Promoter Hypermethylation Contributes to Frequent Inactivation of a Putative Conditional Tumor Suppressor Gene *Connective Tissue Growth Factor* in Ovarian Cancer

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Abstract

Connective tissue growth factor (CTGF) is a secreted protein belonging to the CCN family, members of which are implicated in various biological processes. We identified a homozygous loss of *CTGF* (6q23.2) in the course of screening a panel of ovarian cancer cell lines for genomic copy number aberrations using in-house array-based comparative genomic hybridization. *CTGF* mRNA expression was observed in normal ovarian tissue and immortalized ovarian epithelial cells but was reduced in many ovarian cancer cell lines without its homozygous deletion (12 of 23 lines) and restored after treatment with 5-aza 2′-deoxycytidine. The methylation status around the *CTGF* CpG island correlated inversely with the expression, and a putative target region for methylation showed promoter activity. *CTGF* methylation was frequently observed in primary ovarian cancer tissues (39 of 66, 59%) and inversely correlated with *CTGF* mRNA expression. In an immunohistochemical analysis of primary ovarian cancers, CTGF protein expression was frequently reduced (84 of 103 cases, 82%). Ovarian cancer tended to lack CTGF expression more frequently in the earlier stages (stages I and II) than the advanced stages (stages III and IV). CTGF protein was also differentially expressed among histologic subtypes. Exogenous restoration of CTGF expression or treatment with recombinant CTGF inhibited the growth of ovarian cancer cells lacking its expression, whereas knockdown of endogenous CTGF accelerated growth of ovarian cancer cells with expression of this gene. These results suggest that epigenetic silencing by hypermethylation of the *CTGF* promoter leads to a loss of CTGF function, which may be a factor in the carcinogenesis of ovarian cancer in a stage-dependent and/or histologic subtype-dependent manner. [Cancer Res 2007;67(15):7095–105]

Introduction

Epithelial ovarian cancer is the leading cause of death from gynecologic tumors (1), due to its aggressive nature and the fact that the majority of patients are diagnosed in advanced stages of the disease. The lack of preventive strategies, early diagnostic methods, and effective therapies to treat recurrent ovarian cancer creates a pressing need to understand the molecular mechanisms responsible for the development and progression of ovarian cancer and to identify molecular markers and targets for diagnosis as well as therapy (2). Sporadic ovarian cancers display defects in many genes, including *AKT, EGFR, ERBB2, RAS, PIK3CA, MYC, DOC-2/DAB2, SNCG*, and *TP53*, as well as a myriad of cytogenetic abnormalities (3). These defects result from both genetic and epigenetic changes and can occur at varying frequencies in different pathologic subtypes, which are morphologically and biologically heterogeneous, both early and late in the transformation process (2). Because there have been no known tumor suppressor genes (TSG) other than *TP53* showing high frequencies of somatic mutations in ovarian cancer, further efforts for the identification of putative TSGs are needed.

Several typical TSGs were originally pinpointed by mapping regions of biallelic loss in cancer cells (4–6), although the homozygous deletion of those genes is a rare event and other mechanisms, including aberrant methylation of CpG sites within the promoter region (7), may predominantly contribute to their functional inactivation. Therefore, scanning the entire genome for homozygous deletions with high resolution is believed to be useful for a precise and rapid identification of tumor suppressors. Indeed, we have applied in-house bacterial artificial chromosome (BAC)–based arrays (8) for an array-based comparative genomic hybridization (array-CGH) analysis of various human cancers and identified candidate TSGs mainly inactivated through homozygous loss or promoter hypermethylation from homozygously deleted regions (9, 10).

In ovarian cancer, the aberrant DNA methylation of known TSGs, such as *p16INK4A* (11), *RASSFIA* (12), *BRCA1* (13), and *hMLH1* (14), has been reported. However, the importance of epigenetic changes to TSGs in ovarian cancer remains largely unknown, and it is possible that more genes frequently inactivated through DNA methylation and involved in the pathogenesis of ovarian cancer will be identified. In the report presented here, during the course of a program to screen a panel of ovarian cancer cell lines for copy number aberrations in a genome-wide manner using our in-house BAC array (8), we have identified a homozygous loss of *connective tissue growth factor* (*CTGF/CCN2*) at 6q23.1, whose expression was absent in some ovarian cancer cell lines without homozygous loss, although it

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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was present in normal ovary. Because (*a*) reduced expression of CTGF and its clinicopathologic significance (15, 16) and DNA methylation of the genomic sequence around *CTGF* (17–19) in several cancers were reported but (*b*) a detailed target sequence for DNA methylation contributing gene silencing has never been shown and (*c*) the effect of down-regulated CTGF expression on ovarian carcinogenesis remains unknown, we further determined the expression and methylation status of *CTGF* and their clinicopathologic and functional significance using cell lines and primary tumors of ovarian cancer.

Materials and Methods

Cell lines and primary tumors. Of the 24 ovarian cancer cell lines used (Supplementary Table S1), ES-2 was obtained from the American Type Culture Collection; OVISE, OVMANA, OVTOKO, OVKATE, OVSAHO, and RMUG-S were from the Japanese Collection of Research Bioresources (Osaka, Japan); and HT, MH, KK, KF28, and KFr13 were from the National Defense Medical College (20, 21). Other lines (HTOA, HUOA, HMKOA, MCAS, HMOA, HNOA, RMG-I, RMG-II, RMUG-L, W3UF, HIOAnu, and HTBOA) were described previously (22). As a control, the normal ovarian epithelial cell–derived cell line OSE-2a (23) was kindly provided by Dr. Hidetaka Katabuchi (Kumamoto University School of Medicine, Kumamoto, Japan). All cell lines were maintained in appropriate medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The status of the *TP53* gene (exons 5–8) mutation was determined as described previously (24). To analyze restoration of genes of interest, cells were cultured with or without various concentrations of 5-aza 2′-deoxycytidine (5-aza-dCyd) for 5 days and/or 100 ng/mL trichostatin A (TSA) for the last 12 h.

Primary tumor samples were obtained during surgery from 114 patients being treated at the National Cancer Center Hospital in Tokyo, with written consent from each patient in the formal style and after approval by the local ethics committees. Samples from 66 of these patients were frozen immediately in liquid nitrogen and stored at −80°C until required, whereas samples from 103 of the patients were embedded in paraffin for immunohistochemistry. None of the patients had received preoperative radiation or immunotherapy. All patients underwent complete surgical staging, including i.p. cytology, bilateral salpingo-oophorectomy, hysterectomy, omentectomy, and pelvic/para-aortic lymphadenectomy. Aggressive cytoreductive surgery was done in patients with advanced disease. Surgical staging was based on the International Federation of Gynecology and Obstetrics (FIGO) staging system: stage I, 57 patients; stage II, 11 patients; stage III, 34 patients; and stage IV, 12 patients.

Array-CGH analysis. A MCG Cancer Array-800 (8) was used for the array-CGH. Hybridizations were carried out as described elsewhere (9). Hybridized slides were scanned with a GenePix 4000B (Axon Instruments), and acquired images were analyzed with GenePix Pro 6.0 imaging software (Axon Instruments). Average ratios that deviated significantly (>2 SD) from 0 (log 2 ratio, <−0.4 and >0.4) were considered abnormal.

Screening for homozygous deletions by genomic PCR using cell lines. We screened DNAs from 24 ovarian cancer cell lines for homozygous losses by genomic PCR. All primer sequences used in this study are listed in Supplementary Table S2.

Reverse transcription-PCR and quantitative real-time reverse transcription-PCR. Single-stranded cDNAs were generated from total RNAs and amplified with primers specific for each gene (Supplementary Table S2). The *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was amplified at the same time to allow estimation of the efficiency of cDNA synthesis. For reverse transcription-PCR (RT-PCR), PCR products were electrophoresed in 3% agarose gels (9). Quantitative real-time RT-PCR experiments were done with an ABI Prism 7900 Sequence Detection System (Applied Biosystems) as described previously (24). Each assay was done in triplicate.

