Fig. S1

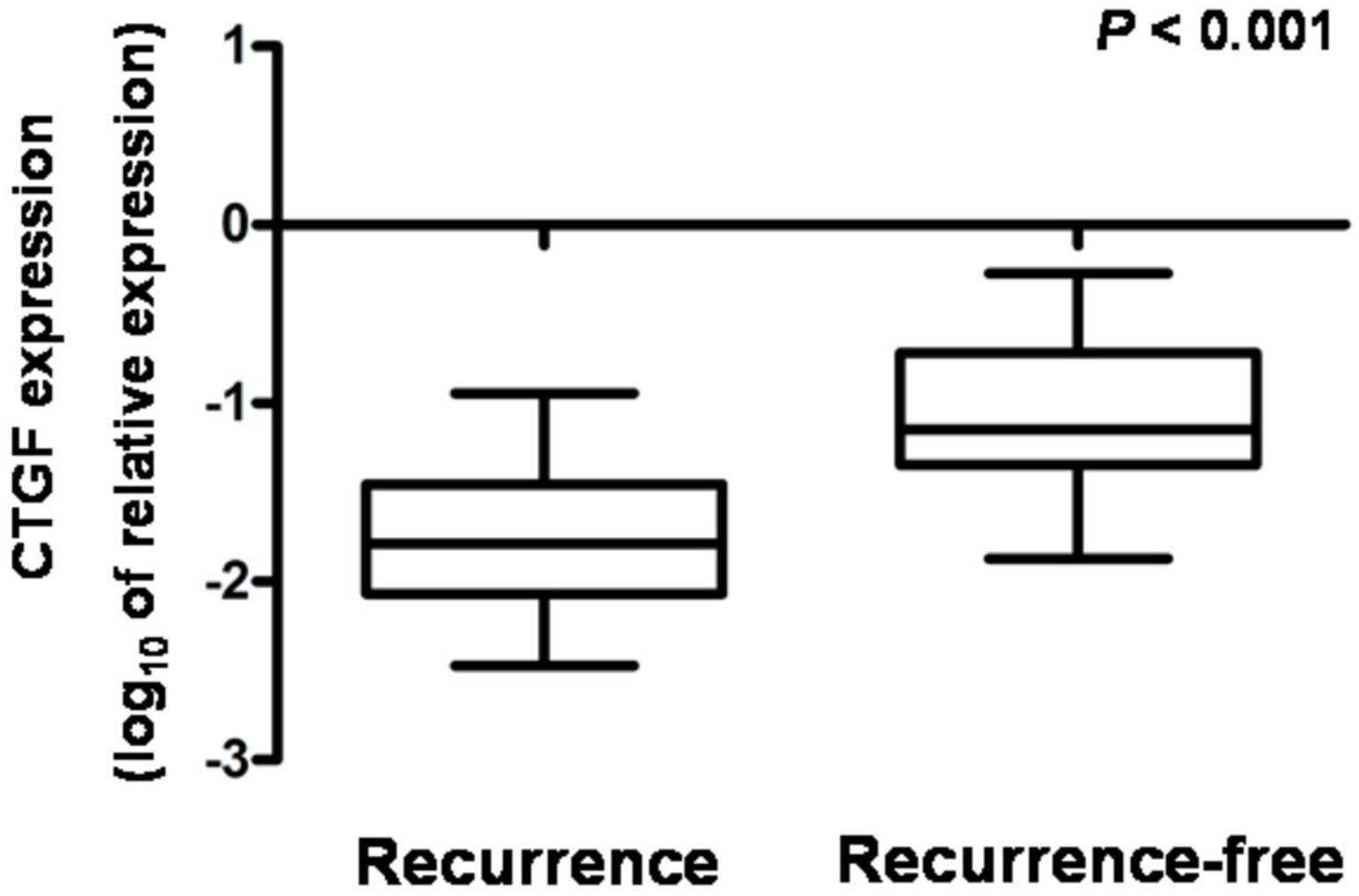
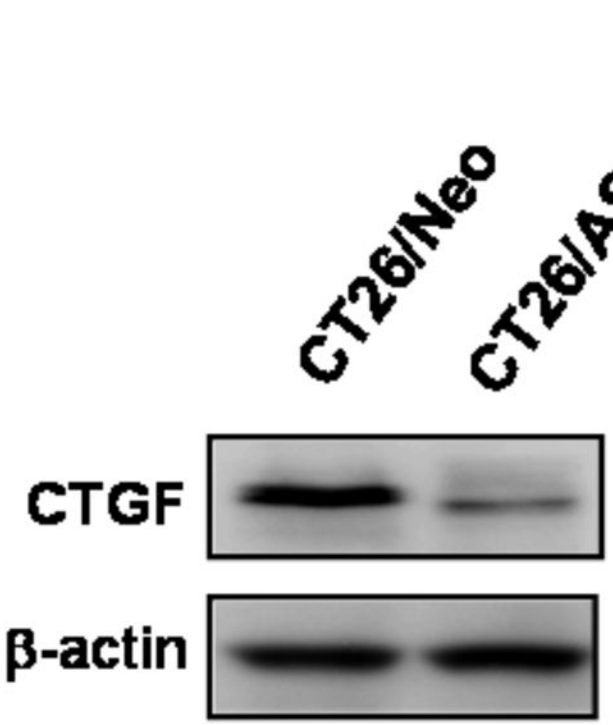

