Connective Tissue Growth Factor Is Overexpressed in Esophageal Squamous Cell Carcinoma and Promotes Tumorigenicity through β-Catenin-T-cell Factor/Lef Signaling*

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Connective tissue growth factor (CTGF or CCN2), a member of the CCN family, is involved in diverse biological processes such as cell adhesion, proliferation, and angiogenesis. In this study, we show that overexpression of CTGF occurred in a significant proportion of esophageal squamous cell carcinoma (ESCC) samples that were of a high tumor grade and metastatic. Forced expression of CTGF in Eca109 ESCC cells accelerated their growth in culture and significantly increased tumor formation in nude mice, whereas RNA interference-mediated knockdown of CTGF in ESCC cells significantly inhibited cell growth and colony formation, as well as tumorigenicity in vivo. Moreover, overexpression of CTGF in ESCC cells resulted in the accumulation and nuclear translocation of β-catenin, leading to activation of β-catenin-T-cell factor (TCF)/Lef signaling. Up-regulation of c-Myc and cyclin D1, two target genes of β-catenin-TCF/Lef signaling, was also observed in the CTGF-overexpressing cells. These effects of CTGF in ESCC cells were abolished by transfection with either dominant negative β-catenin or dominant negative TCF4. Furthermore, we identified a β-catenin-TCF/Lef-binding site (TBE) in the promoter region of CTGF and found that CTGF is a transcriptional target of β-catenin-TCF/Lef signaling. Taken together, these results revealed that the interaction of CTGF and β-catenin-TCF/Lef forms a positive feedback loop, which could contribute to the tumorigenicity of ESCC.

Esophageal cancer is the ninth most frequently occurring malignancy in the world, and recent evidence shows that the incidence of this malignancy is increasing (1). Two major types of esophageal cancers are esophageal adenocarcinoma and esophageal squamous carcinoma (ESCC)‡; the latter is most prevalent in China and other Asian countries (2, 3). The development of this malignancy rises from the stepwise accumulations of multiple genetic alterations, 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 (4–6), retinoblastoma (7), and survivin (8), deletion of p16 INK4A (9), and amplification and overexpression of cyclin D1 (10), MMP7 (11), vascular endothelial growth factor, and epidermal growth factor receptor (12) have been observed in ESCC. However, the precise mechanisms of ESCC at the molecular level are still poorly understood.

Connective tissue growth factor (CTGF) is a cysteine-rich protein that was first identified in conditioned medium of human umbilical vein endothelia cells (13). It belongs to the CCN family of genes, which includes five other members as follows: Cyr61 (cysteine-rich protein 61, CCN1), Nov (nephroblastoma overexpressed gene, CCN3), WISP-1 (Wnt-1-induced secreted protein 1, CCN4), WISP-2 (CCN5), and WISP-3 (CCN6) (14, 15). These proteins share a similar structure as follows: NH₂-terminal signal peptide and four conserved domains with sequence similarities to insulin-like growth factor-binding proteins, von Willebrand type C factor, thrombospondin 1, and a cysteine knot characteristic of other growth factors, including platelet-derived growth factor, nerve growth factor, and transforming growth factor (TGF)-β (16).

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‡ The abbreviations used are: ESCC, esophageal squamous cell carcinoma; CTGF, connective tissue growth factor; TCF, T-cell factor; TBE, β-catenin-TCF/Lef-binding site; PBS, phosphate-buffered saline; siRNA, small interfering RNA; RT, reverse transcription; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; r-h, recombinant human; DN, dominant negative; TGF-β, transforming growth factor-β; GFP, green fluorescent protein; RNAi, RNA interference; BrdUrd, bromodeoxyuridine; ChIP, chromatin immunoprecipitation.
Expression and Function of CTGF in ESCC

The expression of CTGF is regulated by various signals, including phosphatidylinositol 3-kinase/AKT (17) and TGF-β signaling pathways (18). Previous studies revealed that CTGF, mainly acting as a downstream mediator of the TGF-β pathway, is expressed in various pathological conditions, including fibrosis (19, 20), scleroderma (21), atherosclerosis (22), and renal diseases (23). Moreover, CTGF plays a role in many other biological processes such as cell proliferation, survival, migration, differentiation, angiogenesis, and wound healing (24). Recent studies have demonstrated that CTGF may have an important role in a variety of human cancers. Overexpression of CTGF is found in prostate cancers (25), gliomas (26), and breast cancers (27). Also, suppression of CTGF expression decreases the survival and myogenic differentiation of human rhabdomyosarcoma cells (28). Paradoxically, in lung adenocarcinomas (29) and colon cancers (30), overexpression of CTGF inhibits invasion and metastasis of the cancer cells both in vitro and in vivo. Also in cartilaginous tumors, inverse correlations exist between malignant phenotypes and the level of CTGF (31).

In this study, we found that CTGF was overexpressed in ESCC, and its expression was associated with the clinical features of these patients. Furthermore, we demonstrated that the oncogenic activity of CTGF was mediated through the activation of β-catenin-TCF/Lef signaling pathway, and CTGF itself was a direct transcriptional target of this pathway. We propose that the cross-talk between CTGF and β-catenin-TCF/Lef signaling forms a positive feedback loop that may contribute to the tumorigenicity of ESCC.