Methylation analysis. Genomic DNAs were treated with sodium bisulfite and subjected to PCR using primer sets designed to amplify regions of interest (Supplementary Table S2). For the combined bisulfite restriction analysis (COBRA; ref. 26), PCR products were digested with *Bst*UI and electrophoresed. For bisulfite sequencing, PCR products were subcloned and then sequenced.

For the methylation-specific PCR (MSP) analysis, sodium bisulfite–treated DNAs were subjected to PCR using primers specific to the methylated (MSP) and unmethylated (unmethylated specific PCR) forms of DNA sequences of interest (Supplementary Table S2), and PCR products were visualized on 3% agarose gels. DNAs from cell lines recognized as unmethylated by bisulfite sequencing were used as negative controls for methylated alleles, whereas those from cell lines recognized as methylated or CpGenome Universal Methylated DNA (Chemicon International) were used as positive controls.

Promoter reporter assay. DNA fragments around the *CTGF* CpG island were obtained by PCR and ligated into the vector pGL3-Basic (Promega). Reporter assay was done as described elsewhere (9) using each construct or a control empty vector and an internal control pRL-hTK vector (Promega).

Immunohistochemistry and scoring method. Indirect immunohistochemistry was done with formalin-fixed, paraffin-embedded tissue sections as described elsewhere (26). Briefly, antigens were retrieved by autoclave pretreatment in high pH buffer (DAKO) for 10 min at 95°C. After blocking in 2% normal swine serum, the slides were incubated with an anti-CTGF antibody (1:100 dilution; Santa Cruz Biotechnology) overnight at 4°C and then reacted with a Histofine simple stain, MAX PO(G) (Nichirei). Antigen-antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. The slides were counterstained with Mayer's hematoxylin.

A formalin-fixed HTBOA cell line expressing *CTGF* mRNA, in which >50% of cells showed cytoplasmic staining of CTGF protein, and RMUG-S cell line lacking *CTGF* mRNA expression, in which none of the cells showed cytoplasmic staining of CTGF protein, were used as positive and negative controls, respectively. Specificity of the antibody was verified by Western blotting (9) as well as an absorption test using synthetic peptide (Santa Cruz Biotechnology; Supplementary Fig. S1). The percentage of the total cell population that expressed CTGF was evaluated for each case at ×200 magnification. Expression of CTGF was graded as either positive (≥10% of tumor cell cytoplasm showing immunopositivity) or negative (<10% of tumor cell cytoplasm showing immunopositivity or no staining). Two observers, who were blinded to the clinical outcomes of the patients, evaluated the slides independently; if a significant discrepancy emerged between their judgments, a consensus was reached after discussion.

Transient transfection, Western blotting, and colony formation assay. A plasmid expressing COOH-terminal 3× Myc-tagged CTGF (pCMV-3Tag-4-CTGF) was obtained by cloning the PCR product of the full coding sequence of *CTGF* in-frame along with the Myc epitopes into the vector pCMV-3Tag-4 (Stratagene). pCMV-3Tag-4-CTGF, or the empty vector (pCMV-3Tag-4-mock), was transfected into cells for colony formation assays (9). The expression of CTGF protein in transiently transfected cells was confirmed 48 h after transfection by Western blotting as described elsewhere (9). Cells were stained with crystal violet after 2 weeks of incubation in six-well plates with appropriate concentrations of G418.

Treatment with recombinant CTGF. To assess the effect of CTGF on growth of ovarian cancer cell lines, cells were treated with 2.5 μg/mL of recombinant human CTGF (Peprotech EC) or PBS for 72 h. The numbers of viable cells after transfection were assessed by a colorimetric water-soluble tetrazolium salt (WST) assay (24). The cell cycle in CTGF-treated cells was analyzed using fluorescence-activated cell sorting (FACS) as described elsewhere (24). For Western blotting, 24-h serum-starved cells were pretreated with or without 2.5 μg/mL CTGF for 1 h and then stimulated with 25 ng/mL of recombinant human epidermal growth factor (EGF; Sigma) for 15 min. Phosphorylation status of extracellular signal-regulated kinase 1/2 (ERK1/2) was evaluated using anti–phospho-ERK1/2 (P-ERK1/2) and anti-ERK1/2 antibodies (Cell signaling Technology).

Transfection with synthetic small interfering RNA. *CTGF*-specific small interfering RNA (siRNA; *CTGF*-siRNA) was purchased from Santa Cruz Biotechnology. A control siRNA for the luciferase gene (CGUACGCG-GAAUACUUCGA, *Luc*-siRNA) was synthesized by Sigma. Each siRNA

(50 nmol/L) was transfected into ovarian cancer cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. The numbers of viable cells 24 to 96 h after transfection were assessed by WST assay.

Statistical analysis. Differences between subgroups were tested by the Mann-Whitney *U* test. Correlations between *CTGF* methylation or expression in primary ovarian cancers and the clinicopathologic variables pertaining to the corresponding patients were analyzed for statistical significance by χ^2 or Fisher's exact test. For the analysis of survival, Kaplan-Meier survival curves were constructed for groups based on univariate predictors, and differences between the groups were tested with the log-rank test. Differences were assessed with a two-sided test and considered significant at the $P < 0.05$ level.

Results

Array-CGH analysis of ovarian cancer cell lines. We assessed copy number alterations among the 24 ovarian cancer cell lines by array-CGH using the same batch of MCG Cancer Array-800 slides for all of them. Copy number gains and losses were seen to some degree in all 24 lines (data not shown). Figure 1*A* documents the frequencies of copy number gains and losses across the entire genome of each cell line. Our array-CGH analysis predicted frequent copy number gains for 3q and 20q and frequent losses for 4q, 13q, 15q, 17p, 18q, Xp, and Xq (Supplementary Table S3), which were mostly consistent with those of our earlier conventional CGH analysis of ovarian cancer cell lines (22) and were similar to published results of conventional CGH analyses of primary ovarian cancers (27–29).

Because the most common genetic aberrations had already been identified in ovarian cancer cell lines and primary tumors, we paid attention to more remarkable patterns of chromosomal abnormalities, such as high-level amplifications (log 2 ratio, >2) and homozygous deletions (log 2 ratio, <−2), which are likely to be landmarks of oncogenes and TSGs, respectively (Table 1). High-level amplifications were detected in two cell lines, and three clones (genes) were presented. Homozygous deletions were detected in three cell lines, and five clones (genes) were presented. Among those genes, *MTAP* and *CDKN2A/p16* located of 9p21.2, *TGFBR2* at 3p24.1, and *SMAD4* at 18q21.1 are known as TSGs inactivated in various human cancers. On the other hand, the homozygous loss at 6q23, the location of *CTGF* (Fig. 1*B*), observed in RMUG-S cells had not been documented in ovarian cancer before, prompting us to examine whether genes, including *CTGF*, located within this region might be involved in the pathogenesis of ovarian cancer.

Identification of target genes involved in homozygous deletion at 6q23.1. To define the extent of the homozygous deletion in RMUG-S cells and to identify other cell lines harboring cryptic homozygous loss in this region, we did genomic PCR experiments with 10 genes, *MOXD1, CTGF, ENPP1, ENPP3, CRSP3, ARG1, AKAP7, EPB41L2, KIAA1913*, and *L3MBTL3* (Fig. 2*B*), which are located around RP11-69I8 (Fig. 1*C*) according to information archived by genome databases.[9] We detected a complete loss of *CTGF, ENPP1, ENPP3, CRSP3, ARG1, AKAP7, EPB41L2*, and *KIAA1913* only in RMUG-S cells (4.2%), whereas *MOXD1* and *L3MBTL3* were retained in this cell line, indicating that the size of the homozygous deletion is ~2.2 Mb at maximum.

