Connective tissue growth factor is activated by gastrin and involved in gastrin-induced migration and invasion

Sabin Bhandari[1], Ingunn Bakke[1], J Dinesh Kumar[2], Vidar Beisvåg[1], Arne K. Sandvik[1,3], Liv Thommesen[1], Andrea Varro[2], Kristin G. Nørsett[1,4]

[1] Department of Cancer Research and Molecular Medicine, NTNU, Norwegian University of Science and Technology, Trondheim, Norway

[2] Department of Cell and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Liverpool, United Kingdom

[3] Department of Gastroenterology and Hepatology, St. Olav's University Hospital, Trondheim, Norway

[4] Central Norway Regional Health Authority (RHA), Stjørdal, Norway

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Correspondence to:

Kristin G. Nørsett

Department of Cancer Research and Molecular Medicine,

NTNU, Norwegian University of Science and Technology

N-7489 Trondheim, Norway

kristin.norsett@ntnu.no

Phone: +47 40220080

Abstract

Connective tissue growth factor (CTGF) has been reported in gastric adenocarcinoma and in carcinoid tumors. The aim of this study was to explore a possible link between CTGF and gastrin in gastric epithelial cells and to study the role of CTGF in gastrin induced migration and invasion of AGS-G_R cells. The effects of gastrin were studied using RT-qPCR, Western blot and assays for migration and invasion. We report an association between serum gastrin concentrations and CTGF abundancy in the gastric corpus mucosa of hypergastrinemic subjects and mice. We found a higher expression of *CTGF* in gastric mucosa tissue adjacent to tumor compared to normal control tissue. We showed that gastrin induces expression of *CTGF* in gastric epithelial AGS-G_R cells via MEK, PKC and PKB/AKT pathways. CTGF inhibited gastrin induced migration and invasion of AGS-G_R cells. We conclude that CTGF expression is stimulated by gastrin and involved in remodeling of the gastric epithelium.

Key words: gastrin, connective tissue growth factor, gastric epithelial cells, migration, invasion

Highlights:

- CTGF is overexpressed in gastric cancer and adjacent tissue compared to normal gastric tissue.
- Gastrin induces expression of CTGF in gastric epithelial cells.
- CTGF inhibits gastrin induced migration and invasion of AGS-G_R cells.

Introduction

The CCN proteins are a family of matricellular proteins involved in many physiological and pathological processes, including carcinogenesis. The connective tissue growth factor (CTGF), also known as CCN2, is believed to interact with a wide range of molecules by virtue of its multi-domain structure. Such interactions enable CTGF to modulate and integrate different biological cues into a cognate biological response. CTGF is a prominent mediator of transforming growth factor β (TGFβ) signalling [1]. It interacts with components of extracellular matrix (ECM) and various cell surface receptors regulating cell and cell-matrix signalling [1]. CTGF is involved in tuning of cell adhesion, migration, extracellular matrix production, survival and tumorigenesis [2]. Furthermore, CTGF promotes migration of many mesenchymal cell types and has been identified in a variety of tumors, including gastric cancer [2-4].

The gastric hormone gastrin is well known for its role in stimulating gastric acid secretion and organization of the gastric mucosa by promoting epithelial cell proliferation, migration and invasion [5]. Gastrin is identified to regulate expression of molecules that exert important effects on extracellular matrix remodelling, including plasminogen activator inhibitors (PAIs) [6] and matrix metalloproteinases (MMPs) [7].

CTGF has been suggested to be involved in the neoplastic transformation of gastrointestinal neuroendocrine cells and has been reported in carcinoids [8, 9]. Here we have assessed the hypothesis that gastrin regulates gastric CTGF expression and explored a potential role of CTGF in gastrin induced migration and invasion of gastric epithelial cells.

Materials and methods

Cells, plasmids and reagents. AGS-G_R cells were maintained as previously described [10]. CTGF expression vector was obtained from Open Biosystems (Thermo Scientific, Rockford, IL, USA) and empty pCMV vector from Addgene (Cambridge, UK). siRNA for CTGF, STAT3 and control siRNA were respectively obtained from Invitrogen (Carlsbad, CA, USA), Qiagen (Germantown, MD, USA) and Ambion (Austin, TX, USA). Human recombinant CTGF was obtained from Peprotech (London, UK). Gastrin (G-17) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The inhibitors Ro 31-8220 (3-[1-[3-(Amidinothio) propyl]-1H-indol-3-yl] -3-(1-methyl-1H-indol-3-yl) maleimide, Bisindolylmaleimide IX, Methanesulfonate) and AKT inhibitor XVIII, SC 66 ((2E, 6E)-2, 6-bis (4-Pyridylmethylene) cyclohexanone) were obtained from Millipore (Merck KGaA, Darmstadt, Germany). BIRB 796 (Doramapimod), PD184352 ($C_{17}H_{14}ClF_2IN_2O_2$) and SB203580 ($C_{21}H_{16}FN_3OS$) were obtained from Axon Medchem (Groningen, NL).

Patients and mice. Four subjects with normal (24.3 ± 2.2 pM) and four patients with high (67 ± 12.7 pM) plasma gastrin concentrations were selected from a cohort, aged 18 and over, who had clinical indications for undergoing upper gastrointestinal endoscopy. The study was approved by the Liverpool Local Research Ethics Committee and by the Royal Liverpool and Broadgreen University Hospitals NHS Trust and all patients gave written, informed consent. Corpus biopsies were collected in RIPA buffer and extracts were prepared as previously described [11]. In addition, four INS-Gas mice with elevated plasma gastrin (348.8 ± 184.1pM) and FYB/N wild type controls with normal plasma gastrin (73.5 ± 12.8pM) concentrations were maintained in an appropriately controlled environment with a 12-12 h light/dark cycle and were fed a commercial

pellet diet with water *ad libitum* as previously described [7] [12]. Animals were killed by increasing CO_2 concentration. Gastric corpus extracts were prepared from unfasted animals in RIPA buffer as previously described [11]. All animal experiments were approved by the University of Liverpool Animal Welfare Committee, and were conducted in compliance with Home Office requirements and the UK Animals (Scientific Procedures) Act 1986.

The human materials used for microarray gene expression analysis were biopsies from gastric adenocarcinoma (n=61) and adjacent (non-tumor) mucosa (n=21) from patients, and age-sex matched normal mucosa form healthy individuals (n=16), collected at St.Olavs Hospital, Trondheim, Norway. The patients gave written informed consent and the study was approved by the Regional Medical Research Ethics Committee of Central Norway (Approval No. 018-02). Samples were kept frozen at -80°C until further processing.