P < 0.001
CTGF expression
(log10 of relative expression)
1
0
-1
-2
-3
Recurrence
Recurrence-free

Fig. S2



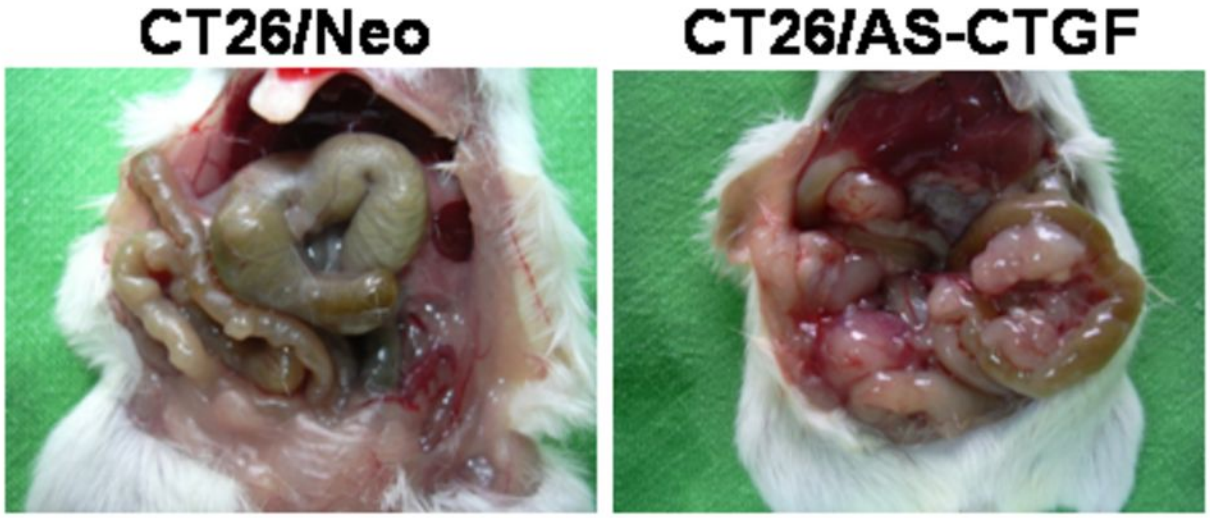