EXPERIMENTAL PROCEDURES

Cell Culture—ESCC cell line Eca109 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) supplemented with 10% fetal bovine serum, 10 units/ml penicillin-G, and 10 mg/ml streptomycin. 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 10 units/ml penicillin-G, and 10 mg/ml streptomycin. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2.

Plasmid Construction and Transfection—To generate the CTGF expression vector, the open reading frame of human CTGF cDNA was cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen) and was fused to a COOH-terminal Myc tag. The CTGF expression vector and empty pcDNA3.1 were transfected into Eca109 cells, using Lipofectamine 2000 reagent (Invitrogen). The transfected cells were selected in 2% agarose gels containing ethidium bromide, and their quality was then determined by visibility of 18 S and 28 S RNA bands under UV light. 2 μg of total RNA with high quality was processed directly to CDNA with the reverse transcription kit (Promega, Madison, WI), following the manufacturer’s instructions, in a total volume of 25 μl. The primer pair used for amplification of the human CTGF gene was as follows: forward primer, 5'-CGACTGGAGAGACGTTGGT-3', and reverse primer, 5'-AGGCTTTGGGATTTGGAG-3'. As an internal standard, a fragment of human β-actin was amplified by PCR using the following primers: forward primer, 5'-ATGATTGCTTCTGTCGAGC-3', and reverse primer, 5'-ACTCTTGCTTGGCTGATCCAC-3'. Amplification reactions were performed in a 20-μl volume of the LightCycler-DNA Master SYBR Green I mixture from Roche Applied Science as follows: with 10 pmo1 of primer, 2 mM MgCl2, 200 μM dNTP mixture, 0.5 units of Taq DNA polymerase, and universal buffer. All of the reactions were performed in triplicate in an iCycler iQ System (Bio-Rad), and the thermal cycling conditions were as follows: 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; 72 °C for 10 min. To confirm specificity of amplification, the PCR products from each primer pair were subjected to a melting curve analysis and electrophoresis in 2% agarose gel.

Western Blot Analysis—Cells were plated into 35-mm dishes and cultured to 80% confluence. The cells were then scraped and lysed in RIPA buffer, and cellular lysates were centrifuged at 10,000 × g (4 °C for 10 min). Protein concentrations were determined using Bradford reagent (Sigma) according to the manufacturer’s instructions. Equal amounts of total cellular protein were mixed with loading buffer (62.5 μl Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 2% mercaptoethanol, and bromphenol blue), boiled for 5 min, and subjected to 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% fat-free dry milk for 1 h at room temperature and incubated overnight with primary antibodies in TBST with 1% bovine serum albumin. After washing with TBST, the membranes were further incubated for 1 h at room temperature with corresponding horseradish peroxidase-conjugated secondary antibody in appropriate dilution and then washed five times with the same buffer. The immunoreactive protein bands were visualized by ECL kit (Pierce). Antibodies to CTGF, c-Myc, c-Jun, β-catenin, and cyclin D1 were purchased from Santa Cruz Biotechnology, and antibody to β-actin was purchased from Sigma.

Establishment of Eca109/CTGF Pooled Transfects—The CTGF-pcDNA3.1 and empty pcDNA3.1 plasmids were transfected into Eca109 cells using Lipofectamine 2000 reagent. The transfected cells were selected in the presence of G418 (600 μg/ml), and after 2 weeks of selection, cells that are resistant to G418 were pooled together for further analysis.

Crystal Violet Assay—For cell proliferation assays, equal numbers of CTGF overexpressing cells, CTGF siRNA-containing cells, and their corresponding control cells were seeded in 48-well plates and cultured in media supplemented with 1% FBS for 7 days; media were changed every other day. Cellular
growth was stopped after 7 days in culture by removing the media and adding 0.5% crystal violet solution in 20% methanol. After staining for 5 min the fixed cells were washed with phosphate-buffered saline (PBS) and solubilized with 1% SDS. The absorbance at 600 nm was evaluated using a microplate reader. For colony formation assay, at 24 h after transfection with each expression and GFP plasmids, Eca109 cells were trypsinized, washed, and counted, and equal numbers of transfected cells were seeded in 24-well plates. The remaining cells were analyzed by fluorescence-activated cell sorter to determine the percentage of GFP-expressing cells, which represent the transfection efficiency. After 24 h of incubation, plated cells were selected with 600 μg/ml of G418 and allowed to form colonies. After 2 weeks, the colonies were stained with 0.1% crystal violet and photographed.

**MTT Assay**—For cell growth analysis, equal numbers of cells were seeded in 48-well plates and cultured for various durations. Cell numbers were measured by MTT assay according to the manufacturer’s protocol (Roche Applied Science).

**Treatment with r-hCTGF**—ECA109 cells were seeded into 48-well plates at 1500 cells/well. r-hCTGF was purchased from Fitzgerald Industries International, Inc. (San Francisco). Eca109 cells were treated with 0.5 or 1 μg/ml of r-hCTGF, respectively, or 1.5 μg/ml CTGF-specific antibody during culture in medium plus 0.5% serum. The treatments were renewed every 48 h. Cells were harvested and counted after 6 days of culture.

