

# CTGF is overexpressed in papillary thyroid carcinoma and promotes the growth of papillary thyroid cancer cells

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Received: 17 January 2011 / Accepted: 29 March 2011 / Published online: 20 April 2011  
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**Abstract** Connective tissue growth factor (CTGF or CCN2), which belongs to the CCN family, is a secreted protein. It has been implicated in various biological processes, such as cell proliferation, migration, angiogenesis, and tumorigenesis. In this study, we found that CTGF expression level was elevated in primary papillary thyroid carcinoma (PTC) samples and correlated with clinical features, such as metastasis, tumor size, and clinical stage. Overexpression of CTGF in PTC cells accelerated their growth in liquid culture and soft agar as well as protecting PTC cells from apoptosis induced by IFN-gamma treatment. Downregulation of CTGF in PTC cells inhibits cell growth in liquid culture and soft agar and induces the activation of caspase pathway and sensitized PTC cells to apoptosis. Our data suggest that CTGF plays an important role in PTC progression by supporting tumor cell survival and drug resistance, and CTGF may be used as a potential tumor marker for PTC diagnosis.

**Keywords** Connective tissue growth factor · Papillary thyroid carcinoma · Proliferation · Apoptosis · Drug resistance · Tumor marker

## Introduction

Thyroid cancer is one of the few malignancies that are increasing in incidence in the world [1, 2]. The major type of thyroid cancer is papillary thyroid carcinoma (PTC). The development of this malignancy rises from the stepwise accumulations of multiple genetic alterations in key signaling effectors, which lead to the activation of oncogenes and/or the inactivation of tumor suppressor genes. The differential expression of these critical genes and their downstream effectors enables cells to override growth controls and eventually undergo carcinogenesis. Mutations of p53 [3], RAS [4], and B-RAF [5], rearrangement of PPARG [6] and RET/PTC [7], and amplification and overexpression of FGFR and EGFR [8] have been observed in thyroid cancer. However, the precise mechanisms of thyroid cancer at the molecular level are still poorly understood.

Connective tissue growth factor (CTGF), a cysteine-rich protein, belongs to the CCN family, which consists of six members: Cyr61 (cysteine-rich protein 61, CCN1), CTGF/CCN2, Nov (nephroblastoma overexpressed gene, CCN3), WISP-1 (Wnt-1-induced secreted protein 1, CCN4), WISP-2 (CCN5), and WISP-3 (CCN6) [9]. Besides NH<sub>2</sub>-terminal signal peptide, it has four structural domains with sequence similarities to insulin-like growth factor-binding proteins, von Willebrand type C factor, thrombospondin 1, and a cysteine knot [10], each of which is thought to have different biological function [11].

Increasing evidence has indicated that CTGF has a function in tumorigenesis. Its upregulation was observed in prostate cancer [12], glioma [13], breast cancer [14], and esophageal squamous cell carcinoma [15]. In contrast to that, downregulation of CTGF was found in lung cancer [16] and colon cancer [17], in which forced expression of

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CTGF blocked invasion and metastasis of the cancer cells both in vitro and in vivo. Also, CTGF expression level correlates with increased survival in chondrosarcoma patients [18]. These disparities in expression in different type of tumors imply that the function of CTGF may be cell type specific.

In this study, we found that CTGF was overexpressed in papillary thyroid carcinoma. Furthermore, we demonstrated that the overexpression of CTGF in thyroid cancer cell line promoted cell growth and inhibited cell apoptosis. However, knockdown CTGF inhibited cell growth and sensitized the cells to apoptosis as well as upregulated the expression of apoptosis-related molecules. These observations suggest that CTGF might serve as a therapeutic target in thyroid cancer.

## Material and methods

### Primary thyroid tissue samples

Primary tissues were collected from patients who received surgery for papillary thyroid cancer at ZhongShan Hospital, Shanghai. All patients had given informed consent. Dissected samples were frozen immediately after surgery and stored at  $-80^{\circ}\text{C}$  until needed.

### Cell culture

Thyroid cancer cell line BHP and TPC-1 was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) and cultured in RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum, 10 units/ml penicillin-G, and 10 mg/ml streptomycin. Cells were incubated at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  humidified air.