Fig. S3

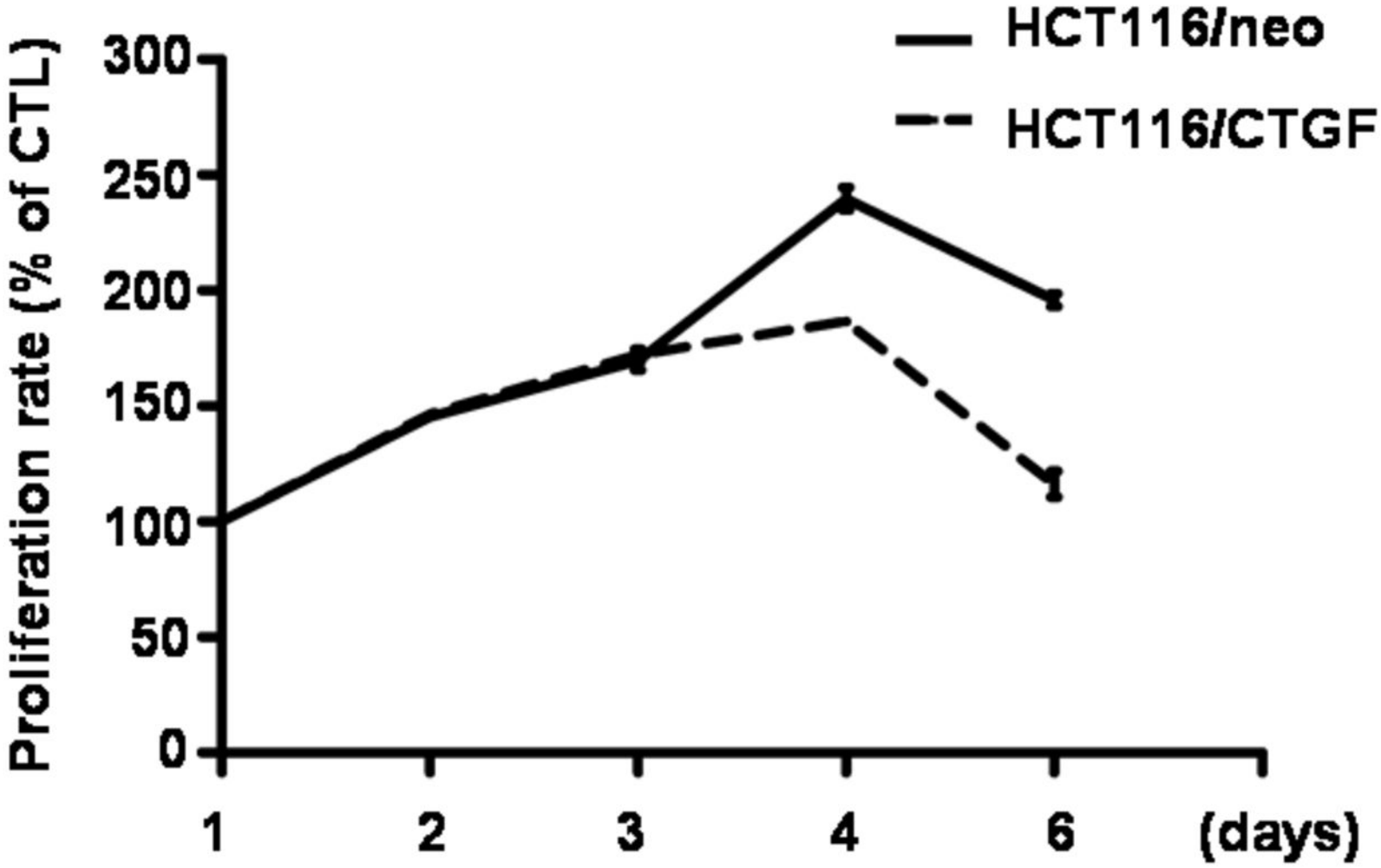

HCT116/neo
HCT116/CTGF
Proliferation rate (% of CTL)
300
250
200
150
100
50
0
1
2
3
4
6
(days)