**Lithium Treatment on Eca109 Cells**—ECA109 cells were plated into 6-well plates at 2 × 10^5 cells/well and cultured for 12 h. The cells were then serum-starved overnight followed by incubation with 50 μM lithium for different times before being lysed by TRIZol reagent. RNA was extracted and transcribed to cDNA. CTGF mRNA level was measured using a pair of specific primer by real time PCR.

**BrdUrd Incorporation Assay**—Eca109/CTGF pooled transfectants and control Eca109/pcDNA3.1 cells were grown to 70% confluence. Cells were treated with bromodeoxyuridine (BrdUrd) labeling reagent (Zymed Laboratories Inc.) for 1 h before they were processed for immunostaining. Cells were stained with anti-BrdUrd primary antibody (mouse IgG) followed by rhodamine red-labeled goat anti-mouse secondary antibody (Invitrogen). Slides for immunostaining were counterstained with the nucleophilic dye 4’,6-diamidino-2-phenylindole. Cells were analyzed by microscopy, and the mean ± S.E. of cells with BrdUrd incorporation were calculated.

**Nuclear Protein Extraction**—Nuclear extracts were prepared by the mini-extraction method as described previously (40). Cells were washed with ice-cold PBS and harvested by being scraped in 1.5 ml of PBS followed by pelleting and resuspending in 400 ml of 10 mM HEPES/potassium hydroxide, pH 7.9, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mM phenylmethylsulfonyl fluoride buffer. The resuspended cells were lysed in 0.5% Nonidet P-40 for 30 min after vortexing for 1 min, followed by centrifugation at 1,000 × g for 5 min to pellet the nuclei. After separation of the cytoplasmic fraction, nuclei were harvested by resuspension in ice-cold buffer containing 20 mM HEPES/potassium hydroxide, pH 7.9, 0.4 mM sodium chloride, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. Tubes were incubated for 1 h on ice and then centrifuged to clear the cellular debris. Nuclear extracts were immediately used for Western blot.

**Luciferase Reporter Assay**—Cells were plated at a subconfluent density and co-transfected with 0.05 μg of the reporter plasmid, 0.5 μg of expression vectors, and 0.05 μg of Renilla luciferase pRL-TK as an internal control for transfection efficiency. Cell lysates were prepared 24 h after transfection, and the reporter activity was measured using the Dual-luciferase reporter assay system (Promega). Transfections were performed in triplicate and repeated three times to reproducibility.

**RNAi-mediated Knockdown of CTGF**—In our experiments, FG12 lentiviral vector, which has an independent open reading frame of green fluorescence protein (GFP), was used to produce small, double-stranded RNA (siRNA) to inhibit target gene expression in ESCC cells, and the information on this vector system has been described in detail by Qin et al. (32). To construct the hairpin siRNA expression cassette, complementary DNA oligonucleotides for siRNA of CTGF (CTGF siRNA) or mutated sequence of CTGF siRNA as control (CTGF siRNA con) were synthesized, annealed, and inserted into FG12. Two CTGF siRNA constructs were used as follows: CTGF siRNA vector 1 (highlighted sequence was the complementary sequence with CTGF mRNA), 5’-ACCGCTGACCTGGAGAA-GAACATCTCAAGAGATGTTCTTCTTCCAGGTTGGATCCC-3’ and 5’-TGGAGGTAATCGCTGAGAAATCTCCTTTGAATGTGCCTTCTTCCA-GGTCAG-3’; and CTGF siRNA vector 2 (highlighted sequence was the complementary sequence with CTGF mRNA), 5’-ACCGATGTACCAGGACATGTCATCAAGAGATGTTCTTCTTCCATGTCTCGTACATTTTTTGGATCCC-3’ and 5’-TGGAGGATTCCAAAATGATCGCCATTGATTTTTGATCCC-3’. CTGF siRNA con vector (highlighted sequence was the random sequence as control that was not related to CTGF mRNA), 5’-ACCGGTACATGACGGATCGTAACGTTCAAGAGACGTTCAGTCGCCATGCTTCAGTATTTTGGATCCC-3’ and 5’-TGGAGGATTCCAAAATGATCGCCATTGATTTTTGATCCC-3’. FG12 vector with CTGF siRNA or CTGF siRNA con was transfected into 293T, and the virus with CTGF siRNA con was harvested from culture medium. The harvested virus was purified by centrifugation at 1,000 × g for 5 min. Cells were washed and resuspended in lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 0.5% Nonidet P-40) at a concentration of 125 mM at room temperature for 5 min. Cells were harvested, washed with ice-cold PBS twice and scrapped into ice-cold PBS containing protease inhibitors (Roche Applied Science). Cells were collected and resuspended in lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 0.5% Nonidet P-40) at a concentration of 125 mM at room temperature for 5 min. Cells were harvested, washed and resuspended in lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 0.5% Nonidet P-40).
The CTGF expression levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase, which was also used as the reference calibrator for CTGF in 36 paired normal esophageal cancer samples. Relative expression levels were shown as a ratio between CTGF and the reference gene—actin. Data were presented as tumor volume (mean ± S.D.).