Gastrin radioimmunoassay. Mouse plasma and human serum samples were assayed for total amidated gastrin concentrations by radioimmunoassay using antibody L2 (which reacts with G-17 and G-34 but not progastrin or Gly-gastrins) and ^{125}I-G-17 as previously described [13].

Immunohistochemistry. Tissue sections were deparaffinised, rehydrated, quenched for endogenous peroxidase before antigen retrieval in boiling citrate buffer. The sections were incubated with mouse anti-CgA (M0869, Dako, Glostrup, Denmark) (1:1500) or goat anti-CTGF (sc-14939, Santa Cruz Biotechnology, Dallas, TX, USA) (1:200) overnight at 4˚C. Reactions were visualized using room temperature incubation with the rabbit/mouse EnVision-HRP/DAB+kit (K5007, Dako), or biotinylated anti-goat (BA-5000, Vector Laboratories, Birlingame, CA, USA) (1:150) for 1h, ABC reagent (PK-6100, Vectastain Elite ABC Kit

(Standard), Vector Laboratories) for 30 min, and DAB chromogen (K5007, Dako). Hematoxylin was used as counterstain. Serial staining was done on the shared surface of neighbouring sections.

RNA extraction. AGS-G_R cells ($2x10^5$) were plated in 6-well plates in complete medium. After 48h, cells were treated with G-17 (5nM, 1h) in serumfree medium. In the experiments with kinase inhibitors, cells were pretreated with inhibitors for 1h prior to G-17 treatment. RNeasy Mini kit (Qiagen, Germantown, MD, USA) was used for total RNA extraction, following instructions provided by the manufacturer. Assessment of RNA integrity, quality and quantity were performed with a Nanodrop spectrophotometer (Nanodrop Technologies, Rockland, DE, USA).

Total RNA from the frozen stomach samples was isolated and purified using an Ultra-Turrax rotating-knife homogenizer and the mirVana miRNA Isolation Kit (Ambion, Thermo Fisher Scientific Inc., DE, USA) according to the manufacturer's instructions.

cDNA synthesis and quantitative real-time PCR (RT-qPCR). Aliquots of 1µg of total RNA were reverse transcribed using Transcriptor First strand cDNA synthesis Kit (Roche Applied Science, Mannheim, Germany). cDNAs were amplified with the FastStart Universal SYBR Green Master (Rox) (Roche). The thermal cycling program used was: 5min at 95°C, then 40 cycles of 30s at 95°C, 30s at 60°C and 30s at 72°C followed by melting curve analysis. Relative *CTGF* mRNA abundance was calculated by $2^{-\Delta\Delta C_T}$ method [14]. All PCR reactions were performed in triplicates on StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and normalized by comparison to β-actin. Primer and probe sequences are shown in Suppl. Table 1.

Microarray gene expression analysis. cRNA was prepared with Ambion's Illumina® TotalPrep™-96 RNA Amplification Kit, using 300 ng total as input material. For each sample, the biotin-labelled cRNA concentrations were checked (NanoDrop, Thermo Fisher Scientific) and 750 ng hybridized to HumanHT-12 Expression BeadChips (Illumina, CA, USA). Only probes abiding to the Illumina detection p value of 0.01 in at least one sample were included in further analyses, using the limma (v. 3.12.1) Bioconductor package [15] for paired t-tests. A false discovery rate adjusted P value <0.05 was taken as significant. The microarray data are available from ArrayExpress (accession no. E-MTAB-1338).

Western blot. For the patient samples, western blot analysis was performed as described in [7] using antibodies to CTGF (Santa Cruz Biotechnology). AGS-G_R cells ($2x10^5$ or $3x10^5$ /well) were plated in 6-well plates and grown for 48h. After G-17 (5nM, 1h) stimulation, the cells were harvested in 200 µl RIPA (Pierce, Thermo Scientific). Proteins were resolved using NuPAGE Novex 10% Bis-Tris Gels (Invitrogen), transferred to polyvinylidene difluride (PVDF) membrane (Millipore, Merck KGaA, Darmstadt, Germany) and probed with anti-CTGF (1:400) (Santa Cruz Biotechnology) followed by HRP-conjugated secondary antibody, detected by SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce, Thermo Scientific) and visualized with Odyssey FC Dual Mode imaging system (LI-COR, Lincoln, NE, USA). The blots were quantified with Image studio version 3.1 (LI-COR, Lincoln). Blots were reprobed for β-actin to normalise for protein loading.

Transient transfection. AGS-G_R cells ($3.5x10^5$/well) were plated in 6-well plates and transfected after 24h with 2.5µg of plasmid (pCMV-CTGF or control pCMV) or siRNA and 12.5µl

Metafectene PRO transfection reagent (Biontex Laboratories GmbH, Martinsried, Germany) per well.

Migration and invasion assays. Biocoat Cell Culture Inserts (migration assays) or Matrigel-coated inserts (invasion assays) (BD Biosciences, Bedford, MA, USA) were performed according to the manufacurer's instructions and as described in [6].

Statistical analysis. Results are presented as means ± SEM. Comparisons between data were made using paired Students *t*-test and were considered significant at $P<0.05$.

Results

Gastric CTGF is increased in hypergastrinemia and in gastric adenocarcinomas.

To investigate if gastric mucosal CTGF is associated with elevated plasma gastrin, gastric corpus biopsies selected from individuals with either low or high gastrin were processed for detection of CTGF by Western blot. In both cases, there was a major band with size 50 kDa (Fig. 1A). In subjects with high plasma gastrin, the abundance of detected protein was significantly increased compared to subjects with normal plasma gastrin ($P<0.05$) (Fig. 1A and B). We then examined CTGF by Western blot of gastric corpus extracts from an animal model of hypergastrinemia, the INS-Gas mouse [16]. Again, there was a major band of 50 kDa and a significant increase of this protein in hypergastrinemic mice compared to normal wild type mice ($P<0.05$) (Fig. 1C and D).

