition. In addition, luteolin may serve as a potential important anticancer agent for thyroid cancer progression by blocking the BANCR/TSHR signaling.

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Disclosure of conflict of interest

None.

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