### Results

**CTGF Is Overexpressed in ESCC and Correlated with Clinical Features of ESCC Patients**—To study the expression pattern of the CTGF gene in esophageal cancers, levels of CTGF mRNA were quantified by RT-PCR in 36 pairs of tumors and their matched normal esophageal tissues (Fig. 1A). Expression levels were shown as a ratio between CTGF and the reference gene—actin to correct for the variation in the amounts of RNA. To determine whether β-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 36 paired normal and esophageal cancer samples. The levels of expression of the CTGF gene were comparable with those when β-actin was used as the reference gene (data not shown). Up-regulation

- 35-
- 50-
- 65-

| Gender | CTGF negative \( (n = 9) \) | CTGF positive \( (n = 27) \) |
|--------|-----------------------------|-----------------------------|
| Female | 7                           | 4                           |
| Male   | 2                           | 23                          |

| Tumor position | CTGF negative \( (n = 9) \) | CTGF positive \( (n = 27) \) |
|----------------|-----------------------------|-----------------------------|
| High           | 2                           | 6                           |
| Medium         | 6                           | 11                          |
| Low            | 1                           | 10                          |

| Tumor grade | CTGF negative \( (n = 9) \) | CTGF positive \( (n = 27) \) |
|-------------|-----------------------------|-----------------------------|
| I            | 6                           | 3                           |
| II           | 2                           | 9                           |
| III          | 1                           | 15                          |

| Metastasis | CTGF negative \( (n = 9) \) | CTGF positive \( (n = 27) \) |
|------------|-----------------------------|-----------------------------|
| No         | 7                           | 8                           |
| Yes        | 2                           | 19                          |

| Drink | CTGF negative \( (n = 9) \) | CTGF positive \( (n = 27) \) |
|-------|-----------------------------|-----------------------------|
| No    | 4                           | 21                          |
| Yes   | 5                           | 6                           |

| Age | CTGF negative \( (n = 9) \) | CTGF positive \( (n = 27) \) |
|-----|-----------------------------|-----------------------------|
| 35- | 5                           | 4                           |
| 50- | 1                           | 16                          |
| 65- | 3                           | 7                           |

| Gender | Tumor position | Tumor grade | Metastasis | Drink | Age |
|--------|----------------|-------------|------------|-------|-----|
| Female | High           | I           | No         | No    | 35- |
| Male   | Medium         | II          | Yes        | Yes   | 50- |
|        | Low            | III         |            |       | 65- |

P-40, 25% glycerol, 1.5 mM MgCl\(_2\)) containing proteinase inhibitors. The lysate was subjected to sonication to shear chromatin to 200–1,000-bp fragments. One-third of the lysate was incubated with 5 μl NaCl at 65 °C to reverse the cross-linking, phenol/chloroform-extracted, ethanol-precipitated, and kept at –80 °C as an input control for PCR analysis. The remaining two-thirds of the lysate were subjected to immunoprecipitation using anti-β-catenin antibody (Santa Cruz Biotechnology) or mouse IgG (Pierce). Immunoprecipitated complexes were collected by using protein G-Sepharose beads. Precipitants were sequentially washed with lysis buffer twice, followed by washing once with wash buffer. After the final wash, 300 μl of elution buffer (0.1 M NaHCO\(_3\), 1% SDS) was added, and beads were rotated at room temperature for 15 min. NaCl (5 μl) was added to reverse the formaldehyde cross-linking. The DNA was extracted with phenol/chloroform, ethanol-precipitated, and resuspended in double-distilled water for PCR analysis. A set of primers specific for the human CTGF promoter was used for PCR amplification (35 cycles). Primer set was 5’-TTGCTCTCT GTAGAACAATGGA-3’ and 5’-TCAAGATGCCTACCTG-TAAAAC-3’; the expected products (~250 bp) were resolved on 1.5% agarose gels.

**Tumorigenicity Assay**—Ten 8-week-old nude mice were separated into two groups. For the first group, 1 × 10\(^6\) CTGF-overexpressing and their control cells were subcutaneously injected on opposite flanks of the same mouse. For the second group, 1 × 10\(^6\) CTGF knockdown cells and their control cells were injected similarly in a cohort of mice. For each group, five nude mice were used for the study. The resulting tumors were measured once a week, and tumor volumes (mm\(^3\)) were calculated using the standard formula: length × width × height × 0.5326. Tumors were harvested 4 weeks after injection and individually weighed. Data were presented as tumor volume (mean ± S.D.) and tumor weight (mean ± S.D.). Statistical analysis was performed using the Student’s t test.
Expression and Function of CTGF in ESCC

A significant association existed between expression of CTGF mRNA in esophageal tumors versus the gender, tumor grade, metastasis, and age of the patient (Table 1). For gender, expression of CTGF was significantly higher in males than in females \( (p = 0.001) \). Levels of CTGF expression were increased significantly in higher stage tumors compared with lower stage tumors \( (p = 0.003) \). For metastasis, expression of CTGF was significantly higher in metastatic than in nonmetastatic esophageal cancers \( (p = 0.011) \). However, tumor position, family history, and alcohol consumption showed no significant correlations with the level of expression of CTGF.