### Plasmid construction and transfection

The human CTGF cDNA was cloned into the eukaryotic expression vector pCMV-tag2B (Invitrogen) and fused with a C-terminal Flag tag. The insert was confirmed by sequencing. The CTGF expression vector and empty pCMV-tag2B were transfected into BHP cells using Lipofectamine 2000 reagent (Invitrogen). The transfected cells were selected with G418 at the concentration 800  $\mu\text{g}/\text{ml}$ , and resistant clones were further confirmed by Western blotting.

### Real-time PCR analysis

The CTGF and  $\beta$ -actin mRNA were analyzed by real-time reverse transcriptase PCR. Briefly, amplification reactions were performed in a 20- $\mu\text{l}$  volume of the LightCycler-DNA

Master SYBR GreenI mixture from Roche Applied Science. PCR reaction included components as follows: 10 pmol of primer, 2 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  dNTP mixture, 0.5 units of Taq DNA polymerase, and universal buffer. The thermal cycling conditions were as follows:  $95^{\circ}\text{C}$  for 3 min; 40 cycles of  $95^{\circ}\text{C}$  for 30 s,  $58^{\circ}\text{C}$  for 20 s, and  $72^{\circ}\text{C}$  for 30 s;  $72^{\circ}\text{C}$  for 10 min. The specificity of amplification was examined by melting curve analysis and electrophoresis in 2% agarose gel. All of the reactions were performed in triplicate in an iCycler iQ System (Bio-Rad). The relative mRNA level was presented as unit values of  $2^{[\text{Ct}(\beta\text{-actin})-\text{Ct}(\text{gene of interest})]}$ . The primer sequences for the human CTGF gene were as follows: forward primer, 5'-CGACTGGAAGACACGTTTGG-3', and reverse primer, 5'-AGGCTTGGAGATTTGGGAG-3'. As an internal standard, a fragment of human  $\beta$ -actin was amplified by PCR using the following primers: forward primer, 5'-GATCATTGCTC CTCCTGAGC-3', and reverse primer, 5'-ACTCCTGCTTG CTGATCCAC-3'.

### Extract protein from tissue samples

Frozen tissue was pulverized and placed in a 1.5-ml round bottom microcentrifuge tube. Then, 500  $\mu\text{l}$  lysis buffer with inhibitors to tissues was added. Tissues were homogenized with a minipestle-homogenizer using 15 strokes, 3 s per stroke on ice, and centrifuged at 12,000g for 15 min at  $4^{\circ}\text{C}$ . Supernatant (without lipid layer) was removed and transferred into another 1.5-ml tube. Tissues were centrifuged again at 12,000g for 15 min at  $4^{\circ}\text{C}$ , and the supernatant was transferred to another tube. The supernatant fraction contains the extracted proteins.

### Western blot analysis

Western blot analysis was done as previously described [15]. Primary antibodies to CTGF, Flag-tag, caspase-3, and caspase-9 were purchased from Santa Cruz Biotechnology, and antibody to  $\beta$ -actin was purchased from Sigma. Secondary antibodies (rabbit anti-mouse IgG (Sigma) and goat anti-rabbit IgG (Cell Signaling Technology)) were used at a dilution of 1:500. Primary antibodies were diluted in TBST containing 1% BSA and  $\text{NaN}_3$ . The immunoreactive protein bands were visualized by ECL kit (Pierce).

### Immunohistochemistry

For immunohistochemistry, cancerous and corresponding normal thyroid tissues were frozen in a cryostat chamber, and 10-mm sections were collected on glass slides. The sections were fixed in ice-cold acetone for 30 min, washed in 0.01 M PBS for  $3 \times 5$  min, blocked for 1 h in 0.01 M PBS supplemented with 0.3% Triton X-100 and 5% normal serum, and then incubated with goat anti-human CTGF