Loss of *CTGF* expression and its restoration after DNA demethylation in ovarian cancer cell lines. Next, we determined mRNA expression levels of *CTGF, ENPP1, ENPP3, CRSP3, AKAP7, EPB41L2*, and *KIAA1913* by in all 24 ovarian cancer cell lines, normal ovary, and the normal ovarian epithelial cell–derived immortalized cell line OSE-2a. We excluded *ARG1* from the analysis because our preliminary experiment (data not shown) and the information archived by the genome databases[10] showed almost no expression of this gene in normal ovary. Among seven genes we tested, *CRSP3, EPB41L2*, and *AKAP7* were expressed in most of the ovarian cancer cell lines and normal ovary (Fig. 1*C*), suggesting that these genes are unlikely to be targets for inactivation in ovarian cancer cells. On the other hand, *CTGF, ENPP1, ENPP3*, and *KIAA1913* were frequently silenced even in ovarian cancer cell lines without their homozygous loss, suggesting that the loss of expression of those genes might result from mechanisms other than genomic deletion. Because aberrant methylation in 5′ regulatory region harboring a larger than expected number of CpG dinucleotides (CpG island) is a key mechanism by which TSGs can be silenced (7), we searched for the CpG island around transcription start sites of those genes using the CpGPLOT program[11] and identified it only in *CTGF* but not in the other three genes, prompting us to focus on *CTGF* for further analyses. *CTGF* showed a complete loss of expression in the RMUG-S cell line and a reduced expression in another 12 lines without its homozygous loss (12 of 23, 52%; Fig. 1*C*). The other 11 ovarian cancer lines, normal ovary, and OSE-2a cells did express *CTGF* mRNA. Only one of the five lines that had shown a hemizygous loss around *CTGF* in array-CGH analysis exhibited a decline in the expression of this gene (data not shown).

To investigate whether DNA demethylation could restore the expression of *CTGF* mRNA, we treated ovarian cancer cells lacking *CTGF* expression with 5-aza-dCyd for 5 days. Induction of *CTGF* mRNA expression occurred after treatment with 5 μmol/L 5-aza-dCyd in HNOA and in RMG-II cells (Fig. 1*D*). In addition, we observed an enhancement of *CTGF* mRNA expression by 5-aza-dCyd given along with TSA in both lines, although treatment with TSA alone had no effect on the expression, suggesting that histone deacetylation does play some role in the transcriptional silencing of *CTGF* among methylated ovarian cancer cells. Restoration of *CTGF* expression by the treatment with 5-aza-dCyd was also observed in the rest of the ovarian cancer cell lines with reduced expression of this gene except RMUG-S (Supplementary Fig. S2*A*).

Methylation of the *CTGF* CpG island in ovarian cancer cell lines. To show the potential role of the methylation within CpG island in silencing of *CTGF*, we first assessed the methylation status of each CpG site around the *CTGF* CpG island (regions 1–3 in Fig. 2*A*) in ovarian cancer cell lines with or without *CTGF* expression and the OSE-2a cells, by means of bisulfite sequencing (Fig. 2*A*). Regions 2 and 3 tended to be extensively methylated in *CTGF*-nonexpressing cell lines (HTOA, HUOA, RMUG-L, RMG-I, HNOA, and KF28), whereas region 1 was hypomethylated in almost all cell lines tested. In addition, regions 2B, the 3′ part of region 2, and 3 are extensively methylated in some *CTGF*-expressing ovarian cancer lines (KK and OVISE), whereas region 2A, the 5′ part of region 2, tended to be hypermethylated in the nonexpressing

[9] http://www.ncbi.nlm.nih.gov/ and http://genome.ucsc.edu/

[10] http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene and http://www.lsbm.org/database/index.html