Fig. S4, left

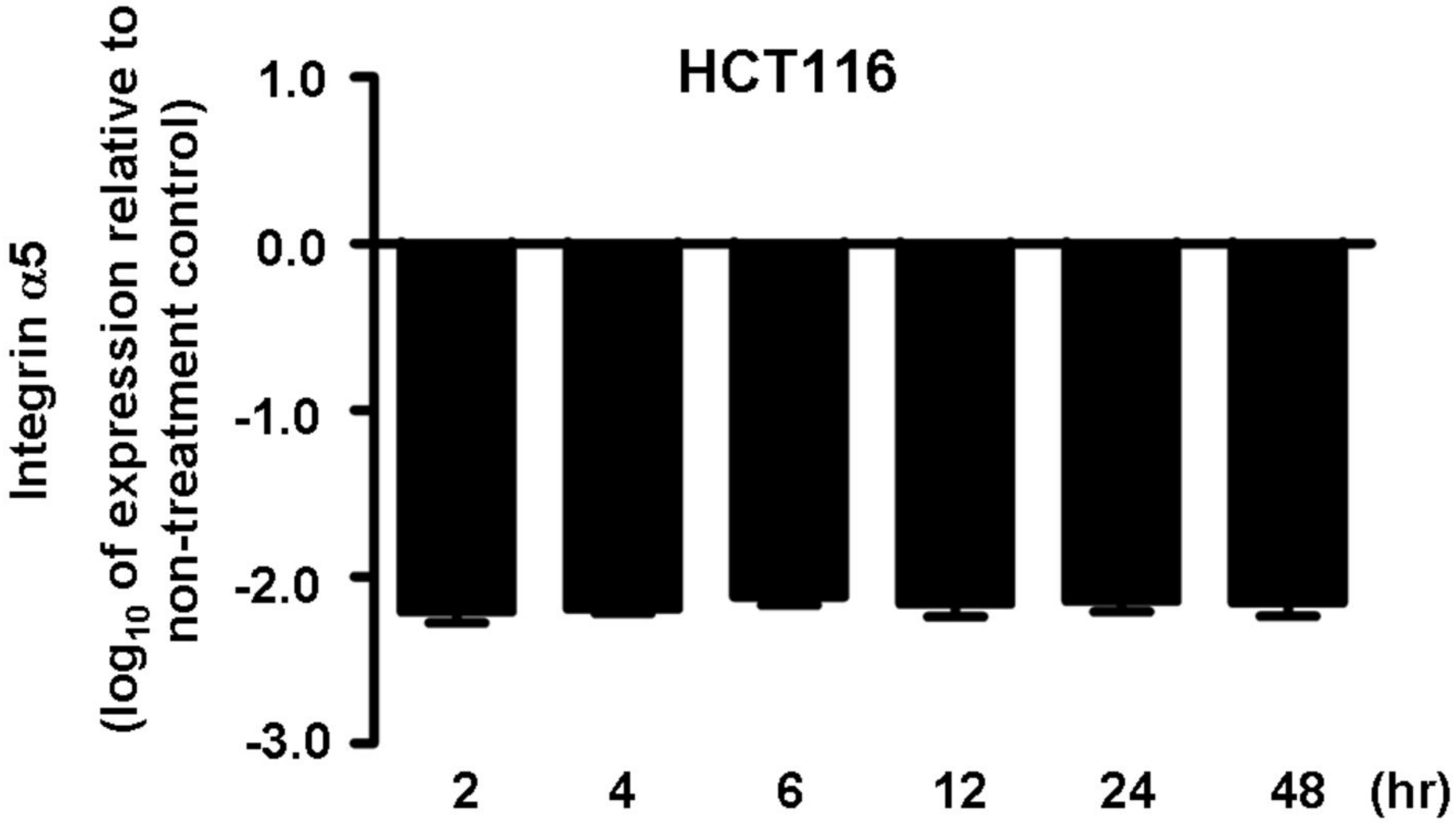