(polyclonal, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody at 4°C overnight. After brief washes in 0.01 M PBS, sections were incubated for 2 h in 0.01 M PBS with horseradish peroxidase-conjugated rabbit anti-goat IgG (1:1,000; Chemicon, Temecula, CA, USA), followed by visualization with 0.003% H<sub>2</sub>O<sub>2</sub> and 0.03% DAB in 0.05 M Tris-HCl (pH 7.6). Negative controls consisted of substitution of the primary antibody with normal serum at the same dilution. Immunohistochemistry for each individual was performed at least three times, and all sections were counterstained with hematoxylin. Scoring of immunohistochemistry staining was carried out independently by three pathologist blinded to the patient's clinical parameters. All stained sections were scored both in the tumor and adjacent nontumor areas at least in ten high-power field areas with a minimum of 300 preserved cells assessed in each area. The percentage of cells expressing target protein was estimated by dividing the number of positive cells by the number of total cells per high-power field area. Thyroid cells bearing obvious brown signal in the cytoplasm compared with negative control were defined as positive cells. Paired-samples *t* test was used to evaluate the difference between protein levels of CTGF in cancer samples compared with matched normal thyroid tissues. Results were considered statistically highly significant at  $P < 0.01$ . All statistical analyses were performed using the program SPSS for Windows (SPSS, Chicago, IL).

#### MTT assay

In vitro cell growth was measured by MTT assay. Before treatment, cells were plated in 96-well plates with a concentration of 2,000 cells per well. To measure cell growth, 20  $\mu$ l 5 mg/ml MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added into the media and cultured at 37°C. After 5 h, 200  $\mu$ l DMSO was added to resolve the generated formazan right after removing the cell media, and the OD540 value of the solvent was measured by an automatic microplate reader. The measurement process was performed every 24 h for 4 or 5 days to generate a cell growth curve.

#### RNAi-mediated knockdown of CTGF

Target sequence for CTGF small interfering RNA was as listed: 5-CTGACCTGGAAGAGAACA-3. The control nucleotide sequence of small interfering RNA was GTACATAGGGACGTAACG, which was the random sequence that was not related to CTGF mRNA. FG12 RNAi vector was used to produce small double-stranded RNA (small interfering RNA) to inhibit target gene expression in thyroid cancer cells.

#### Apoptosis analysis

Cells were trypsinized, washed twice with PBS, centrifuged (2,000g, 5 min), reconstituted with 250  $\mu$ l PBS and 750  $\mu$ l ice-cold ethanol (4°C, 1 h). This was followed by repeated washed with PBS twice, resuspended in PBS containing propidium iodide and RNase for 1 h at 37°C, and analyzed by flow cytometry.

#### Soft agar assay

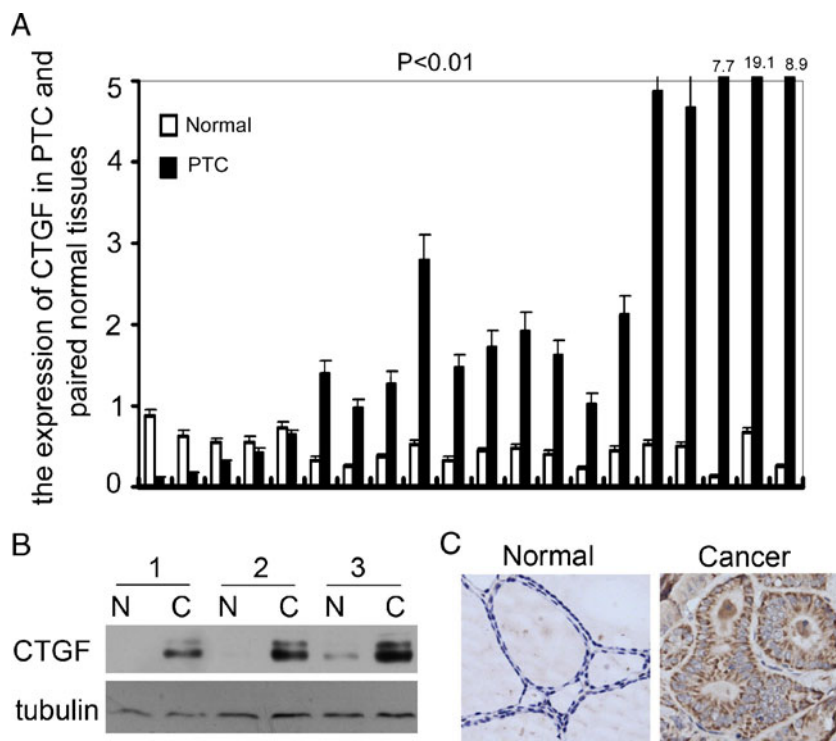
For base agar, 1% agar (DNA grade) was melted in microwave and cooled to 40°C in a water bath, and 2 $\times$  DMEM/F12+additives were warmed to 40°C in water bath followed by mixing equal volumes of the two solutions to give 0.5% agar + 1 $\times$  DMEM/F12 + additives. Then, 1.5 ml was added to Petri dish. For top agar, 0.7% agar (DNA grade) was melted in microwave and cooled to 40°C in a water bath, and 2 DMEM/F12 + additives were warmed to the same temperature. Cell were trypsinized, counted, and added 0.1 ml of cell suspension to 10-ml centrifuge tubes. We labeled 35-mm Petri dishes with base agar appropriately. For top agar, 3 ml 2 $\times$  DMEM/F12 + Additives was added, and 3 ml 0.7% agar was stored to a tube and mixed gently, and 1.5 ml to each replicate plate was added. Assay was incubated at 37°C in humidified incubator for 10–14 days. Plates were stained with 0.5 ml of 0.005% Crystal Violet for 1 h, and colonies were counted using a dissecting microscope.