[11] http://www.ebi.ac.uk/emboss/cpgplot/

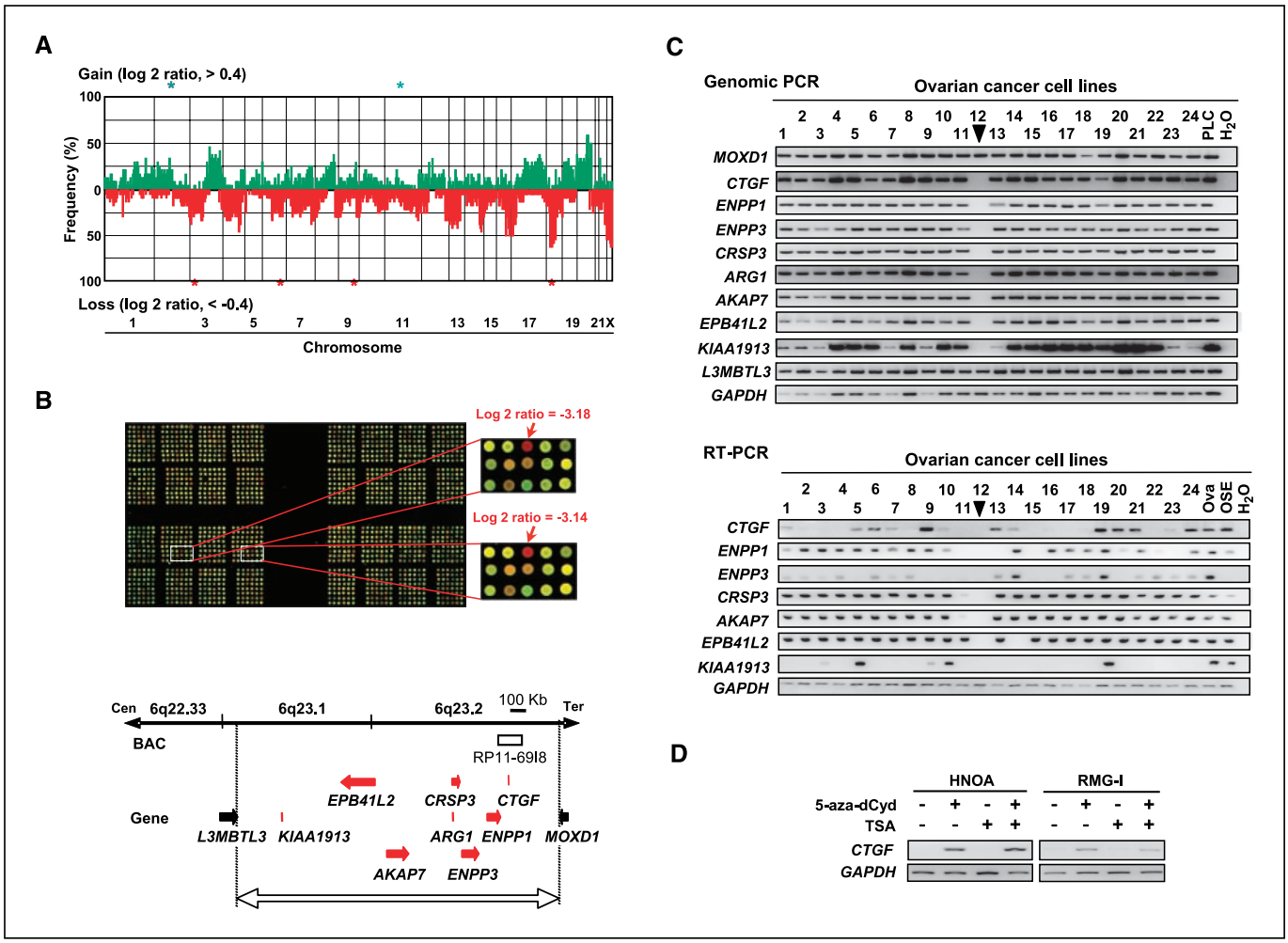

Figure 1. *A,* genome-wide frequencies of copy number gains (>0; *green*) and losses (<0; *red*) in 24 ovarian cancer cell lines. Clones are ordered as chromosomes 1 to 22 and X, and within each chromosome based on the University of California Santa Cruz (UCSC) mapping position (version May 2004). *Green asterisks,* clones with at least one high-level amplification; *red asterisks,* clones with at least one homozygous deletion (Table 1). *B,* identification of the 6q23.2 homozygous deletion in ovarian cancer cell line. *Top,* representative duplicate array-CGH image of the RMUG-S cell line. A homozygous deletion (copy number ratio as log 2 ratio) of the BAC clone at 6q23.2 was detected as a clear red signal (*red arrows*). *Bottom,* map of 6q23 covering the region homozygously deleted in the RMUG-S cell line. A BAC (RP11-69I8) was homozygously deleted in the array-CGH analysis (*vertical white bar*). Homozygously deleted region in RMUG-S cells, as determined by genomic PCR analysis (*vertical white closed arrow*). Ten genes located within this region (*red or black arrows*) show homozygously deleted or retained genes, respectively, and positions and directions of transcription. *C,* genomic and RT-PCR analyses of genes located around the 6q23 homozygously deleted region in ovarian cancer cell lines. *Top,* homozygous deletions of *CTGF, ENPP1, ENPP3, CRSP3, ARG1, AKAP7, EPB41L2*, and *KIAA1913*, but not *MOXD1* and *L3MBTL3*, were detected in one ovarian cancer cell line (RMUG-S; *arrowhead*) by genomic PCR. *1,* HT; *2,* HTOA; *3,* HUOA; *4,* KF28; *5,* MH; *6,* OVKATE; *7,* OVSAHO; *8,* KFr13; *9,* HMKOA; *10,* MCAS; *11,* RMUG-L; *12,* RMUG-S; *13,* KK; *14,* OVISE; *15,* OVMANA; *16,* OVTOKO; *17,* RMG-I; *18,* RMG-II; *19,* ES-2; *20,* W3UF; *21,* HIOAnu; *22,* HMOA; *23,* HNOA; *24,* HTBOA; PLC, peripheral leukocytes. *Bottom,* mRNA expression of *CTGF, ENPP1, ENPP3, CRSP3, AKAP7, EPB41L2*, and *KIAA1913* in ovarian cancer cell lines and the normal ovary (*Ova*) and normal ovarian epithelial cell–derived cell line OSE-2a (*OSE*), detected by RT-PCR analysis. *Arrowhead,* the cell line with the homozygous deletion indicated in the genomic PCR analysis. Expression of *CRSP3*, *AKAP7*, and *EPB41L2* mRNAs was observed to some degree in most ovarian cancer cell lines, whereas *ENPP1, ENPP3, CTGF*, and *KIAA1913* showed frequent silencing. Notably, 12 of the 23 cell lines (50%) without a homozygous deletion of *CTGF* showed decreased expression. *D,* results of RT-PCR to reveal restored *CTGF* expression in HNOA and RMG-I cells after treatment with 5-aza-dCyd (5 μmol/L) for 5 d with or without TSA (100 ng/mL) for 12 h.

ovarian cancer lines (HTOA, HUOA, RMUG-L, RMG-I, HNOA, and KF28) but hypomethylated in the *CTGF*-expressing ovarian cancer lines (KK, OVISE, and HTBOA) and OSE-2a cells. Consequently, methylation of region 2A was likely to be inversely correlated with the expression status of *CTGF*, suggesting that region 2A may be crucial to regulate the basal transcription level of *CTGF*.

To compare the methylation and expression status of *CTGF* in a larger number of ovarian cancer lines, we did COBRA. Consistent with the results of bisulfite sequencing, no methylated allele was detected in region 1 among most of the lines tested regardless of the *CTGF* expression status (Fig. 2*B*). On the other hand, most of the ovarian cancer cells lacking *CTGF* expression, except OVMANA and OVTOKO, had a methylated allele without an unmethylated allele in region 2, whereas most of the ovarian cancer cell lines and OSE-2a cells expressing *CTGF* had an unmethylated allele with or without methylated allele. Notably, OVISE cells expressing *CTGF* were found to have only an allele methylated in region 2 by COBRA. In this cell line, bisulfite sequencing showed that region 2B containing two *Bst*UI sites was highly methylated but region 2A was hypomethylated (Fig. 2*A*), indicating that region 2A is a critical target site for epigenetic events affecting *CTGF* expression. However, mechanisms other than DNA methylation, including

histone modification, epigenetic silencing of transcription factors or upstream components of signaling pathway activating CTGF expression, and microRNAs (30), may also contribute to the direct or indirect silencing of *CTGF*. Indeed, restoration of *CTGF* expression by TSA and/or 5-aza-dCyd was also observed in OVMANA cell line only having unmethylated allele and OVTOKO cell line having unmethylated allele and partially methylated alleles (Fig. 2*B*; Supplementary Fig. S2*B*).

Promoter activity of the sequence around the *CTGF* CpG island. Because the sequence around the *CTGF* CpG island seems to be a target for methylation and closely related to gene silencing, we tested sequences around the CpG island for promoter activity, using three fragments covering this region (fragments 1–3 in Fig. 2*A*) and three types of ovarian cancer cell lines: RMUG-S with a homozygous deletion of *CTGF*, KK expressing *CTGF*, and KF28 lacking *CTGF* expression. Because region 1 is unlikely to be a critical target for methylation, we excluded it from the analysis. Fragments 1 and 3 containing region 2A showed a remarkable increase in transcriptional activity, whereas fragment 2 not containing region 2A showed very weak activity in all types of cell lines (Fig. 2*C*), suggesting that region 2A may contain critical sequence(s) for gene silencing.

Methylation of the *CTGF* promoter region in primary ovarian cancer tumors. To determine whether the aberrant methylation of *CTGF* also takes place in primary tumors, we did MSP with primer sets targeting the sequence around the most frequently methylated sites around region 2A in a panel of 66 primary ovarian cancer tumors (Fig. 2*D*). Specificity and sensitivity of MSP and the comparison of sensitivity between MSP and COBRA were shown in Supplementary Fig. S3. Consistent with the results of the bisulfite sequencing and COBRA (Fig. 2*A* and *B*), a representative cell line lacking *CTGF* expression (RMUG-L) was methylated, whereas the *CTGF*-expressing cell line (OSE-2a) was unmethylated, as expected. We detected *CTGF* hypermethylation in 39 of the 66 primary ovarian cancer tissues (59%; Fig. 2*D*; data not shown). To confirm the results of the MSP analysis quantitatively, we did bisulfite sequencing in some of representative cases. Aberrant hypermethylation was observed in ovarian cancer tissues, which showed a methylation pattern in the MSP analysis, whereas tumors with an unmethylated pattern in the MSP analysis showed hypomethylation (Fig. 2*D*). To confirm that the methylation of *CTGF* is associated with gene silencing in primary ovarian cancer, we then examined the expression status of *CTGF* mRNA using real-time RT-PCR with cDNA prepared from 43 primary ovarian cancer tumors except for mucinous type tumors, which contain a larger amount of noncancerous cell contamination compared with other types of ovarian cancer. We found that primary tumors showing methylation of the *CTGF* region 2A by MSP expressed the gene at a significantly lower level than tumor without methylation ($P = 0.041$, Mann-Whitney U test; Fig. 2*D*), suggesting that the methylation of *CTGF* promoter and the gene silencing through this mechanism were not an artifact arising during the passage of ovarian cancer cell lines *in vitro*, but rather may be a true cancer-related event during the pathogenesis of ovarian cancer. However, no clear association between the methylation status of *CTGF* region 2A and the clinicopathologic characteristics was observed (Supplementary Table S4).