**Forced Expression of CTGF in Eca109 ESCC Cell Line Stimulated Their Growth and Colony Formation**—To examine the effects of CTGF on the growth of esophageal cancer cells, Eca109 cells were stably transfected with either a pcDNA/CTGF containing full-length CTGF or an empty vector pcDNA3.1 as a control. Five G418-resistant clones were screened for CTGF expression by Western blot analysis. Two pcDNA/CTGF stable transfected clones with high expression of CTGF (Eca109/CTGF1 and -3) are shown in Fig. 2A, which were used for further study. The effect of CTGF on cell growth was evaluated by crystal violet and MTT assays. The rate of growth of Eca109/CTGF was 1.5–2.5-fold greater than the Eca109/V control cells in both crystal violet and MTT assays (Fig. 2B and C).

Moreover, clonogenic assay showed that Eca109/CTGF cells formed more colonies than did the wild-type control cells (Fig. 2F). To avoid a nonspecific toxicity by forced expression of CTGF, we assessed the effect of r-hCTGF on the growth of ESCC cell line Eca109. Treatment with r-hCTGF for 6 days promoted cell growth in a dose-dependent manner compared with the control.

**FIGURE 2. Effects of CTGF on cell growth.** A, Eca109 cells were stably transfected with either the empty pcDNA3.1 vector or the CTGF expression vector. Eca109/CTGF clones were selected for their high expression of CTGF as shown by Western blot analysis. Equal loading was ascertained using β-actin as an internal control. B, crystal violet cell growth assay. Eca109/V and Eca109/CTGF cells were plated in 1% FBS. Cellular growth was stopped after 7 days \( (d) \), and crystal violet-stained cells were evaluated and reported in the plot. Results represent the mean ± S.D. of three experiments. C, cell growth rates of Eca109/CTGF1 and -3 and Eca109/V in 1% FBS were measured by MTT assay. D, treatment of Eca109 cells with r-hCTGF. Eca109 cells were cultured in RPMI 1640 + 0.5% FBS in the presence of indicated concentration of r-hCTGF or CTGF antibody. Untreated cells were used as controls. Cell number was counted after 6 days of incubation. Each experiment was performed in triplicate, and results represent the mean ± S.D. of three experiments. Statistical significance was determined with a Student’s t test; *, \( p < 0.05 \); **, \( p < 0.01 \). E, expression of cyclin D1 in Eca109/V and Eca109/CTGF clone 1 and 3 cells is shown by Western blot. F, clonogenic growth assay. Eca109 cells were transfected with either the CTGF expression vector (pcDNA3.1/CTGF) or empty vector PcDNA3.1 as well as enhanced GFP for the transfection efficiency control and grown in G418 to select neo-resistant colonies (2 weeks). Colonies were fixed, stained, and photographed with an inverted phase contrast microscope. Each experiment was performed in triplicate. G, expression of cyclin D1 and c-Myc in Eca109 parent cells, Eca109/pcDNA3.1, and Eca109/CTGF pooled cells. H, BrdUrd incorporation assay. BrdUrd labeling reagent was added to the cultures, and 1 h later plates were fixed and stained by 4′,6-diamidino-2-phenylindole and anti-BrdUrd antibodies. Cells were analyzed by microscopy, and the means ± S.E. of cells with BrdUrd incorporation were calculated.

Expression of CTGF gene occurred in 27 of 36 (75%) esophageal cancers compared with the paired normal esophageal tissues. Univariate analysis showed that mRNA levels of CTGF was significantly different between paired normal and cancer samples \( (p = 0.001) \). In addition, elevated levels of CTGF protein were found in human ESCC tissues compared with the paired normal tissue from the patients as shown by immunohistochemical staining (Fig. 1B) and Western analysis (Fig. 1C). Statistical analysis revealed that the expression of CTGF was significantly higher in males than in females \( (p = 0.001) \). Levels of CTGF expression were increased significantly in higher stage tumors compared with lower stage tumors \( (p = 0.003) \). For metastasis, expression of CTGF was significantly higher in metastatic than in nonmetastatic esophageal cancers \( (p = 0.011) \). However, tumor position, family history, and alcohol consumption showed no significant correlations with the level of expression of CTGF.
Expression and Function of CTGF in ESCC

A

| Component                  | DNA  | Eca109/CTGF | Eca109/pcDNA3.1 |
|----------------------------|------|-------------|-----------------|
| nuclear β-catenin          | +    | -           | -               |
| total β-catenin            | -    | +           | +               |
| nucleoporin 62             | -    | -           | -               |

B

| Component                  | Eca109/CTGF | Eca109/pcDNA3.1 |
|----------------------------|-------------|-----------------|
| GSK3β pSer9               | +           | -               |
| GSK3β                     | +           | +               |

C

| Component                  | Eca109     | Eca109/PCDN3.1 |
|----------------------------|------------|---------------|
| nuclear β-catenin          | -          | +             |
| total β-catenin            | +          | +             |
| nucleoporin 62             | -          | -             |
| GSK3β                     | +          | +             |

D

| Component                  | Eca109     | 293T          |
|----------------------------|------------|---------------|
| TOPflash                   | +          | -             |
| FOPflash                   | -          | +             |
| pcDNA3.1                   | -          | -             |
| CTGF                       | -          | -             |