The expression pattern of CTGF in human gastric mucosa (n=10) was also observed by immunohistochemistry, showing expression mostly in epithelial cells. Some scattered, single cells strongly expressed CTGF (Fig. 1F), and these were also positive for the neuroendocrine marker CgA in serial sections (Suppl. Fig. 1). To analyse the expression of *CTGF* in human gastric adenocarcinoma tissues, microarray gene expression analysis was performed. Comparisons of gene expression patterns between tumor, adjacent mucosa and normal mucosa showed upregulated expression of *CTGF* in adjacent mucosa (log2 fold change 2.8, $P<0.001$) and in tumor (log2 fold change 3.1, $P<0.001$) compared to normal controls. No significant regulation was detected between tumor *vs* adjacent mucosa. *CTGF* overexpression was verified using RT-qPCR (*CTGF* in adjacent mucosa (log2 fold change 3.9, $P<0.001$) and in tumor (log2 fold change 3.1, $P<0.001$) compared to normal controls) and also confirmed at protein level using

immunohistochemistry. Both tumors (n=17) and adjacent mucosa (n=5) showed increased expression of CTGF compared to normal control mucosa (Fig. 1F).

Gastrin induces expression of CTGF transcripts via MEK, PKC and PKB/AKT.

To study the molecular mechanisms by which gastrin induces CTGF, RT-qPCR and Western blot experiments were performed. Gastrin induced a concentration dependent increase in CTGF expression that was maximal 1h and 6h after stimulation, respectively, for CTGF mRNA and protein (Fig. 2). The MEK inhibitor PD 184352 repressed the gastrin induced expression of *CTGF* in a dose dependent manner (Fig. 3A). The response was also inhibited by Ro-31-8220 (Fig. 3B), which is an inhibitor to PKC, and by the specific AKT Inhibitor XVIII, SC66 (Fig. 3C), indicating activation of MEK 1/2, PKC and AKT by gastrin and its involvement in transducing signals required for expression of *CTGF*. The p38MAPK inhibitors SB203580 and BIRB 796 had no effect on *CTGF* expression (Suppl. Fig. 2).

A previous study has recognized STAT-3 as an important regulator of TGF-β induced CTGF expression [17]. However, gastrin stimulated *CTGF* was not influenced by knocking down STAT-3 in AGS-G_R cells (Suppl. Fig. 3).

CTGF suppresses gastrin induced migration and invasion.

Since CTGF is implicated in control of cell migration and invasion we examined the role of CTGF on gastrin stimulated migration and invasion of AGS-G_R cells in Boyden chambers. Addition of exogenous recombinant CTGF significantly inhibited the gastrin induced migration and invasion (Fig. 4A and B). Knockdown of CTGF resulted in a significant increase of migration and invasion and gastrin treatment further enhanced this effect (Fig. 4C and D). Ectopic expression of CTGF dramatically reduced the number of migrating and invading cells as

a consequence of gastrin treatment (Fig. 4E and F). Collectively, these results suggest that CTGF inhibits the migratory and invading potential of AGS-G_R cells. Downregulation and overexpression of CTGF mRNA and protein was verified by qPCR and Western blotting (Suppl. Fig. 4).

Discussion

A high expression of CTGF has been reported in gastric cancer [3, 4, 18] and this is confirmed in the present study. In addition, our results (microarray and IHC) show that CTGF is upregulated in adjacent (non-tumor) mucosa from patients with gastric adenocarcinoma compared to normal tissue, but not in tumor compared to adjacent tissue, implicating a likely role of CTGF in early gastric cancer development. This observation supports previous suggestion that CCN family proteins play a role in the initiation stage of gastric cancer development [19].

A 50 kDa major band was observed when analyzing hypergastrinemic patients and mice tissue by Western blot using a CTGF antibody. The 50 kDa band of CTGF, corresponding to the band observed in the current study, has previously been described [20]. It is likely that the 50 kDa band is a stable aggregate of CTGF fragments, or CTGF fragments tightly associated to other growth factors. The CTGF belongs to a big family of CCN proteins, consisting of a large number of processing products. Further studies are needed to identify the CTGF fragments observed.

In the present study, we found that gastrin induces expression of CTGF. AGS-G_R cells treated with gastrin were shown to express CTGF in a time and dose dependent manner, both at mRNA and protein level. The results suggest involvement of PKC, PKB/AKT and MEK/ERK pathways in transducing the gastrin signal to induce *CTGF* gene expression. These pathways are known to be activated by gastrin [21]. However, the pathways have not previously been shown to be involved in gastrin induced expression of *CTGF*. Furthermore, observations form this study show that CTGF attenuates gastrin induced migration and invasion of AGS-G_R cells.

It is clear that gastrin stimulates expression of a number of genes involved in mucosal organization and protection [5]. The fact that gastrin may upregulate expression of molecules with apparently opposite effects on matrix modelling such as PAIs and MMPs indicate that the mechanisms involved are likely to be complex. CTGF probably influence the balance of opposite effects. *CTGF* mRNA has been shown to correlate with both PAI-1 and tissue inhibitors of MMPs (TIMPs), both markers of tissue turnover [22]. Previous studies document a complex network of both similar and unique signalling cascades leading to induction of PAI-1 and CTGF in response to modifications in cytoskeletal structure [23].

Previous studies have reported that CTGF may be positively involved in gastric cancer progression. High CTGF expression has been shown to correlate with more lymph node metastasis, more peritoneal dissemination, and a shorter survival of gastric cancer patients [3, 4, 24]. Downregulation of CTGF in gastric cancer cells has been shown to reduce peritoneal dissemination in a nude mouse model [24]. Previous studies *in vitro* have demonstrated that knockdown of CTGF decreases proliferation and colony formation of gastric cancer cells. After CTGF knockdown, gastric cancer cells also show less migration/invasion abilities with decreased protein expression and proteolytic activity of both MMP-1 and MMP-9 [24]. Moreover, CTGF has been shown to promote gastric cancer cell adhesion to peritoneum [25]. However, in our model system using AGS-G_R cells, CTGF was demonstrated to inhibit gastrin induced migration and invasion. Our results are compatible with previous results showing that CTGF inhibits gastric cancer peritoneal metastasis by blocking integrin-dependent adhesion [26]. Differences in *in vitro* migration/invasion results may be due to variable use of serum in the experimental setups [18].

Taken together, these results indicate that CTGF may play different roles in different cancers and different settings/model systems. CTGF may play a role both in the initial stage of GC carcinogenesis and in later stages (invasion and metastasis) [19].

Conflict of interest

None.

Author contributions:

SB, LT, AKS, IB, VB, AV and KGN contributed to the conception and design of experiments. SB, IB, DK, VB and KGN performed the experiments. SB, LT, AKS, AV, DK, VB, IB and KGN contributed to the analysis and interpretation of data. KGN initiated and organized the work. SB and KGN wrote the manuscript draft, while all authors have contributed and approved the final manuscript.