Association between expression level of CTGF and clinicopathologic characteristics in primary cases. To further clarify the clinical significance of the *CTGF* gene in ovarian cancer, the expression level of CTGF protein in primary ovarian cancer tissues was evaluated by immunohistochemistry using a CTGF-specific antibody (Supplementary Fig. S1). The results of the immunohistologic staining were classified as level 0 (negative staining), level 1 (1–10% of tumor cells stained), level 2 (10–50% of tumor cells stained), and level 3 (>50% of tumor cells stained). A high level of immunoreactivity for CTGF (level 3) was detected in normal ovarian epithelium (Fig. 3*A*). The CTGF protein was predominantly found in the cytoplasm or the membrane of normal or tumor epithelial cells. Although some ovarian cancer specimens showed high levels of CTGF (Fig. 3*B*), no or very weak immunoreactivity (levels 0 and 1) for CTGF was frequently observed in other ovarian cancer specimens (Fig. 3*C*). A low (levels 0 and 1) and high expression levels (levels 2 and 3) of CTGF were found in 84 of 103 (82%) cases and 19 of 103 (18%) cases, respectively. The relationship between the expression level of CTGF protein and the clinicopathologic characteristics is summarized in Table 2. In contrast to the *CTGF* mRNA level,

Table 1. High-level amplifications (log 2 ratio, >2.0) and homozygous deletions (log 2 ratio, <−2.0) detected in 24 ovarian cancer cell lines by array-CGH analysis using MCG Cancer Array-800

Alteration	BAC	Locus*		Cell line ($N = 24$)		Known candidate target gene†
		Chromosome band	Position	*n*	Name	
High-level amplifications (log 2 ratio, >2.0)	RP11-438012	2q14.2	chr2: 120,629,082–120,846,427	1	OVISE	*GL12*
	RP11-300I6	11q13.3	chr11: 69,162,462–69,323,966	1	ES-2	*CCND1, FGF3*
	CTD-2234J21	11q13.3	chrl1: 69,307,612–69,307,884	1	ES-2	*CCND1, FGF3*
Homozygous deletions (log 2 ratio, <−2.0)	RP11-7I16	3p24.1	chr3: 30,541,893–30,705,070	1	KFr13	*TGFBR2*
	RP11-69I8	6q23	chr6: 132,249,163–132,410,700	1	RMUG-S	None
	RP11-70L8	9p21.3	chr9: 21,732,608–21,901,258	1	HTBOA	*CDKN2A, MTAP*
	RP11-145E5	9p21.3	chr9: 21,792,634–22,022,985	1	HTBOA	*CDKN2A, MTAP*
	RP11-10I6	18q21.1	chr18: 46,348,632–46,493,352	1	RMUG-S	*SMAD4*

* Based on UCSC Genome Browser, May 2004 Assembly.

† Putative oncogenes or tumor suppressor genes located around BAC.

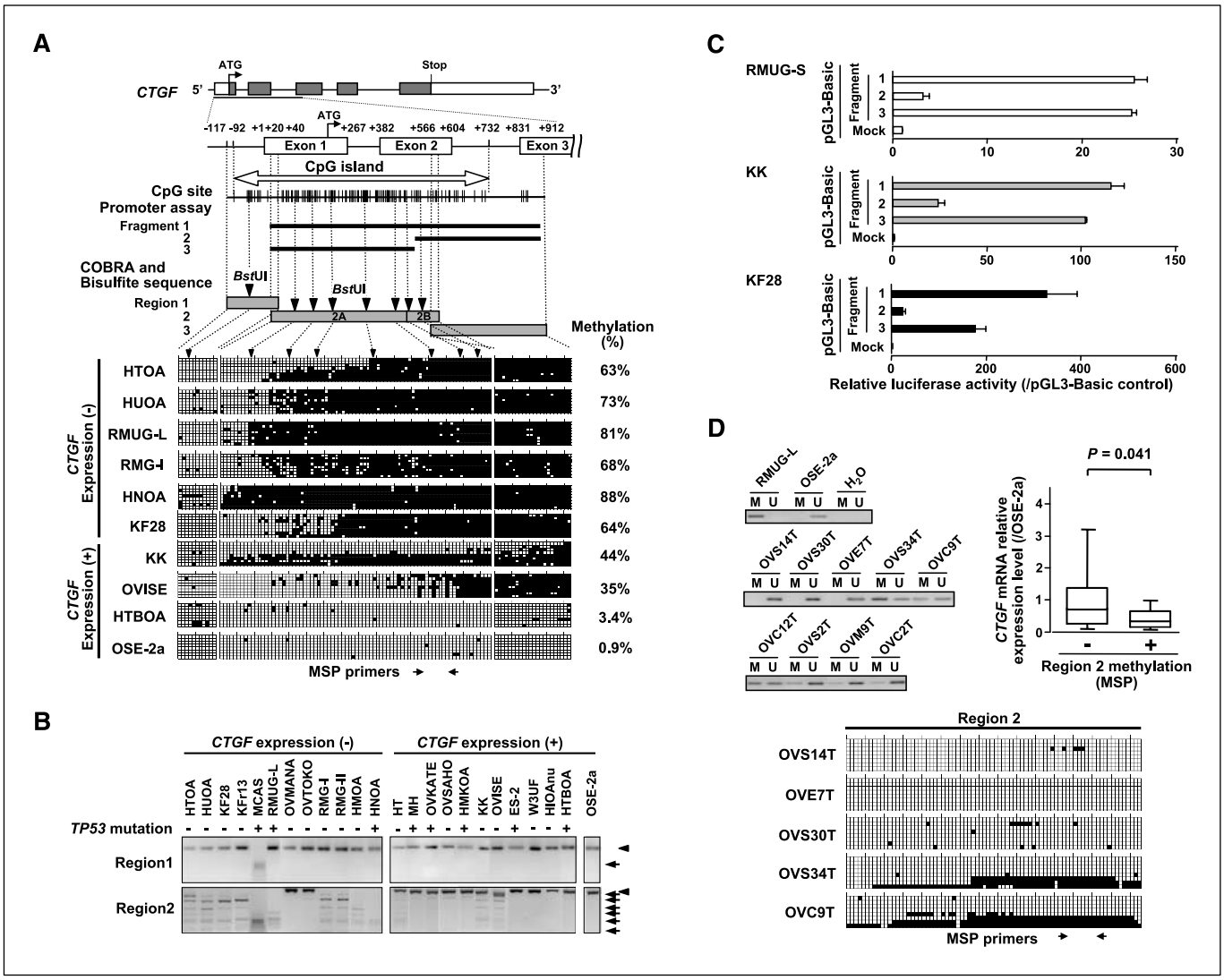

Figure 2. Methylation status of the *CTGF* CpG-rich region in ovarian cancer cell lines. *A,* schematic map of the CpG-rich region containing the CpG island (*closed white arrow*) around exon 1 of *CTGF* and representative results of bisulfite sequencing. CpG sites (*vertical ticks on the expanded axis*). Exons (*open box*). The transcription-start site is marked at +1. The fragments examined in a promoter assay (*thick black lines*). The regions examined in the COBRA and bisulfite sequencing (*horizontal gray bars*). Restriction sites for *Bst*UI (for the COBRA; *black downward arrowheads*). Representative results of bisulfite sequencing of the *CTGF* CpG-rich region examined in *CTGF*-expressing ovarian cancer cell lines (+) and *CTGF*-nonexpressing ovarian cancer cell lines (−). Each square indicates a CpG site: *open squares,* unmethylated; *solid squares,* methylated. PCR primers for MSP (*arrows*). *B,* representative results of the COBRA of the *CTGF* CpG island in ovarian cancer cell lines after restriction with *Bst*UI. *Arrows,* fragments specifically restricted at sites recognized as methylated CpGs; *arrowheads,* undigested fragments indicating unmethylated CpGs. *C,* promoter activity of the *CTGF* CpG-rich region. pGL3-Basic empty vectors (mock) and constructs containing one of three different sequences around the highly methylated region of *CTGF* (fragments 1–3 with a 157, 584, and 346 bp size, respectively, in *A*) were transfected into RMUG-S, KK, and KF28 cells. Luciferase activity was normalized versus an internal control. *Columns,* mean of three separate experiments, each done in triplicate; *bars,* SD. *D, top left,* representative results of a MSP analysis of the *CTGF* promoter region in primary ovarian cancer tissues. Parallel amplification reactions were done using primers specific for unmethylated (*U*) or methylated (*M*) DNA. *Top right,* correlation between methylation status of *CTGF* determined by MSP and mRNA expression status determined by RT-PCR in 43 primary tumors except for mucinous type tumors. Statistical analysis used the Mann-Whitney *U* test. *Horizontal bars in the boxes,* median values; *vertical bars,* range; *horizontal boundaries of the boxes,* first and third quartiles. *Bottom,* methylation status of the *CTGF* promoter region determined by bisulfite sequencing in tumor samples. *Arrows,* the positions of primers for MSP.