FIGURE 3. CTGF stimulated β-catenin-TCF/Lef signaling pathway. Lysates from pcDNA3.1 vector-transfected (Eca109/V) and CTGF stable-transfected (Eca109/CTGF) cells were run on 4–15% polyacrylamide gel and transferred onto polyvinylidene difluoride membrane. Wnt-1 signaling regulatory proteins β-catenin (A) and both total GSK-3β and phosphorylated GSK-3β (B) were examined by individual specific antibodies. β-Actin served as a loading control. C, expression of nuclear and total β-catenin, total GSK-3β and phosphorylated GSK-3β in Eca109 parent cells, Eca109/pcDNA3.1 and Eca109/CTGF pool cells. β-Actin and nucleoporin 62 served as a loading control. D, TOPflash reporter activity in Eca109 and 293T cells. Cells were co-transfected with either TOPflash or FOPFlash reporter plasmid as well as either pcDNA3.1/CTGF or pcDNA3.1. β-Catenin-TCF/Lef-mediated transcription was determined by the TOPflash luciferase activity. FOPFlash contained a mutation in TCF/LEF-1-binding sites and was used as control. Reporter activities were normalized to internal control Renilla. Data represent results from triplicate dishes in two separate experiments. E, immunoblotting to examine expression for two β-catenin-TCF/Lef signaling pathway target proteins, c-Myc and c-Jun. Equal amounts of proteins for Eca109/V control cells, Eca109/CTGF1, and -3 cells were used in Western blot analysis and probed with c-Myc and c-Jun antibody. F, dominant negative β-catenin (DN β-catenin) and dominant negative TCF4 (DN TCF4) blocked up-regulation of cyclin D1 in Eca109/CTGF cells.

trol group. However, the CTGF-neutralizing antibody significantly attenuated the enhanced cell growth caused by r-hCTGF (Fig. 2D). Furthermore, Western analysis showed that cell cycle-related protein cyclin D1 was up-regulated in the two stable clones compared with Eca109/V control cells (Fig. 2E). To rule out the possibility of clonal variations in this study, Eca109/CTGF pooled transfects were constructed. Consistently, the expression of cyclin D1 was also up-regulated compared with the parent Eca109 cells and Eca109/pcDNA3.1 pool transfects, which mirrored the observations in Eca109/CTGF stable cell clones (Fig. 2G). At the same time, we used BrdUrd incorporation assay to investigate the role of CTGF in cell proliferation. We found that Eca109/CTGF pooled transfects showed more BrdUrd incorporation than control cells (Fig. 2H). These results indicated that the forced expression of CTGF in ESCC cells enhanced their cell growth.

CTGF Stimulates Nuclear Translocation of β-Catenin and Activates β-Catenin-TCF/Lef Signaling—Previous studies have shown that β-catenin-TCF/Lef signaling is aberrant in esophageal carcinomas (33). In our earlier studies, we showed that Cyr61, one member of the CCN family, can stimulate the nuclear localization of β-catenin in glioma (34). To investigate whether overexpression of CTGF altered β-catenin-TCF/Lef signaling, Western blot and RT-PCR were performed to examine the expression of β-catenin-TCF/Lef-dependent genes, such as c-jun and cyclin D1. We found that the endogenous expression levels of cyclin D1, c-Myc, and c-Jun were elevated significantly in Eca109/CTGF stables compared with Eca109/V control cells (Fig. 2F and Fig. 3E). Similar results were observed in Eca109/CTGF pooled transfects (Fig. 2G). To examine further these results, Eca109/CTGF cells were transfected transiently with a dominant negative β-catenin (DN β-catenin) and a dominant negative TCF4 (DN TCF4). Western analysis showed that the up-regulation of cyclin D1 in Eca109/CTGF cells was abolished by DN β-catenin and DN TCF4 (Fig. 3F). These results suggested that CTGF stimulated β-catenin-TCF/Lef signaling causing an up-regula-
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respective. Whereas the level of total GSK-3β was similar in the two cell types, the phosphorylated GSK-3β was markedly increased in the Eca109/CTGF stable clone cells compared with Eca109/V cells (Fig. 3B). To determine whether the elevated level of phosphorylated GSK-3β altered the subcellular localization of β-catenin, we examined the levels of β-catenin within the nucleus from either Eca109/CTGF stable clone cells or Eca109/V cells. As expected, overexpression of CTGF dramatically altered the subcellular localization of β-catenin with greater levels of β-catenin localized to the nucleus in Eca109/CTGF cells compared with Eca109/V cells (Fig. 3A). Furthermore, considering the potential clonal variation, we repeated the same experiments using Eca109/CTGF pooled transfects, and similar results could be recapitulated (Fig. 3C).

The luciferase reporters TOPflash and FOPflash, which have a minimal thymidine kinase promoter and either wild-type (TOP) or mutated (FOP) binding sites for the β-catenin/TCF/Lef complex, have been widely used to characterize the transcriptional activity of β-catenin/TCF/Lef complex (36). These reporter constructs were transfected into either Eca109 or 293T cells together with either the CTGF expression construct or pcDNA3.1, respectively, and luciferase activity was determined. In both Eca109 cells and 293T cells, overexpression of CTGF increased TOPflash activity, whereas CTGF exerted little effect on the FOPflash reporter (Fig. 3D). Taken together, these results suggest that CTGF stimulates β-catenin-TCF/Lef signaling and enhances expression of proteins associated with cell proliferation.