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Figure legends

Fig. 1. *Increased CTGF in corpus biopsies from hypergastrinemic humans and mice, in human gastric adenocarcinomas and adjacent tissue compared to normal controls.*

(A) Western blot showing the major CTGF band in gastric corpus biopsies from four subjects with serum gastrin <30 pm (Hi-gas, -) and four subjects with hypergastrinemia (Hi-gas, +). (B) Abundance of the CTGF band estimated by densitometry of Western blots by the data shown in A. Means ± SEM. (C) Western blot showing the major CTGF band (50 kDa) in gastric corpus biopsies from four wild-type mice and four INS-Gas hypergastrinemic mice. (D) Abundance of the major CTGF band estimated by densitometry of the Western blot of data shown in C. Means ± SEM. (E) Gene expression analysis of *CTGF* (ILMN_2115125, Illumina Human HT-12), showing increased *CTGF* levels in human gastric adenocarcinoma and adjacent non-tumor) tissue compared to age-sex matched normal control gastric tissue. The symbols represent individual mRNA expression levels (log2 intensities) in the designated groups, with the line indicating median. (F) Immunohistochemical staining showing expression of CTGF (brown) in mucosa from normal control and adjacent non-tumor gastric tissue. The strongest expressing cells probably represent neuroendocrine cells (Suppl. Fig. 1). Scale bars 50 µm.

Fig. 2. *Time and dose dependent induction of CTGF by gastrin in AGS-G_R cells.*

(A) Expression of *CTGF* mRNA in response to G-17 (5 nM) was maximal after 1h. Mean ± SEM of three independent experiments. * $P<0.05$. All measurements were performed in triplicates.

(B) G-17 (5 nM) enhances CTGF protein abundance relative to β-actin in AGS-G_R cells. Data represent one of three biological replicas.

(C) Progressive increase in expression of *CTGF* with concentrations of G-17 (1h) from 0.5 to 10 nM. Mean ± SEM of three independent experiments. * P<0.05. All measurements were performed in triplicates.

(D) Dose dependent effects of G-17 (treated for 4h) on CTGF protein abundance relative to β-actin. Data represent one of three biological replicas.

Fig. 3. *Inhibition of G-17 stimulation of CTGF by the inhibitors PD 184352, Ro 31-8220 and XVIII, SC66.*

(A) Suppression of G-17 (5 nM, 1h) induced *CTGF* transcript in response to various concentrations of PD 184352. (B) Ro 31-8220 inhibits G-17 (5 nM, 1h) induced expression of *CTGF* in a dose dependent fashion.

(C). The AKT Inhibitor XVIII, SC66 inhibits G-17 (5 nM, 1h) induced expression of *CTGF*. Means ± SEM of three independent experiments. * P<0.05. All measurements were performed in triplicates.

Fig. 4. *CTGF inhibits gastrin induced migration and invasion of AGS-G_R cells.*

(A) Exogenously added CTGF attenuates both basal and G-17 (0.3 nM, 18h) induced migration of AGS-G_R cells. Means ± SEM of three independent experiments. * P<0.05. All measurements were performed in triplicates.

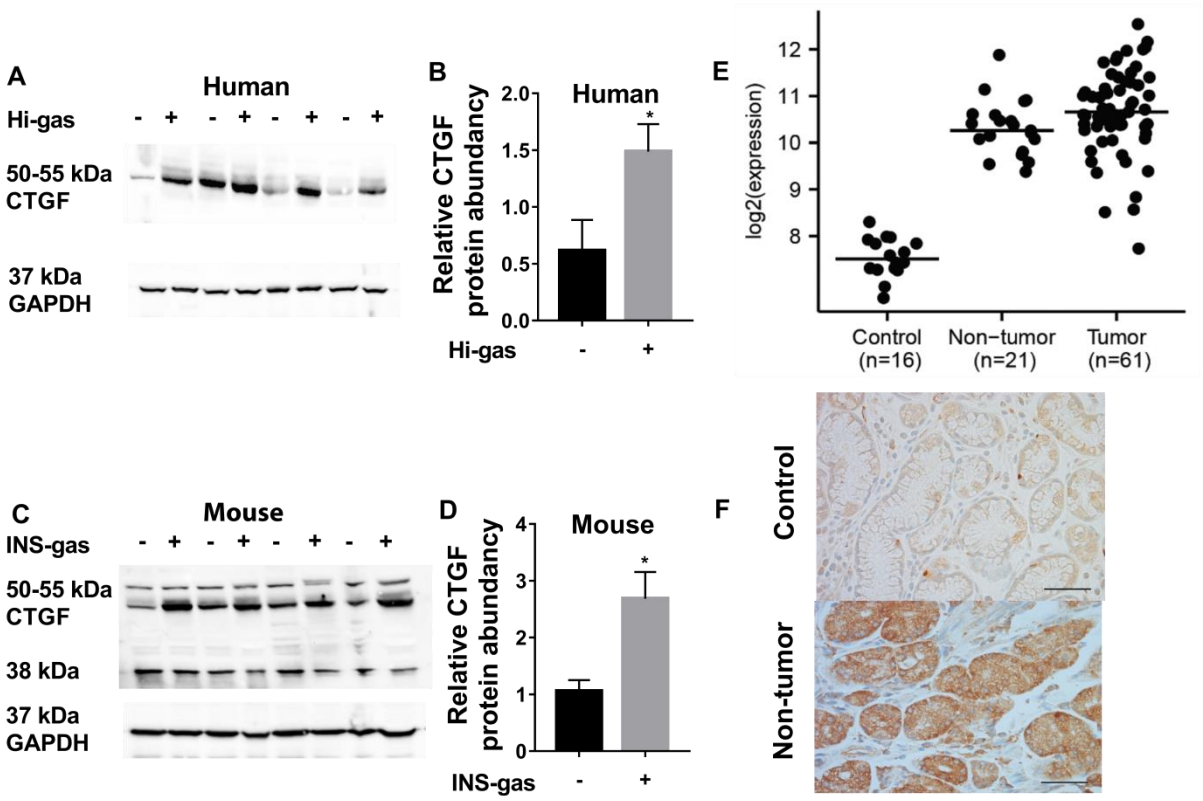
(B) Addition of recombinant CTGF cells attenuates G-17 (0.3 nM, 24h) induced invasion of AGS-G_R cells. Means ± SEM of two independent experiments. * P<0.05. All measurements were performed in triplicates.