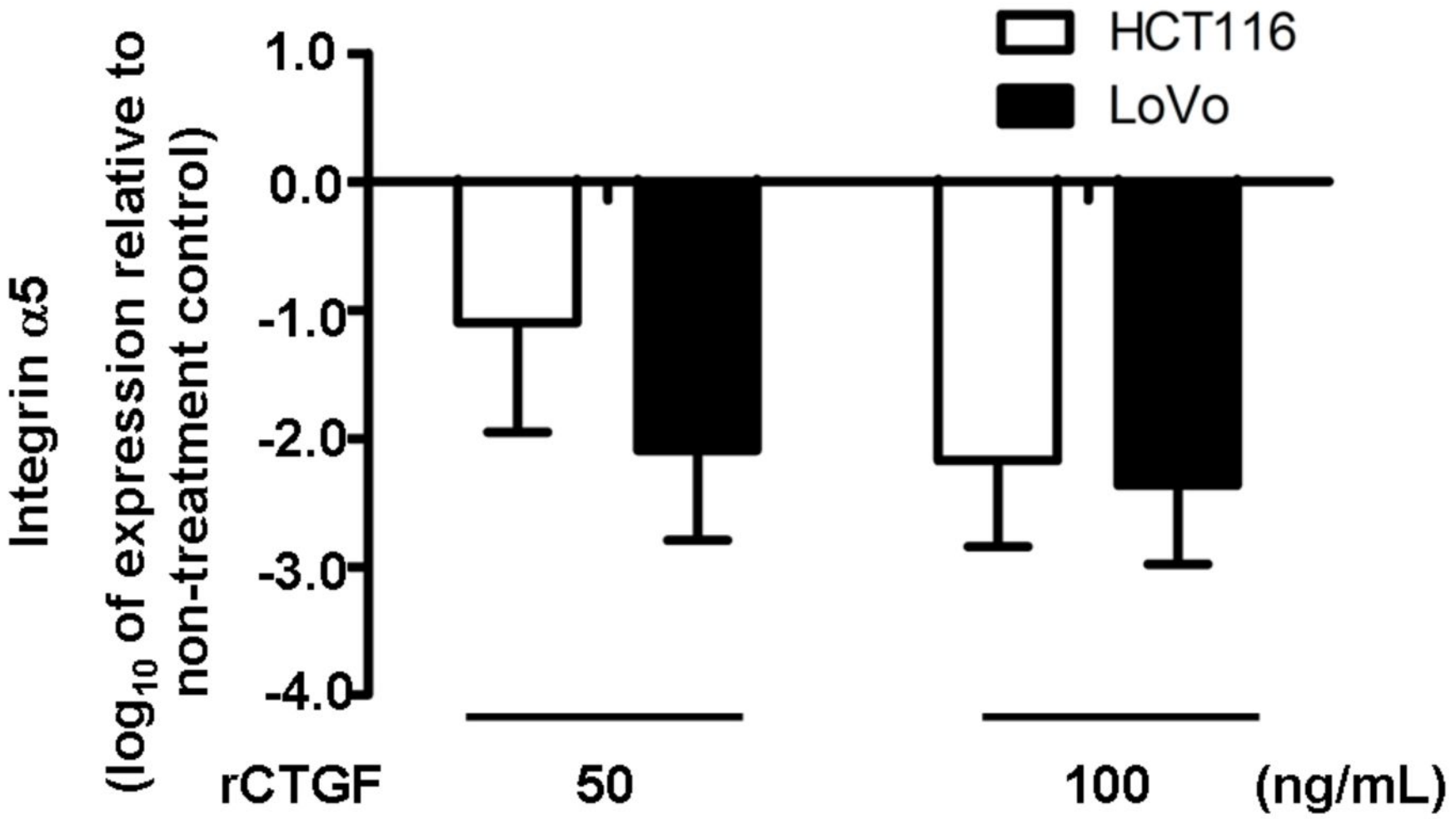