CTGF protein expression was not clearly associated with the methylation status of *CTGF* region 2, even in tumors other than mucinous type tumors (P = 0.215, Fisher's exact test; data not shown). CTGF protein expression was significantly associated with tumor stage: ovarian cancer tended to lack CTGF expression in the earlier stages (stages I and II) but tended to exhibit CTGF expression in the advanced stages (stages III and IV; P = 0.027, χ^2 test). CTGF protein was also differentially expressed among histologic subtypes. However, no significant relationship was found between the level of CTGF expression and the age of patients, the result of surgery, or the result of peritoneal cytology. In overall survival, no significant difference was observed between the patients with lower levels of CTGF and those with higher levels of CTGF in all stages and in stage III and IV disease (P = 0.158 and 0.148, respectively, log-rank test; data not shown). In stage I and II disease, however, no deaths occurred in patients with higher levels of CTGF expression during the study period, whereas 17.5% (10 of 57 cases) of patients with lower levels of expression died, although a statistical analysis could not be done due to no deaths in one group (Fig. 3*D*). Those findings suggest

that the incidence of the inactivation of CTGF and its role in tumorigenesis may differ with the stage and/or histologic subtype of this disease.

Suppression of cell growth induced by CTGF in ovarian cancer cells. The frequent silencing of *CTGF* in cell lines and primary tumors of ovarian cancer suggests that CTGF is likely to be a functional tumor suppressor for this disease. To investigate whether restoration of CTGF expression would suppress growth of ovarian cancer cells in which the gene had been silenced, we did colony formation assays using an expression construct of the full-coding sequence of *CTGF* (Fig. 4*A*). Two weeks after transfection and subsequent selection of drug-resistant colonies, the numbers of larger colonies produced by *CTGF*-transfected cells decreased compared with those of cells containing empty vector, regardless of mutation status of the *TP53* gene (Fig. 2*B*).

To avoid a nonspecific toxicity by forced expression of CTGF, we assessed the effect of recombinant human CTGF on growth of the nonexpressing ovarian cancer cells (Fig. 4*B, top*). Treatment with recombinant CTGF for 72 h reduced cell viability in HNOA and HMOA cell lines compared with vehicle (PBS) alone. In FACS analysis using HMOA cell line (Fig. 4*B, bottom*), treatment with recombinant CTGF resulted in an accumulation of cells in G_0-G_1 phase and a decrease in S and G_2-M phase cells but no increase in sub-G_1 phase cells compared with vehicle alone, suggesting that CTGF may arrest ovarian cancer cells at the G_1-S checkpoint (G_0-G_1 arrest) without inducing apoptosis.

To further examine the mechanisms of CTGF-induced growth inhibition in ovarian cancer cells, we investigated the effect of CTGF on EGF-dependent phosphorylation of ERK1/2 in HMOA cell line because (*a*) the overexpression of the EGF receptor is associated with poor prognosis of ovarian cancer (31) and (*b*) the suppressive effect of CTGF overexpression on EGF-dependent phosphorylation has been reported in non–small cell lung cancer (NSCLC) cell line (16). In serum-starved HMOA cells, ERK1/2 was remarkably phosphorylated with EGF treatment and the level of phosphorylation was decreased by pretreatment with CTGF (Fig. 4*C*).

To confirm a growth-suppressive effect of CTGF, we knocked down endogenously expressed CTGF by transient transfection of *CTGF*-siRNA to KK and ES-2 cell lines retaining expression of CTGF (Fig. 4*D*). Transfection of *CTGF*-siRNA accelerated cell growth in those cell lines compared with *Luc*-siRNA–transfected counterparts. Because transfection of *CTGF*-siRNA to RMUG-S cell line lacking *CTGF* expression showed no effect on cell growth compared with *Luc*-siRNA, growth-promoting effect of *CTGF*-siRNA observed in KK and ES-2 cells may not be caused by off-target effects of siRNA used in this study.

Discussion

In this study, we identified a homozygous deletion of *CTGF* at 6q23.2 in ovarian cancer cell lines by array-CGH analysis using an in-house BAC array. Expression of *CTGF* was detected in normal ovary and a normal ovarian epithelial cell–derived cell line but frequently silenced through methylation of CpG sites around the *CTGF* CpG island exhibiting promoter activity in our panel of ovarian cancer cell lines, suggesting that *CTGF* may be one of targets for inactivation in ovarian cancer, although possible involvement of other target gene(s) for the homozygous loss at 6q23.1 remains unclear. Hypermethylation of the *CTGF* promoter region was frequently detected in primary ovarian cancers. Lower CTGF protein levels were frequently observed in primary ovarian cancers, although the clinical significance of CTGF expression might differ among disease stages and histologic subtypes. In addition, the transient transfection of *CTGF* or treatment with recombinant CTGF had an inhibitory effect on growth of *CTGF*-nonexpressing ovarian cancer cells, whereas knockdown of CTGF using siRNA accelerated growth of

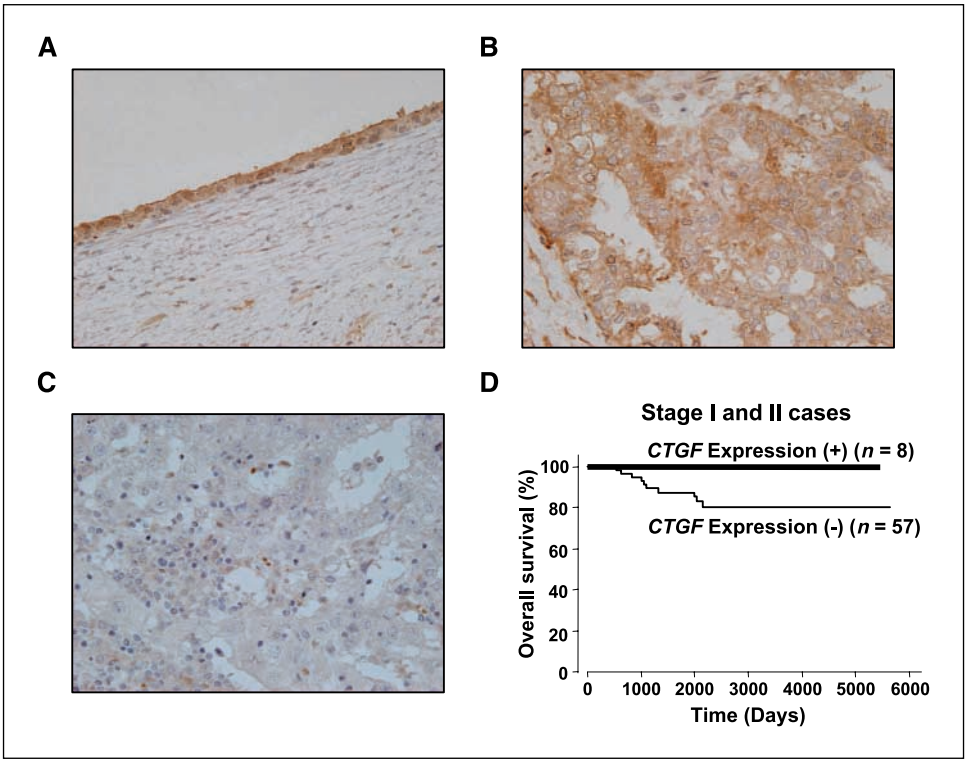

Figure 3. Immunohistochemical analysis of CTGF expression in primary ovarian cancer tumors. *A,* representative CTGF immunohistochemical staining of normal human ovarian epithelial cells. High CTGF expression is shown in normal ovarian epithelial cells. Magnification, ×200. *B* and *C,* representative CTGF immunohistochemical staining of primary ovarian cancer cells. High (*B*) or almost no (*C*) expression of CTGF was observed in primary ovarian cancer cells. In normal epithelial cells and ovarian cancer cells, CTGF is localized distinctly in the apical cytoplasm. Magnification, ×200. *D*, Kaplan-Meier curve for overall survival rates of patients with stage I and II ovarian cancer. There were no deaths in patients with stage I and II ovarian cancers showing higher CTGF levels.