(C) Migration of unstimulated AGS-G_R cells is stimulated by G-17 (0.3 nM, 18h), and the response is increased in CTGF siRNA treated cells. Means ± SEM of three independent experiments. * $P<0.05$. All measurements were done in triplicates.

(D) siRNA knockdown of CTGF enhances gastrin induced invasion of AGS-G_R cells. Means ± SEM of three independent experiments. All measurements were done in triplicates.

(E) Ectopic expression of CTGF inhibits G-17 (0.3 nM, 18h) induced migration of AGS-G_R cells. Means ± SEM of eight independent experiments. * $P<0.05$. All measurements were done in triplicates. (F) Ectopic expression of CTGF inhibits G-17 (0.3 nM, 24h) induced invasion. Means ± SEM of five independent experiments. * $P<0.05$. All measurements were done in triplicates.

Fig. 1.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Hi-gas - + - + - + - +
50-55 kDa CTGF
37 kDa GAPDH
B
Human
Relative CTGF protein abundancy
2.0
1.5
1.0
0.5
0.0
*
Hi-gas - +
E
log2(expression)
12
11
10
9
8
Control (n=16)
Non−tumor (n=21)
Tumor (n=61)
C
Mouse
INS-gas - + - + - + - +
50-55 kDa CTGF
38 kDa
37 kDa GAPDH
D
Mouse
Relative CTGF protein abundancy
4
3
2
1
0
*
INS-gas - +
F
Control
Non-tumor

Fig. 2.

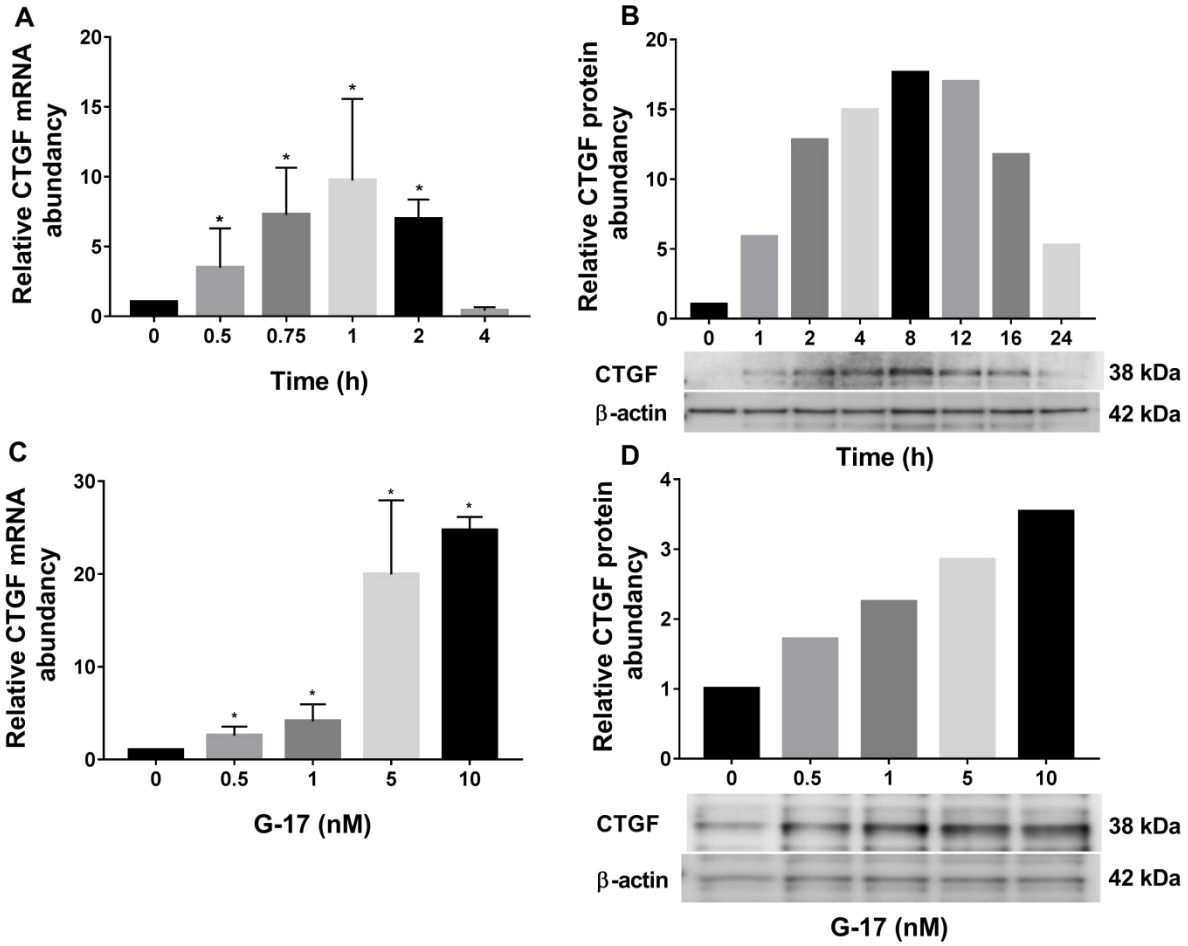

A
Relative CTGF mRNA abundancy
20
15
10
5
0
0
0.5
0.75
1
2
4
Time (h)
B
Relative CTGF protein abundancy
20
15
10
5
0
0
1
2
4
8
12
16
24
CTGF
38 kDa
β-actin
42 kDa
Time (h)
C
Relative CTGF mRNA abundancy
30
20
10
0
0
0.5
1
5
10
G-17 (nM)
D
Relative CTGF protein abundancy
4
3
2
1
0
0
0.5
1
5
10
CTGF
38 kDa
β-actin
42 kDa
G-17 (nM)

Fig. 3.