HCT116
Integrin α5
(log10 of expression relative to non-treatment control)
1.0
0.0
-1.0
-2.0
-3.0
2
4
6
12
24
48
(hr)

Fig. S4, right


HCT116
LoVo
1.0
0.0
-1.0
-2.0
-3.0
-4.0
Integrin α5
(log10 of expression relative to non-treatment control)
rCTGF
50
100
(ng/mL)

Supplementary Figure legends

Figure S1. Association between CTGF mRNA expression and recurrence with metachrnous PC in a subset of patients who had no PC at the initial surgery. The figure shows the relative CTGF expression was significantly decreased in patients with recurrent PC ($P < 0.001$).

Figure S2. Peritoneal dissemination of CT26/Neo and CT26-AS-CTGF transfectants. (*Left*): immunoblot analysis of CTGF and β-actin in CT26 transfectants. (*Right*): abdominal cavity photographs from BALB/c female mice that had been injected intraperitoneally with CT26/Neo or CT26/AS-CTGF cells ($1x10^5$/mouse, n = 6/group), respectively. Mice were sacrificed upon appearing moribund or after 6 weeks. Subsequently, the extent of peritoneal dissemination and the number of nodules were determined.

Figure S3. The growth properties of the vector (Neo)- and CTGF-transfected HCT116 cells in monolayer cultures. Data are the mean ± SE. All experiments were carried out in triplicate on separate occasions with similar results.

Figure S4. Integrin α5 mRNA expression was analyzed by real-time PCR in colon

cancer cells. (*Left*): integrin α5 mRNA expression of HCT116 cells treated with 100ng/mL rCTGF in different time courses. At least triplicates of each reaction were performed. (*Right*): qPCR analysis of integrin α5 expression in HCT116, LoVo cells treated with 50, 100 ng/mL rCTGF for 48 hours respectively, and the mRNA expression in non-treatment was considered as the control.

Supplementary Table S1. Risk factors of intraperitoneal recurrence in 99 patients without peritoneal dissemination at initial operation.

Feature		Positive [n (%)]	P
Sex			
	Male	8/43 (18.6%)	0.804
	Female	12/56 (21.4%)	
Tumor site			
	Right	7/27 (25.9%)	0.409
	Left	8/42 (20%)	
	Rectum	5/30 (16.7%)	
Tumor differentiation			
	Well	0/3	0.307
	Moderate	16/85 (18.8%)	
	Poor	4/11 (36.4%)	
Stage			
	II	9/38 (23.7%)	0.287
	III	10/44 (22.7%)	
	IV	1/17 (5.9%)	
Lymph node			
	N0	8/46 (17.4%)	0.520
	N1	6/33 (18.2%)	
	N2	6/20 (30%)	
Intra-tumor invasion$^{\Phi}$			
	Present	7/38 (18.4%)	0.801
	Absent	13/61 (21.3%)	
CEA level (ng/ml)			
	≦3	6/38 (15.8%)	1.000
	>3	6/44 (13.6%)	
CTGF expression			
	Low	17/50 (34%)	0.000*
	High	3/49 (6.1%)	
Tumor depth			
	T3	17/94 (18.1%)	0.0547
	T4	3/5 (60%)	

* Statistical significance (P<0.05)

Supplementary Table S2. Results of peritoneal seeding of CT26/Neo and CT26/AS-CTGF transfectants in BALB/c mice

Characteristics / Cell lines	Peritoneal seeding					
	Diaphragm seeding[#]		Local bowel invasion[#]		Nodule count[&]	
	Involved No.	*P*	Involved No.	*P*	Median No. (range)	*P*
CT26/Neo	0/6	0.015	2/6	0.061	7 (6~9)	0.0001
CT26/AS-CTGF	5/6		6/6		27 (21~37)	

#: P value derived from Fisher's exact test. &: P value from Mann-Whitney test

Supplementary Information

Materials and Methods

Western blot analysis

The cells were washed with PBS containing 5 mmol/L EDTA and 1 mmol/L sodium orthovanadate, scraped into a lysis buffer (20 mmol/L Tris-HCl [pH 8.0], 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μmol/L leupeptin, and 0.15 U/mL aprotinin), and stored for 30 minutes on ice. The lysed cells were then centrifuged at 14,500 *g* for 30 minutes at 4°C, and the supernatant was collected. Proteins in the supernatant were quantified by spectrophotometry and electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P membrane; Millipore Corp, Bedford, MA). After the blot was blocked in PBS with 5% defatted milk and 1% Tween-20, the membrane-bound proteins were probed with primary antibodies against β-actin (Sigma Chemicals), CTGF, or integrin α5 (Santa Cruz Biotechnology). The membrane was washed and then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 30 minutes. Antibody-bound protein bands were detected with enhanced chemiluminescence reagents (Amersham Bioscience, Piscataway, NJ) and photographed using Kodak X-Omat Blue autoradiography film (Perkin Elmer Life Science, Boston, MA).