## Results

### CTGF is overexpressed in papillary thyroid carcinoma

To investigate the expression pattern of CTGF in PTC samples, levels of CTGF mRNA were quantified by RT-PCR in 20 pairs of tumors and their matched normal thyroid tissues (Fig. 1a). Expression levels were shown as a ratio between CTGF and the reference gene  $\beta$ -actin to correct for the variation in the amounts of RNA. To determine whether  $\beta$ -actin is suitable for the calibrator of normalization, a second housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, was also used as a reference calibrator for CTGF in 20 paired normal and thyroid cancer samples. The expression level of the CTGF gene was comparable with those when  $\beta$ -actin was used as the reference gene (data not shown). Upregulation of CTGF gene occurred in 15 of 20 (75%) thyroid cancers compared with the paired normal thyroid tissues. In addition, elevated levels of CTGF protein were found in human thyroid cancer tissues compared with the paired normal tissue from the patients as shown by Western analysis (Fig. 1b). In order to confirm these results, we performed the immunohistochemistry to investigate the

**Fig. 1** Expression of CTGF was elevated in paired PTC samples. **a** Relative expression of CTGF mRNA in paired human PTC samples and normal thyroid tissues. Semiquantitative RT-PCR was performed on 20 paired PTC RNA samples. Data were calculated from triplicates. **b** Expression of CTGF protein in three, randomly picked, paired PTC samples were analyzed by Western blot. **c** Immunohistochemical analyses of CTGF expression in PTC and matched normal thyroid tissues



expression of CTGF in 72 paired thyroid carcinoma samples. It was found that the expression of CTGF was upregulated in the cancer and correlated with tumor metastasis, size, and clinical stage (Fig. 1c and Table 1). These results indicate that it is possible that CTGF might play a very important role in the progression of thyroid cancer.

Forced expression of CTGF in papillary thyroid carcinoma cell line BHP stimulated their growth and colony formation and inhibited cell apoptosis

To examine the effects of CTGF on the growth of papillary thyroid carcinoma cells, BHP cells were stably transfected with either a pCMV-tag2B/CTGF containing full-length CTGF or an empty vector pCMV-tag2B as a control. G418-resistant clones were screened for CTGF expression by Western blot analysis. Two pCMV-tag2B/CTGF stably transfected clones with high expression of CTGF (BHP/CTGF1# and 2#) are shown in Fig. 2a, which were used for further study. The effect of CTGF on cell growth was evaluated by MTT assays. The rate of growth of BHP/CTGF1# and BHP/CTGF2# was about 2.0- and 1.5-fold greater than the BHP/pCMV-tag2B control cells in MTT assay, respectively (Fig. 2b). Next, the effects of CTGF on cell apoptosis were examined using FACS. We found that overexpression of CTGF in BHP cells inhibited cell apoptosis at the basal level (Fig. 4a). This result suggested that the ability of CTGF on promoting cell growth was attributed to its function of inhibiting cell apoptosis.