Table 2. Correlation between clinical background and expression of CTGF protein

	n	Expression of CTGF*	*P* †
		n (%)	
Total	103	19 (18)	
Age (y)			
<60	71	15 (21)	0.388
≥60	32	4 (13)	
FIGO stage			
I and II	66	8 (12)	**0.027**
III and IV	37	11 (30)	
Histologic type			
Serous	42	8 (19)	**0.029**
Mucinous	15	7 (47)	
Clear cell	34	2 (6)	
Endometrioid	12	2 (17)	
Optimal surgery (cm)			
Optimal (<2)	82	16 (20)	1.000
Suboptimal (≥2)	14	2 (14)	
Unknown	7	1 (14)	
Peritoneal cytology			
Positive	48	11 (23)	0.387
Negative	50	8 (16)	
Unknown	5	0 (0)	
Methylation ‡			
Positive	33	6 (18)	0.739
Negative	22	5 (23)	
Unknown	48	8 (17)	

NOTE: Statistically significant values are in boldface type.
* CTGF protein expression was evaluated by immunohistochemical analysis described in Materials and Methods.
† *P* values are from χ^2 or Fisher's exact test and were statistically significant when <0.05 (two sided).
‡ Methylation status was determined by MSP target for region 2A described in Materials and Methods.

CTGF-expressing ovarian cancer cells. These results suggest that loss of epigenetic inactivation of CTGF plays a pivotal role in the tumorigenesis of ovarian epithelial cells.

CTGF is a member of the CCN family, which comprises CTGF, cysteine-rich 61 (Cyr61/CCN1), nephroblastoma overexpressed (Nov/CCN3), Wisp-1/elm1 (CCN4), Wisp-2/rCop1 (CCN5), and Wisp-3 (CCN6). Among them, CTGF is believed to be a multifunctional signaling modulator involved in a wide variety of biological or pathologic processes, such as angiogenesis, osteogenesis, and renal and skin disorders (32–35). In carcinogenesis, CTGF was shown to be a positive regulator: the level of CTGF expression is positively correlated with bone metastasis in breast cancer (36), glioblastoma growth (37), a poor prognosis in esophageal adenocarcinomas (38), the aggressive behavior of pancreatic cancer cells (39), the invasive melanoma (40), and prognosis of chondrosarcoma (41). On the other hand, there is a body of evidence showing antigrowth (16, 42, 43) or antimetastatic (15) activity of CTGF in cancer cells and decreased CTGF expression in the aggressive or metastatic phenotype in various cancers, such as breast, colon, and NSCLCs (15, 16, 45). Given our results showing a tumor-suppressive function of CTGF, the role of CTGF in various cancers seems to vary considerably, depending on the tissues involved, although the exact mechanism has not yet been clarified. The question of how the tissue context is able to determine the action of CTGF in carcinogenesis deserves further investigation.

CTGF is located at 6q23.1, a chromosomal region that is rarely involved in copy number losses (22, 27–29). Indeed, most of the ovarian cancer cell lines used in this study showed normal DNA copy numbers around this region. Among 12 cell lines that showed reduced expression of *CTGF*, 9 lines had promoter hypermethylation, only 1 line showed both hemizygous deletion around this gene and promoter hypermethylation, whereas 2 lines showed neither, suggesting that the inactivation of CTGF might occur frequently through methylation of both alleles during the tumorigenesis of ovarian cancer. DNA methylation around the *CTGF* gene has also been reported in other cancers, such as hepatocellular carcinoma (17, 18) and colon cancer cell lines (19), suggesting that *CTGF* might be a universal target for methylation in various types of cancers. However, (*a*) some ovarian cancer cell lines showed reduced *CTGF* expression without DNA methylation and (*b*) silencing of CTGF protein expression occurs more frequently compared with DNA methylation of the *CTGF* gene in primary ovarian cancer, suggesting that mechanisms other than DNA methylation also contribute to silencing of CTGF in ovarian cancer. Recently, miR-17-92, especially miR-18, was shown to be responsible for CTGF down-regulation in Myc-transduced RAS-transformed mice colonocytes (30). Therefore, further analyses will be needed to clarify all mechanisms for silencing CTGF expression and determine the functional significance of each mechanism in primary ovarian cancer.

In our promoter assays, the *CTGF* CpG island around exons 1 and 2, especially fragment 3 from exon 1 to the middle of exon 2, whose methylation status was inversely related with expression status in ovarian cancer cells, showed clear promoter activity, whereas fragment 2 from the middle of exon 2 to exon 3, which was highly methylated in ovarian cancer cells regardless of expression status, showed weaker promoter activity. It was reported that the commonly methylated region within the *CTGF* CpG island starts from the middle of exon 1 and its methylation seems to be inversely correlated with *CTGF* expression in hepatocellular carcinoma cell lines (17, 18), and exonic methylation is observed in colon cancer cell lines with increased expression of *CTGF* caused by 5-aza-dCyd treatment (19), although methylation status of CpG sites through the entire CpG island and its correlation with gene expression was not clearly shown (17–19). Those results suggest that methylation of CpG sites within fragment 3/region 2A might be responsible for the silencing of *CTGF*, although few studies have shown that promoter activity can occur in fragments, especially CpG islands, not containing a 5′ sequence around transcription start sites (9, 46–48).

In the present study, little or no immunoreactivity for CTGF protein was observed in most primary ovarian cancers, especially in the earlier stages, which is contrary to the previously reported finding that low-level immunoreactivity was usually observed in advanced stage of colorectal cancers (15). In the earlier stages of ovarian cancer, moreover, patients with tumors showing lower CTGF immunoreactivity tended to have a worse survival rate than those showing higher levels of expression. Because we showed that (*a*) normal ovarian epithelia and immortalized ovarian epithelial cell–derived cell line express CTGF and (*b*) induction of *CTGF* expression or treatment of recombinant CTGF inhibited growth of *CTGF*-nonexpressing ovarian cancer cells, it is suggested that frequent silencing of *CTGF* occurred as an early event in ovarian

cancers at least partly through promoter methylation may contribute to the progression to an advanced stage. In the advanced stages, on the other hand, *CTGF* expression might be restored and contribute to more malignant phenotypes, such as invasion and metastasis, although the number of cases was too small to provide any conclusive results in the statistical analysis. In a breast cancer model (36), *CTGF* was identified as one of the genes contributing to bone metastagenicity, and its expression was transcriptionally induced by transforming growth factor β (TGFβ), which can have direct pro-oncogenic effects on tumor cells by stimulating their invasion and metastasis at least partially by inducing epithelial-to-mesenchymal transition in the later stages of carcinogenesis, when cancer cells have become insensitive to TGFβ-induced growth inhibition and apoptosis (36, 49). Therefore, it is possible that CTGF expression might be induced or restored by TGFβ to acquire an invasive/metastatic phenotype in advanced ovarian cancers without *CTGF* methylation. Because the silencing of *CTGF* occurs in a subset of ovarian cancer and may affect various biological functions in a stage-dependent and/or histologic subtype-dependent manner, evaluation of the methylation and/or expression status of *CTGF* with disease stage and/or histologic subtype might be useful for predicting the progression or