Knockdown of CTGF Inhibited Cell Proliferation and Clonogenic Growth Associated with Down-regulation of β-Catenin/TCF Signaling in ESCC Cells—To determine whether endogenous CTGF plays a role in cell growth, we used RNAi-mediated knockdown lentiviral vector to decrease the basal level of CTGF in Eca109 cells. Western blot results showed that two of the target sequences markedly decreased the expression of CTGF compared with the control sequence (Fig. 4A). The RNAi drastically inhibited cell growth of these cells in liquid culture (Fig. 4, B and C) and their capacity for clonogenic growth (Fig. 4D).

We further investigated the effects of silencing of CTGF on β-catenin-TCF/Lef signaling. As expected, expression of two target proteins of the β-catenin/TCF/Lef signaling pathway, cyclin D1 and c-Myc, were markedly decreased after knocking down CTGF (Fig. 4E). These results suggested that a decrease in the expression of CTGF suppressed β-catenin-TCF/Lef signaling.

CTGF Promotes Tumorigenicity of Eca109 Cells in Vivo—To examine the role of CTGF in tumorigenicity of Eca109 cells, Eca109/CTGF, CTGF siRNA, and their control cells were injected into the flanks of 4-week-old nude mice (control on one flank and experimental on other flank of each mouse). Tumor size was measured once a week. The Eca109/CTGF cells, which expressed CTGF at a high level, developed tumors with both a significantly shorter latency (2 weeks after injection) as well as markedly larger size at 4 weeks compared with the tumors from the control Eca109/V cells. In contrast, knockdown of endogenous CTGF significantly inhibited tumorigenicity of Eca109 cells in nude mice (Fig. 5, A–C). Taken together, these results suggested that CTGF stimulated tumorigenicity in vivo.

CTGF Is a Target Gene of β-Catenin/TCF/Lef Signaling—Sequence analysis of the CTGF promoter from −643 to −480 found a TBE (CTTTGTT) at −640, which perfectly matched the TBE consensus site identified in other β-catenin/TCF/Lef signaling target genes (Fig. 6A). This finding strongly suggested that CTGF is a β-catenin/TCF responsive gene. To test more vigorously whether the expression of CTGF is regulated in a β-catenin/TCF-dependent manner, we examined the effect of LiCl on the expression of CTGF. LiCl is an inhibitor of GSK-3β
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FIGURE 5. Effect of CTGF expression on tumor growth. Eca109/V, Eca109/CTGF, CTGF siRNA controls, or CTGF siRNA cells were mixed with Matrigel (1:1) and injected subcutaneously into BNX nude mice (1 × 10^6 cells/flank). A, time course of tumor growth. Tumor volumes were measured every week. Each point represents the mean volume ± S.D. of tumors. At 4 weeks after injection, tumors were removed and weighed. Average tumor volume (B) and weights (C) were measured at autopsy. Results are shown as mean ± S.D. of tumor weights. Statistical significance was determined with a Student's t test; *, p < 0.05; **, p < 0.01; con, control.

that has a critical role in the degradation of β-catenin and thus can act as an effective Wnt signaling activator (37). Upon LiCl treatment, the phosphorylated GSK-3β level (Ser(P)-9) increased in a time-dependent manner (data not shown). Real time PCR analysis showed that CTGF was up-regulated and reached the maximum in 6 h and then decreased subsequently (Fig. 6B). Similarly, we next investigated whether β-catenin-TCF/Lef signaling can modulate the expression of CTGF in 293T cells. 293T cells with a basal level of CTGF were transfected with DN TCF4 for 24 h, and the level of CTGF was analyzed by RT-PCR. As shown in Fig. 6C, expression of CTGF decreased. In conclusion, these results indicate that the expression of CTGF gene can be regulated by β-catenin/TCF signaling in ESCC cells.

The process of confirming the TBE in CTGF promoters began with the cloning of a series of CTGF promoters with 5’ deletions in pGL3–643 construct (Fig. 6D). Each construct was transfected into 293T cells together with DN TCF4 or control plasmid pCS2. Deletions up to position −643 did not significantly relieve the inhibition mediated by the DN TCF4. By contrast, the deletion of nucleotides −643 to −480 significantly alleviated the inhibition of CTGF promoter by the dominant negative construct of TCF4 (Fig. 6E). These data indicated the existence of a TCF-binding element somewhere between the −643 and −480 nucleotides of the CTGF promoter. To explore the biological function of this TBE, we first deleted the nucleotides between the −643 and −630. This deletion construct significantly alleviated the inhibition of the CTGF promoter by the DN TCF4 compared with the pGL3−643/+1 construct (Fig. 6F). Next, we mutated the CTTTGTT sequence to CGGGGTT in the pGL3−643/+1 construct. This mutation also significantly alleviated the inhibition (Fig. 6F).