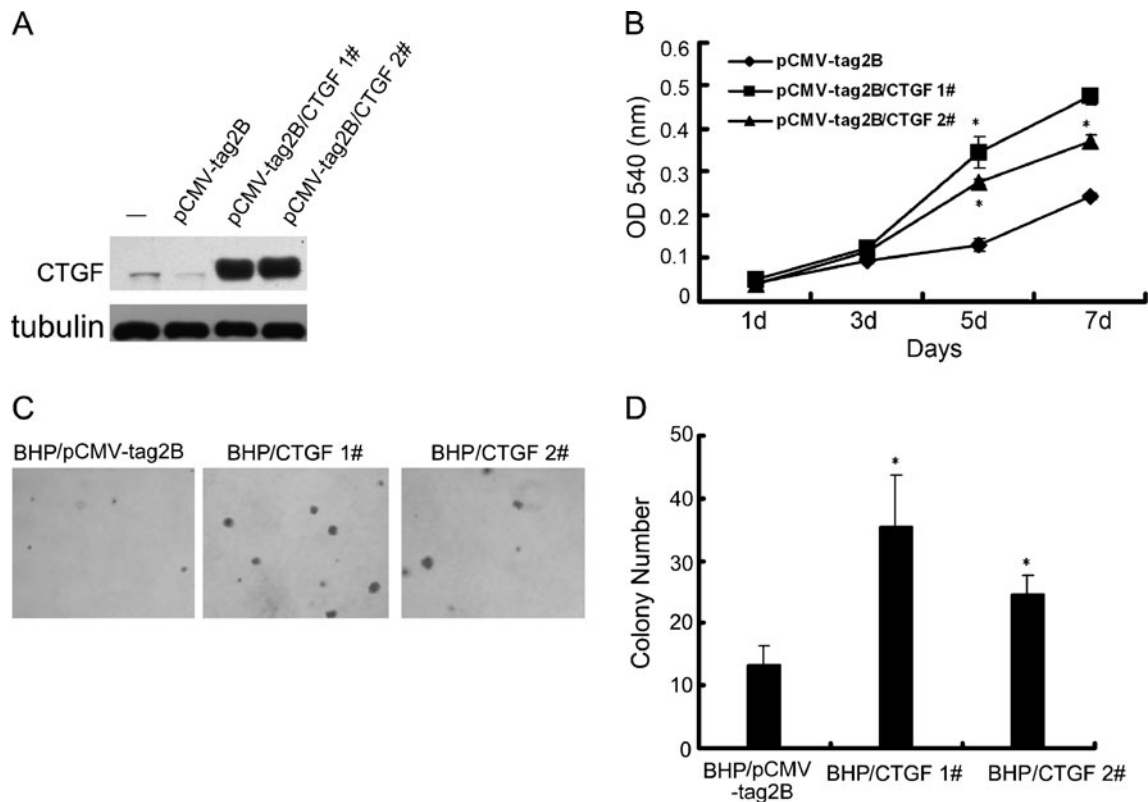
Anchorage-independent assay was a good method to test the tumorigenesis ability of the cancer cells in vitro. In

**Table 1** Relationship between CTGF expression in thyroid carcinoma and the clinical features

Clinical characteristic	CTGF expression		$\chi^2$	P
	Positive	Negative		
Gender				
Male	8	18	14.18	0.000**
Female	35	11		
Metastasis				
Yes	23	5	9.575	0.002**
No	20	24		
Family history				
Yes	8	7	0.322	0.57
No	35	22		
Tumor size <sup>a</sup>				
I	4	14	15.728	0.000**
II	15	9		
III	24	6		
Clinical stage				
I	3	18	27.597	0.000**
II	21	9		
III	19	2		

\* $P < 0.05$ ; \*\* $P < 0.01$

<sup>a</sup> I  $\leq 15$  cm<sup>3</sup>, II 15–25 cm<sup>3</sup>, III  $\geq 25$  cm<sup>3</sup>



**Fig. 2** CTGF promoted the growth of PTC cells. **a** The expression of CTGF in BHP cells, BHP cells transfected with pCMV-tag2B empty vector and CTGF clones (pCMV-tag2B/CTGF 1# and 2#) using anti-CTGF antibody. **b** Cell growth rates of stably overexpressing CTGF clones (pCMV-tag2B/CTGF 1# and 2#) and their control cells were measured by MTT assay. Results represent the mean $\pm$ SD of three

experiments performed in triplicate. **c** Soft agar assays of CTGF overexpressed BHP cells and their control cells. A total of 1,000 cells per well were seeded in soft agar for 2 weeks, and colonies were enumerated. The results represent the mean $\pm$ SD of quadruplicate plates of three independent experiments as shown in **d**. \* $p$ <0.05

order to test whether overexpression of CTGF potentiated the tumorigenesis ability of BHP cells in vitro, anchorage-independent assay was performed. The results showed that BHP/CTGF cells formed more colonies on soft agar than control cells, demonstrating CTGF-enhanced tumorigenesis ability in vitro (Fig. 2c–d).