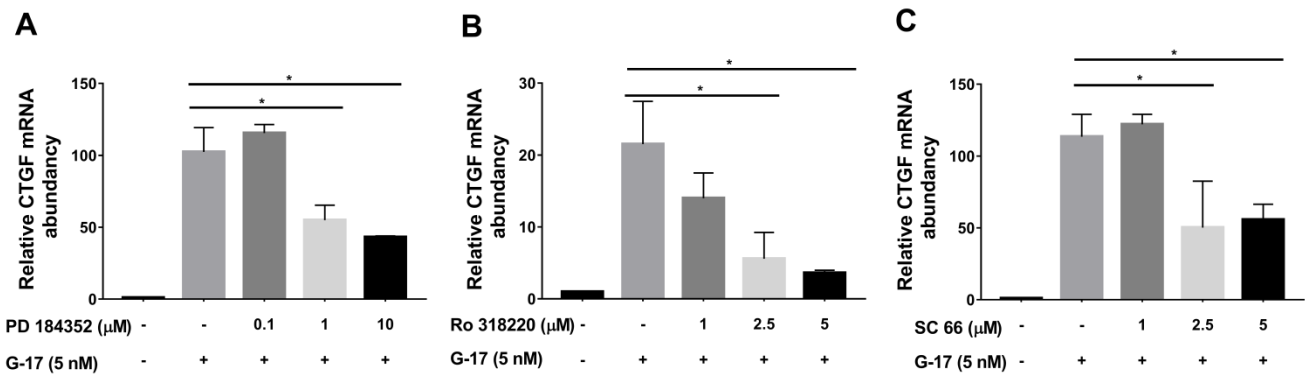
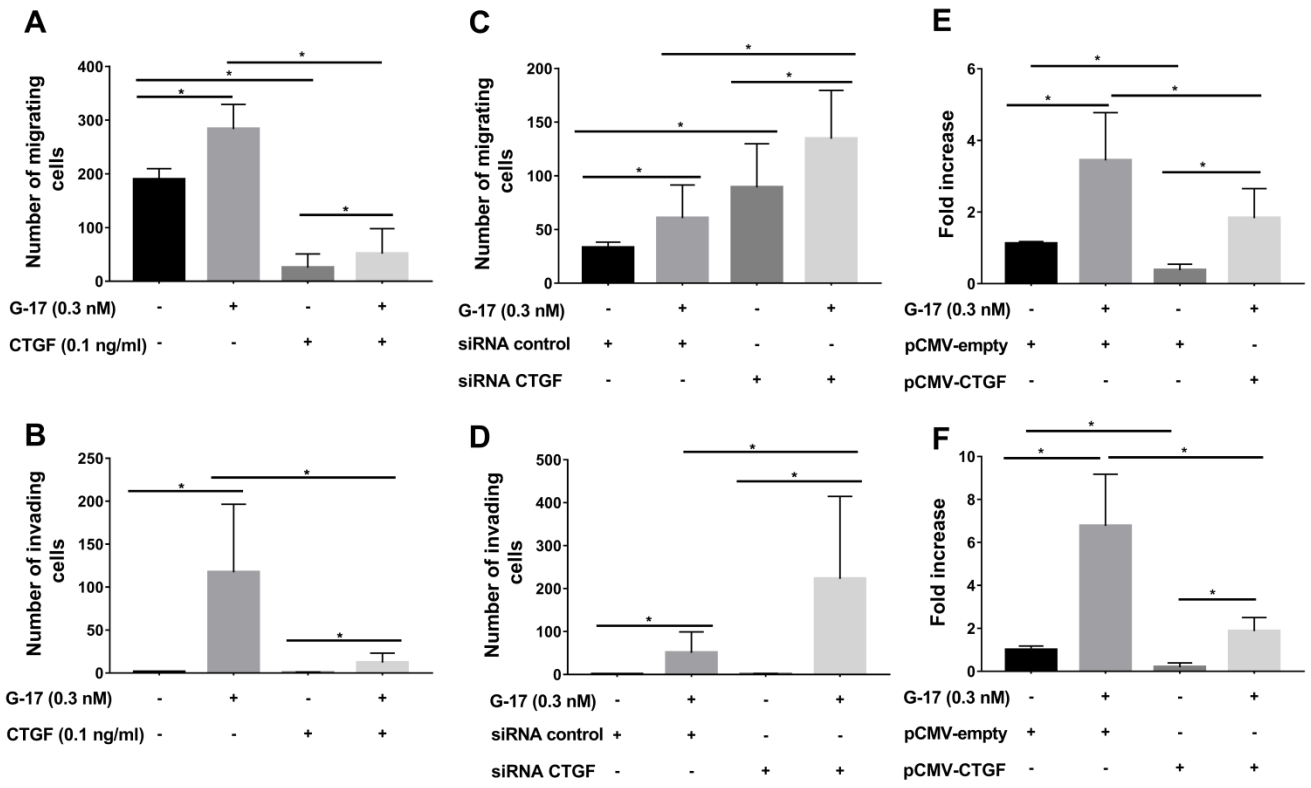

A
Relative CTGF mRNA abundancy
150
100
50
0
*
*
PD 184352 (μM) - - 0.1 1 10
G-17 (5 nM) - + + + +
B
Relative CTGF mRNA abundancy
30
20
10
0
*
*
Ro 318220 (μM) - - 1 2.5 5
G-17 (5 nM) - + + + +
C
Relative CTGF mRNA abundancy
150
100
50
0
*
*
SC 66 (μM) - - 1 2.5 5
G-17 (5 nM) - + + + +

Fig. 4.


A
Number of migrating cells
400
300
200
100
0
G-17 (0.3 nM) - + - +
CTGF (0.1 ng/ml) - - + +
C
Number of migrating cells
200
150
100
50
0
G-17 (0.3 nM) - + - +
siRNA control + + - -
siRNA CTGF - - + +
E
Fold increase
6
4
2
0
G-17 (0.3 nM) - + - +
pCMV-empty + + + -
pCMV-CTGF - - - +
B
Number of invading cells
250
200
150
100
50
0
G-17 (0.3 nM) - + - +
CTGF (0.1 ng/ml) - - + +
D
Number of invading cells
500
400
300
200
100
0
G-17 (0.3 nM) - + - +
siRNA control + + - -
siRNA CTGF - - + +
F
Fold increase
10
8
6
4
2
0
G-17 (0.3 nM) - + - +
pCMV-empty + + - -
pCMV-CTGF - - + +