RNA isolation and RT-PCR

RNA was isolated using TRIzol (Invitrogen, Rockville, MD) from transfected colon adenocarcinoma cells, and reverse transcription was performed using 5 μg of total RNA at a final reaction volume of 20 μL in Moloney murine leukemia virus reverse transcriptase buffer (10 mM dithiothreitol, all 4 deoxynucleoside 5'-triphosphates [dNTPs; at 2.5 mM each], 1 μg of $[dT]_{12–18}$ primer, and 200 U of Moloney murine leukemia virus reverse transcriptase [Promega Corporation]). The reaction mixture was incubated at 65°C for 5 min, and the reaction was terminated by heating at 42°C for 60 min. One microliter of the reaction mixture was then amplified by polymerase chain reaction (PCR) with the following primer pairs: CTGF primers, 5′-GCTTACCGACTGGAAGACACGTT-3′ (sense) and 5′-TCATGCCATGTCTCC-GTACATC-3′ (antisense), to produce a 500-base pair fragment of the CTGF gene; integrin β1 primers 5′-TGTTCAGTGCAGAGCCTTCA-3′ (sense) and 5′-CCTCATACTTCGGATTGACC-3′ (antisense), to produce a 505-base pair fragment of the integrin β1 gene; integrin α2 primers 5′-CACTCGATTTGGTTCAGCAA-3′ (sense) and 5′-GAACCACTTGTCCAAAGGCA-3′ (antisense), to produce a 264-base pair fragment of the integrin α2 gene; integrin α5 primers 5′-CCAGGATGGCTACAATGATG-3′ (sense) and

5′-CCCACAATCAGATCAGGATA-3′ (antisense), to produce a 202-base pair fragment of the integrin α5 gene; and the GAPDH primers, 5′-GAAGGTGAAGGTCGGAGTC-3′ (sense) and 5′-CAGGAGGCATTGCTGATGA-3′ (antisense), were used as the internal control to produce a 450-base pair fragment product of the GAPDH gene. PCR amplification was performed in a reaction buffer containing 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, all 4 deoxynucleoside triphosphates (each at 167 μmol/L), 2.5 U of *Taq* DNA polymerase, and 10 μmol/L primers. The reactions were performed in a Biometra Thermaoblock (Biometra Inc, Miami, FL) with the following program: denaturation for 1 minute at 95°C, annealing for 1 minute at 56°C, and elongation for 1 minute at 72°C for a total of 28 cycles, followed by a final extension at 72°C for 10 minutes. Equal volumes of each PCR sample were then electrophoresed on 1.5% agarose gel, which was then stained with ethidium bromide and photographed under UV illumination.

Real-Time PCR

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) was pursued using FastStart Universal Probe master mix (Roche) and amplified using an ABI Prism 7000 Sequence detector (Applied Biosystems). Those commercial

primers specific for *CTGF* (HS00170014-M1), Integrin α5 (HS00233732-M1) and *β-actin* (4333762T) were designed by Applied Biosystems (TaqMan® Gene Expression Assays); and the results are modified by *β-actin*. Moreover, the experiment was performed three times at least in duplicates. The primer sequence set for CTGF is GAGAACATTAAGAAGGGCAAAAAGT and Integrin α5 GGGAACAGACGG- GGTCAGTGTGCTG. Gene expression was calculated using relative quality expression (reference below). In cancer tissue samples, control group was chosen as the mean normal control expression value of all normal samples. In cancer cell lines, control group was chosen as the non-treatment status. The relative values were then expressed as the mean of the Log10 for each group representing the geometric means (95% confidence interval [CI] of the means). The differences in values between groups were then measured as fold differences.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.

Proliferation Assay

Cells were prepared in density of $3x10^5$ cells/mL in 6 well plates after treatment in different conditions, and washed twice with PBS. After washing, cells were immersed in 0.5 mL/each well MTT reagent which concentration was 1mg/mL and

incubated for 30 mins. The solution was removed gently, and 0.6 mL DMSO, which would dissolve out formazan, was added to the plats of each well. Moreover, the 6 well plates were shaken with 30 rpm speed for 15mins and transferred to 96 well plates, the O.D value was gained by 570 filter of ELISA reader (Multiskan EX, Thermo).