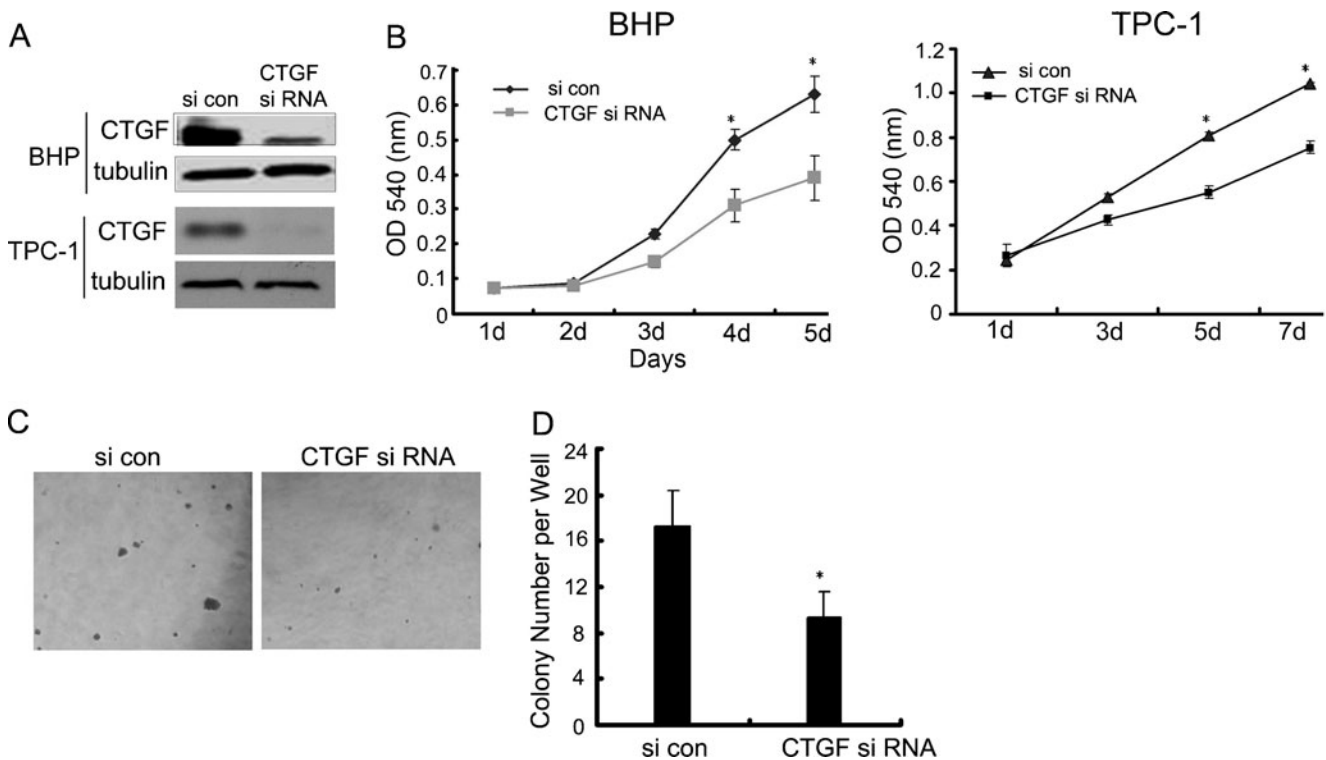
#### Knockdown of CTGF inhibited cell proliferation and promoted cell apoptosis

To determine whether endogenous CTGF played a role in cell growth, we used RNAi-mediated knockdown lentiviral vector to decrease the basal level of CTGF in BHP cells and TPC-1 cells. Western blot results showed that the target interfered RNA markedly diminished the expression of CTGF compared with the control RNA (Fig. 3a). The RNAi-mediated knockdown of CTGF expression dramatically inhibited cell growth of these cells in liquid culture (Fig. 3b) by MTT assay as well as their capacity for clonogenic growth (Fig. 3c–d) by soft agar assay. These data further confirmed the function of CTGF observed in

the gain-of-function assay and implicated their role in tumorigenesis.