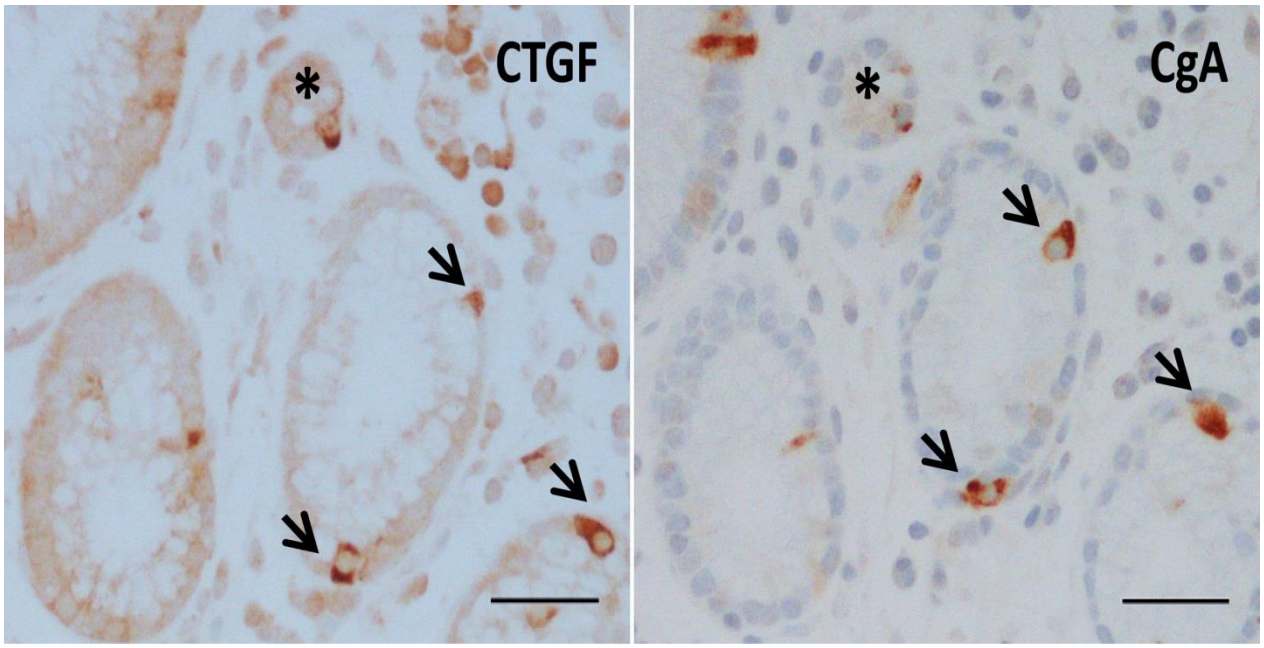
Suppl. Table 1

The table shows primers and TaqMan probe sequences used for *CTGF* and *β-actin* gene.

Gene	
CTGF	S-5'-TGC AGG CTA GAG AAG CAG AG-3'
	AS-5'-GCC CTT CTT AAT GTT CTC TTC C-3'
	P-5'-TTC GCA AGG CCT GAC CAT GC-3'
β-actin	S-5'- TGA GCG CGG CTA CAG CTT-3'
	AS-5'- CC TTA ATG TCA CGC ACG ATT T-3'
	P-5'-ACC ACC ACG GCC CAG CGG-3'

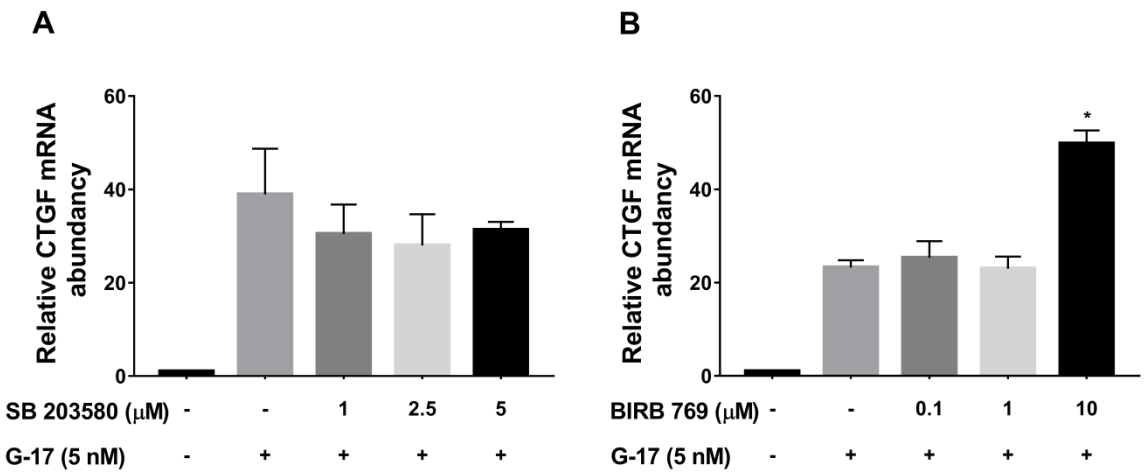
S= sense, AS= antisense, P=TaqMan probe

Supplementary figures



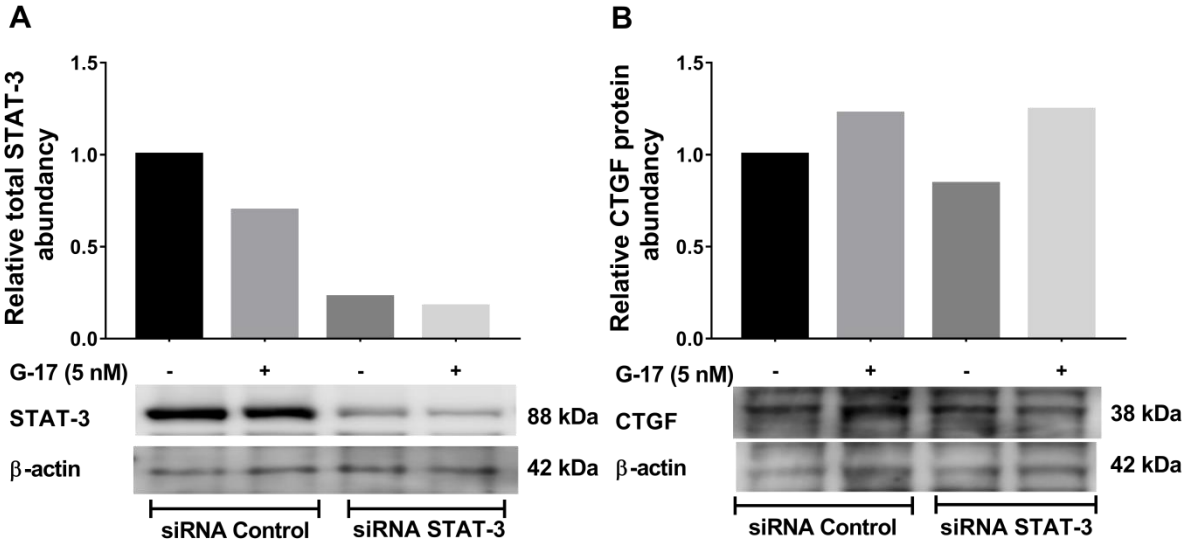
Suppl. Fig. 1. *Colocalization of CTGF and CgA in gastric epithelial cells.*

Immunohistochemical staining showing strong CTGF expression in cells that also express the neuroendocrine marker CgA (arrows) in stained serial sections from normal control gastric mucosa. * = identical positions. Scale bars 50 µm.