More directly, ChIP assay examined the binding of β-catenin to the CTGF promoter. The PCR product representing the DNA sequence of the CTGF promoter was immunoprecipitated by the β-catenin antibody. The results suggested that endogenous β-catenin effectively bound to the DNA sequences of the CTGF promoter (Fig. 6H). Taken together, this consensus TCF-binding motif in CTGF promoter is involved in DN TCF4-mediated transcriptional inhibition.

DISCUSSION

In this study, our results showed that CTGF was highly expressed in ESCC tissues compared with matched normal esophageal tissue from patients, and the level of CTGF was correlated with the clinical parameters of the patients. We subsequently evaluated the function of CTGF in Eca109, an ESCC cell line. Forced expression of CTGF in Eca109 promoted cell growth and colony formation in vitro and stimulated tumor growth in nude mice, whereas knockdown of basal CTGF in Eca109 inhibited cell proliferation and clonogenic growth as well as tumorigenicity in vivo. These results suggested that CTGF might be implicated in the development and progression of ESCC. We also found that expression of cyclin D1 increased with overexpression of CTGF. Cyclin D1 promotes G1/S transition in the cell cycle, explaining in part how CTGF enhances growth of ESCC cells.

The involvement of CTGF in tumorigenesis has been mentioned in several previous investigations. Early studies demonstrated that overexpression of CTGF promoted angiogenesis in prostate cancer (25) and stimulated cell proliferation and metastasis in pancreatic cancer (38). The level of CTGF showed a significant correlation with the tumor stage and survival in glioma (26) and breast cancers (27). However, some other studies found that CTGF inhibited tumor metastasis and invasion in colon (30) and lung cancers (29). Moreover, although CTGF was highly expressed in breast cancer tissues, treating MCF-7...
breast cancer cell with CTGF protein caused apoptosis (39). Altogether, the data suggest that the role of CTGF in tumor progression is dependent on the tumor type. 

An important finding of this study is the characterization of CTGF as a tumorigenic enhancer by stimulating the \( \beta \)-catenin-TCF/Lef signaling pathway. To our knowledge, this is the first
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Report that CTGF can stimulate β-catenin-TCF/Lef signaling. β-Catenin is an important mediator in the Wnt signaling pathway, and activated β-catenin is translocated into the nucleus to form a complex with TCF/Lef, which stimulates the transcription of a set of target genes involved in cell proliferation (40, 41). The nuclear localization of β-catenin is controlled by its phosphorylation state regulated by GSK-3β, and the latter is inhibited by integrin-linked kinase through direct phosphorylation (34). Previous studies have documented that CTGF binds to integrins αvβ3, αvβ5 (29), α6β1 (42), and αIIbβ3 (43). Moreover, Cyr61, another CCN member, has been shown to bind with integrins stimulating the activity of integrin-linked kinase and AKT (34). Therefore, we proposed that CTGF also activated integrin-linked kinase by the binding to integrins and further inhibited the activity of GSK-3β leading to the accumulation of β-catenin in the nucleus. Because β-catenin signaling pathway has been reported to be involved in the development of human cancers (44), and dysfunction of Wnt signaling in cancer of the esophagus has been reported recently (33), the regulation of the β-catenin pathway by CTGF might further explain the mechanism by which CTGF stimulates growth of ESCC cells. Besides CTGF, several other CCN molecules function through the β-catenin pathway in cancers. For example, Cyr61 promotes glioma growth through activating β-catenin-TCF/Lef signaling (34). Considering the similarity of the protein structure between Cyr61 and CTGF, they may function in similar signaling pathways in the development and progression of tumors.

Intriguingly, our results also showed that CTGF was a target gene of β-catenin-TCF/Lef signaling. Previous studies have shown that Wnt3A, which can activate the β-catenin signal pathway, regulates the expression of CTGF during the differentiation of osteoblasts (45). Our results identified a TBE consensus in the CTGF promoter, and confirmed that CTGF is a target gene of β-catenin-TCF/Lef signaling using both the luciferase promoter-reporter assay and the ChIP assay. Similar to our results, another CCN molecule, WISP-1, has also been demonstrated to be a target gene of Wnt-1 and β-catenin (46). The involvement of CTGF in the development of cancer further identified the role of β-catenin in tumor progression and thereby established a Wnt/β-catenin/CTGF pathway in carcinogenesis. Considering CTGF may be a critical mediator of both fibrosis and cancer development, more attention will be paid to investigating the role of Wnt/β-catenin/CTGF pathway in the tumors induced by fibrosis.

In summary, our results suggest that CTGF and Wnt/β-catenin signaling form a positive feedback loop; CTGF can stimulate Wnt/β-catenin signaling and thus contribute to ESCC malignancy. Additionally, Wnt/β-catenin signaling also positively regulates the expression of CTGF, which might further promote the progression of ESCC. These findings not only recapitulated the involvement of the Wnt/β-catenin pathway in cancer development but also expand the regulatory network of Wnt signaling pathway. Given the diverse function of CTGF, the Wnt/β-catenin pathway seems to be involved in more important physiologic and pathophysiologic processes than earlier conceived. Taken together, our work lays the foundation for novel exploration for the mechanisms and functions of CTGF and β-catenin-TCF/Lef signaling pathway in the development and progression of ESCC.

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