CTGF inhibited both basal level and drug-induced cell apoptosis

CTGF has been reported to confer the ability of drug resistance on tumor cells [19]. Clinically, IFN-gamma has the antitumor effects on treating thyroid cancer [20]. Therefore, in the next study, the effect of CTGF on IFN-gamma resistance was investigated. Forced expression of CTGF inhibited cell apoptosis at the basal level with 5.38% apoptotic cells in cells stably expressing CTGF and about 10% apoptotic cells in the control group. Treating the control cells with IFN-gamma led to about 21% apoptotic cells comparing about 6% apoptotic cells in CTGF overexpressed cells. These results indicated that CTGF protected cells from apoptosis (Fig. 4a–b). However, silencing the expression of CTGF sensitized the cells to apoptosis at basal level (Fig. 4c–d). Moreover, knockdown of CTGF activated caspase 3 and caspase 9,



**Fig. 3** Knockdown of CTGF inhibited the growth of PTC cells. **a** Knockdown the expression of CTGF in BHP cells and TPC-1 cells. **b** Cell growth rates of BHP cells and TPC-1 cells, which knocked down the expression of CTGF and their control cells, were measured by MTT assay. Results represent the mean $\pm$ SD of three experiments

performed in triplicate. **c** Soft agar assays of BHP cells which knocked down the expression of CTGF and their control cells. A total of 1,000 cells per well were seeded in soft agar for 2 weeks, and colonies were enumerated. The results represent the mean $\pm$ SD of quadruplicate plates of three independent experiments as shown in **d**. \* $p$ <0.05

which triggered cell apoptosis pathway (Fig. 4e). Taken together, CTGF inhibited both basal level and drug-induced cell apoptosis.

## Discussion

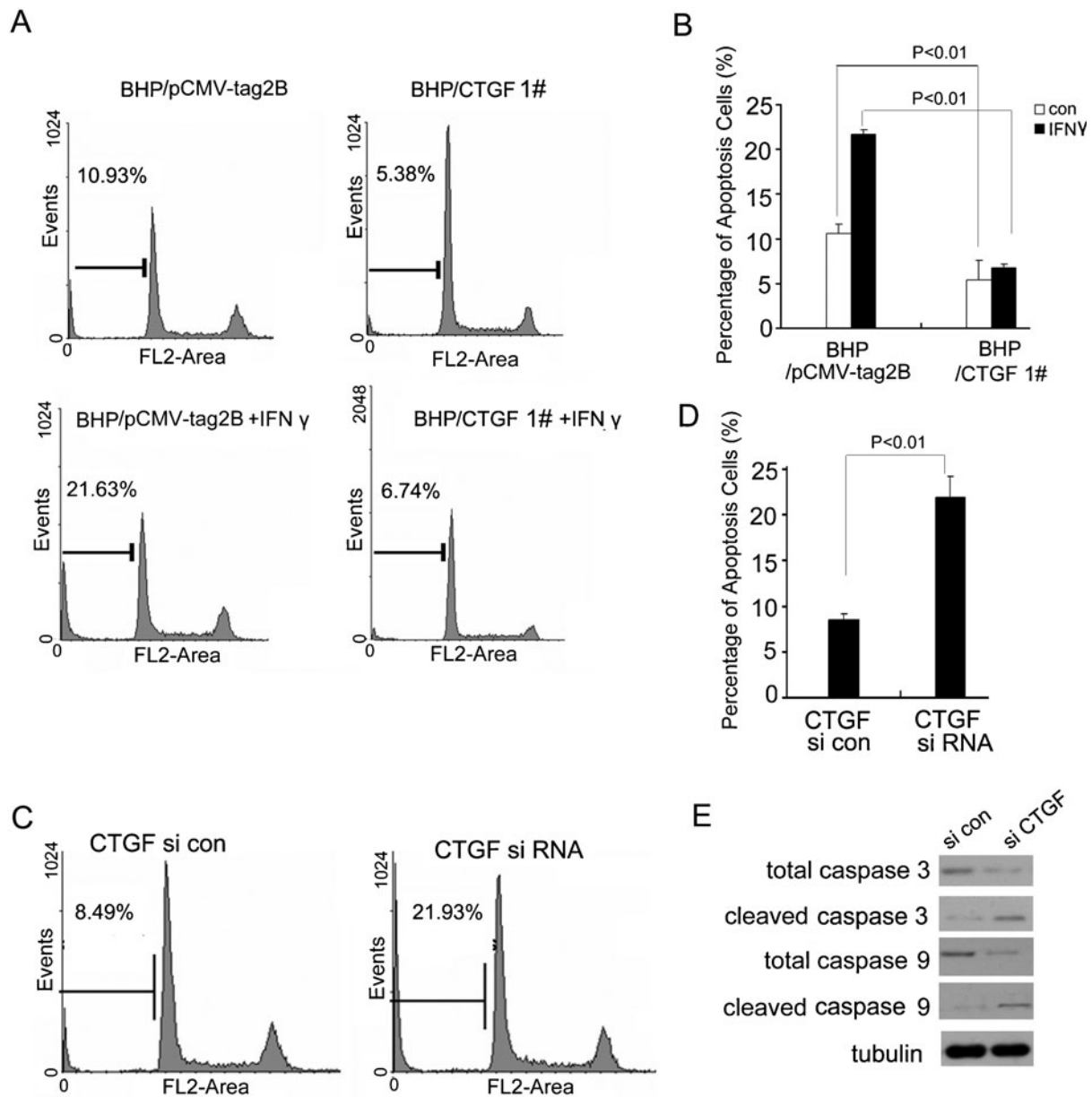
Thyroid cancer includes anaplastic thyroid carcinoma (ATC), papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), and other clinical types. Although DTC (PTC and FTC) has a relative favorable prognosis, it is very common that DTC could transform to ATC [21]. Therefore, it is very urgent to identify novel diagnostic markers and drug-target molecules in DTC.