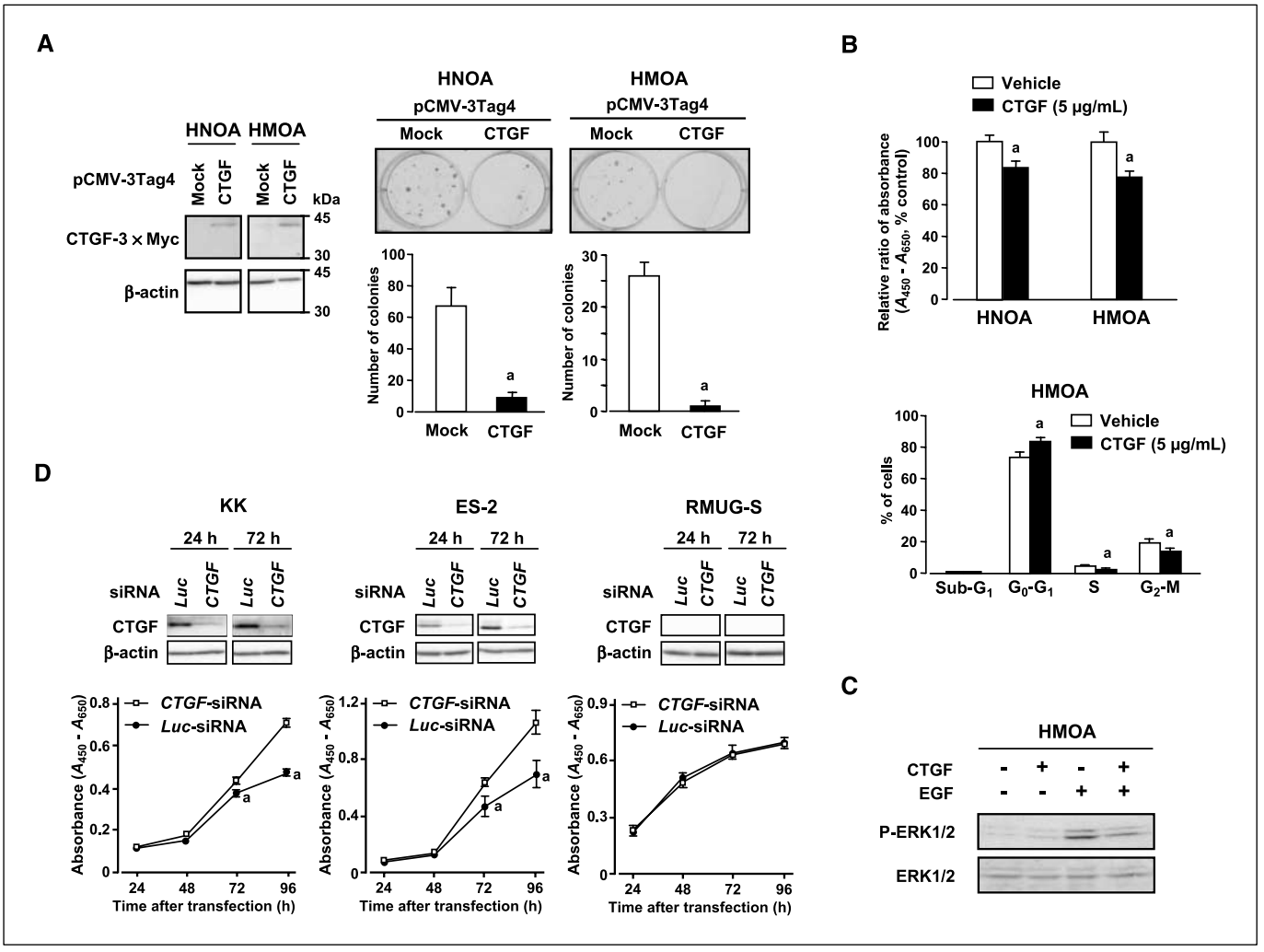

Figure 4. *A,* effects of restoration of CTGF expression on growth of ovarian cancer cells. Colony formation assays were done using ovarian cancer cell lines lacking expression of *CTGF*. Cells were transiently transfected with a Myc-tagged construct containing *CTGF* (pCMV-3Tag4-CTGF), or empty vector (mock), and selected for 2 wks with appropriate concentrations of G418. *Left*, Western blot prepared with 10 μg of protein extract and anti-Myc antibody, showing that cells transiently transfected with pCMV-3Tag4-CTGF expressed Myc-tagged CTGF. *Right, top,* 2 wks after transfection and subsequent selection of drug-resistant colonies, the colonies formed by *CTGF*-transfected cells were less numerous than those formed by mock-transfected cells. *Right, bottom,* quantitative analysis of colony formation. Colonies >2 mm were counted. *Columns,* mean of three separate experiments, each done in triplicate (histogram); *bars,* SD. *a, P* < 0.05, statistical analysis used the Mann-Whitney *U* test. *B,* effects of recombinant human CTGF on growth of ovarian cancer cells. Ovarian cancer cells lacking expression of *CTGF* (HNOA and HMOA) were treated with 2.5 μg/mL of recombinant human CTGF or vehicle (PBS) alone for 72 h. *Top,* cell viability was determined by WST assay in both cell lines; *bottom,* the population in each phase of cell cycle was assessed by FACS using HMOA cell line. Similar result was obtained in HNOA cell line (data not shown). *Columns,* mean of triplicate experiments; *bars,* SD. *a, P* < 0.05, statistical analysis used the Mann-Whitney *U* test. *C,* representative result of Western blotting for P-ERK1/2 and total ERK (ERK1/2) in HMOA cell line. HMOA cells were serum starved for 24 h, pretreated with CTGF (2.5 μg/mL) or vehicle (PBS) for 1 h, and then stimulated with EGF (25 ng/mL) of vehicle (PBS) for additional 15 min. ERK activation was evaluated by the amount of P-ERK determined by Western blotting. Similar result was obtained in HNOA cell line (data not shown). *D,* effect of knockdown of endogenous CTGF on growth of ovarian cancer cells. Fifty nanomol per liter of *CTGF*-specific siRNA (*CTGF*-siRNA) or a control siRNA for the *luciferase* gene (*Luc*-siRNA) were transfected into ovarian cancer cell lines expressing (KK and ES-2) or lacking (RMUG-S) CTGF, and the numbers of viable cells after transfection were assessed at the indicated times by WST assay. *Points,* mean of triplicate experiments; *bars,* SD. *a*, *P* < 0.05, statistical analysis used the Mann-Whitney *U* test.

aggressiveness of this disease. Further examination using a larger set of ovarian cancer cases will be needed to test our supposition that CTGF has two conflicting functions during tumorigenesis and inactivation of CTGF at least partly due to DNA methylation is a frequent and important event in the early progression of ovarian cancer.

In functional analyses, we showed that ectopically expressed CTGF or treatment with recombinant CTGF inhibits growth of *CTGF*-nonexpressing ovarian cancer cells, whereas knockdown of CTGF promotes growth of *CTGF*-expressing ovarian cancer cells. Similar results were obtained in cell lines of other types of cancer, such as NSCLC (16) and breast cancer (43). Chien et al. (16) showed that the growth of NSCLC cell lines expressing wild-type p53 was suppressed by forced expression of CTGF, likewise Cyr61, another member of the CCN family (50), although they have provided no evidence that their growth-inhibitory activity is mediated through p53. In our study, CTGF-induced growth suppression was observed in ovarian cancer cell lines regardless of the mutation status of *TP53*, and mutation of *TP53* was similarly observed in both *CTGF*-expressing and *CTGF*-nonexpressing ovarian cancer cell lines, suggesting that the growth-inhibitory activity of CTGF may not be affected by the mutation status of *TP53* in ovarian cancer. Because CTGF may exert growth-inhibiting activity at least partly through inhibition of the EGF-induced phosphorylation of ERK1/2 in NSCLC (16) and ovarian cancer (Fig. 4*C*), whereas it was shown that *CTGF* expression was inversely correlated with invasiveness/metastasis but not with cell growth in colorectal cancer (15), it is possible that CTGF affects different cellular functions in a cell- or tissue lineage–dependent manner.

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