Suppl. Fig. 2. *p38MAPK inhibitors SB203580 and BIRB 796 had no effect on CTGF expression.* (A) SB 203580 treatment of AGS-G_R cells showed poor inhibition of G-17 (5 nM, 1h) induced *CTGF*. (B) Blockage of p38 MAPK using BIRB 796 had no negative effect on G-17 (5 nM, 1h) induced level of *CTGF*. At higher micromolar concentration of BIRB 796, unspecific side effects are seen.

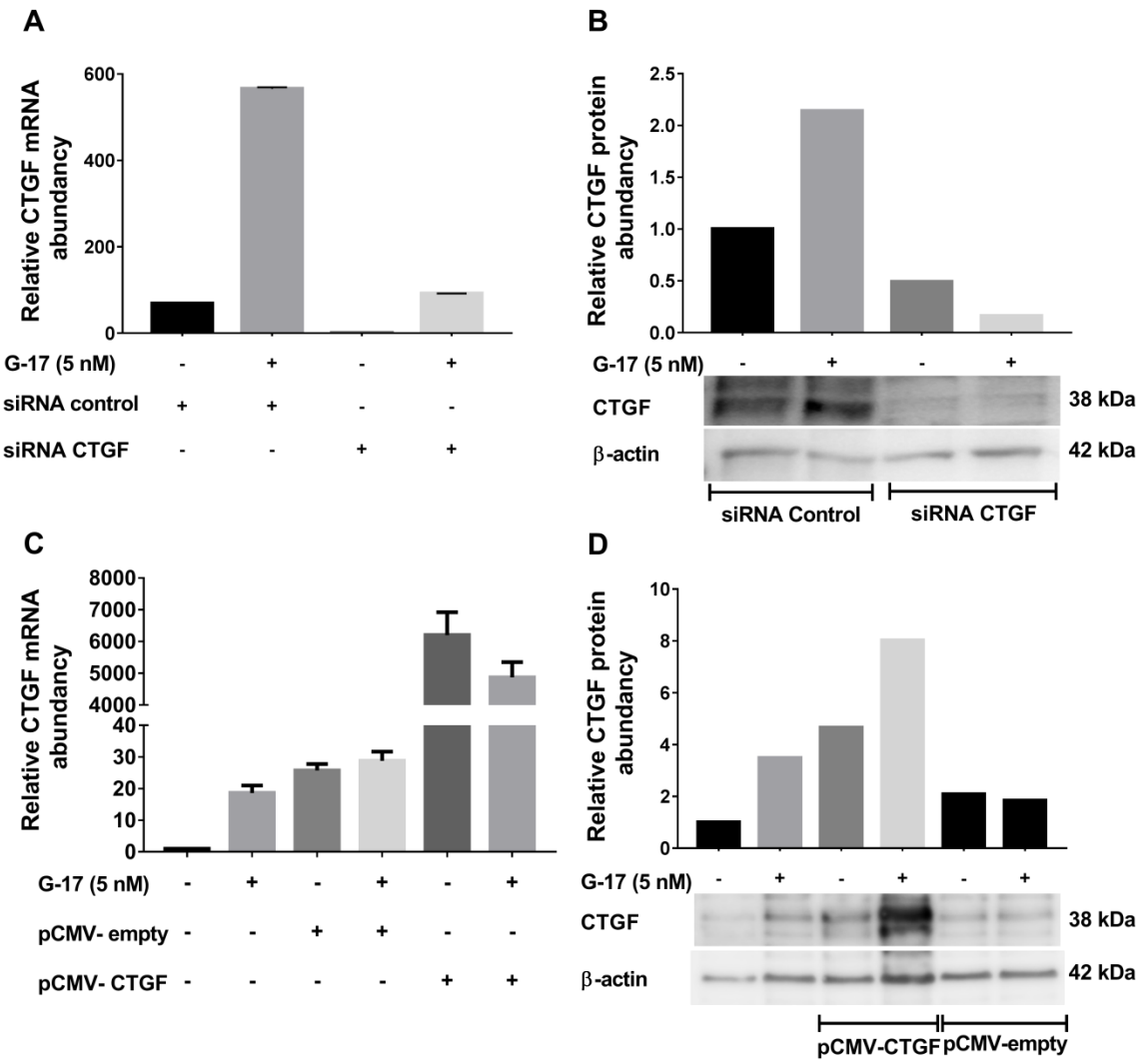
The bars show the mean ± SEM of the three technical replicates. Similar results were obtained from three independent experiments. One representative experiment is shown.



Suppl. Fig. 3. *STAT-3 is not essential for G-17 mediated expression of CTGF.*

(A) Decreased abundance of STAT-3 after knockdown of STAT-3 by siRNA treatment compared with control siRNA. Cells treated with siRNA against STAT-3 revealed 76% knockdown in G-17 (5 nM, 1h) treated sample and 50% in untreated sample. (B) No significant change in expression of CTGF in STAT-3 siRNA knockdown cells compared with siRNA control treated AGS-G_R cells.

The results shown are one representative of two independent experiments. The bars above represent the relative abundance of STAT3/CTGF protein compared to β-actin.



Suppl. Fig. 4. *Verification of knockdown and overexpression of CTGF mRNA and protein.* (A-B) Decreased abundance of *CTGF* mRNA (A) and protein (B) after knockdown of CTGF using siRNA compared with control siRNA. (C-D) Overexpression of *CTGF* mRNA (C) and protein (D) in AGS-G_R transfected with pCMV-CTGF compared to pCMV empty vector and untransfected control cells. Cells were stimulated with G-17 (5 nM, 1h). One representative of two independent experiments is shown. The bars in (C) indicate mean ± SEM of the three technical replicates representing relative *CTGF* mRNA abundancy normalized to β-actin.