In this study, it was found that the expression of CTGF was upregulated in a panel of PTC samples compared to the matched normal tissues. These results indicated that CTGF might serve as a potential biomarker for PTC patients as CTGF was a secreted protein and could be detected easily in the blood. To better understand how CTGF affects formation and progression of PTC, we study PTC cell lines

with forced expression of CTGF. For example, forced expression of CTGF in BHP clones promoted cell growth in liquid culture and clonogenic proliferation in soft agar culture. In addition, CTGF helps BHP cell's resistance to apoptosis induced by IFN-gamma. Finally, knockdown of CTGF stimulates PTC cells to undergo apoptosis by activating caspase pathway.

The involvement of CTGF in tumorigenesis has been examined by several other investigators. Individuals whose gastric carcinoma expressed a high level of CTGF had significantly lower survival rates than those with a low CTGF expression [22]. However, it was noted that patients with lung [23], breast [24], and gallbladder [25] cancers, as well as cartilaginous malignancies [9], whose tumors had high CTGF expression, had significantly better survivals. These data suggest that the role of CTGF in tumor progression depends on the tumor type.

Cancer cells have a growth advantage compared to their normal counterparts because they often have decreased apoptosis allowing them to remain in the proliferation pool. As shown in Fig. 4, the apoptotic pathway appears to be



**Fig. 4** CTGF showed anti-apoptosis activity in BHP cells. **a** Over-expression of CTGF (BHP/CTGF 1#) inhibited both basal level apoptosis and apoptosis induced by IFN-gamma. **b** The quantification

of **a** was shown. **c** Silencing the expression of CTGF sensitized PTC cells to apoptosis. **d** The quantification of **c** was shown. **e** Knockdown of CTGF lead to the activation of caspase pathway

very aberrant when the expression of CTGF in PTC cells was silenced. Recently, Wang et al. reported that CTGF expression was associated with Bcl-xl expression and weak chemotherapy response in breast cancer patients, while overexpression of CTGF caused breast cancer cells to become drug resistant through upregulation of Bcl-xl/cIAP1 [14]. In this study, we found that knockdown of

CTGF also dramatically induced activation of caspase pathway.

Taken together, our findings suggest that prominent level of CTGF provides PTC cells oncogenic activities and drug resistance. At the same time, our study helps explain why high expression of CTGF is observed in PTC samples and identifies CTGF as a therapeutic target.

**Conflicts of interest** None

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