Ovarian Carcinomas: CCN Genes Are Aberrantly Expressed and CCN1 Promotes Proliferation of these Cells

Sigal Gery,[1] Dong Xie,[1] Dong Yin,[1] Hani Gabra,[4] Carl Miller,[1] Heming Wang,[2] Diane Scott,[5] William S. Yi,[1] Miriam L. Popoviciu,[3] Johathan W. Said,[3] and H. Phillip Koeffler[1]

Abstract

Purpose: The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family consists of six matricellular proteins that are involved in various cellular functions, such as proliferation, development, and angiogenesis. The purpose of this study was to explore the possibility that *CCN* genes are involved in ovarian cancers.

Experimental Design: We quantified CCN expression in a series of 59 ovarian cancers using quantitative real-time reverse transcription-PCR. CCN1 protein levels were further determined by immunohistochemistry and Western blot analysis. Overexpression and inhibition of CCN1 expression by small interfering RNA were used to examine its role in ovarian cancer cell proliferation *in vitro* and *in vivo*.

Results: We found dysregulation of levels of the various CCN mRNAs in ovarian cancers compared with their expression in normal whole ovaries. Expression of CCN1 protein was detected in normal ovarian epithelial cells and ovarian tumors as well as in ovarian cancer cell lines. Furthermore, estrogen increased CCN1 mRNA and protein levels in ovarian cancer cells. Ectopic expression of CCN1 enhanced the growth of ovarian cancer cells in liquid culture, whereas inhibition of its expression decreased proliferation and increased apoptosis in these cells. The observed changes in cell growth were accompanied with activation of Akt and extracellular signal-regulated kinase (ERK) signaling pathways. Stable expression of CCN1 in SKOV3 cells significantly increased tumorigenicity in nude mice. Finally, overexpression of CCN1 conferred resistant to carboplatin-induced apoptosis in SKOV3 cells.

Conclusions: This is the first study to show abnormalities in CCN expression in ovarian carcinomas. Furthermore, our results suggest that CCN1 may play a role in ovarian carcinogenesis by stimulating survival and antiapoptotic signaling pathways.

Ovarian cancer is characterized by few early symptoms, resulting in diagnosis at an advanced stage associated with poor survival (1, 2). As a result, it is the most lethal of the gynecologic malignancies. Ovarian cancer develops through multiple genetic changes that are not well characterized; identifying aberrations in oncogenes and tumor suppressor genes remain an important challenge in understanding the pathogenesis of this neoplasm.

Connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family of proteins consists of six members (CCN1-CCN6) also known as cysteine-rich 61 (CCN1); connective tissue growth factor (CCN2); nephroblastoma overexpressed gene (CCN3); and Wnt-1–induced secreted proteins 1, 2, and 3 (CCN4-CCN6). Studies from the past decade showed that CCN proteins are involved in numerous cellular functions, including proliferation, differentiation, and neoplastic transformation (3–5).

CCN1, a prototypical member of the CCN family, is a proangiogenic early response gene. Targeted disruption of this gene in mice resulted in embryonic lethality due to vascular defects, indicating that it serves essential and nonredundant functions during development (6). Results from several studies, including from our laboratory, showed that CCN1 is overexpressed in invasive and metastatic human breast cancer and plays a critical role in estrogen-dependent as well as growth factor–dependent breast cancer progression (7–12). Abnormal expression of CCN1 was also reported in a number of other malignancies, further supporting a role for CCN1 in tumorigenesis (4, 5). Most of the CCN1 functions are mediated via its

Authors' Affiliations: [1]Division of Hematology/Oncology, Cedars-Sinai Medical Center; Departments of [2]Biomathematics and [3]Pathology, University of California at Los Angeles School of Medicine, Los Angeles, California; [4]Section of Molecular Therapeutics, Department of Cancer Medicine, Division of Medicine, Imperial College London Hammersmith Campus, London, United Kingdom; and [5]Cancer Research UK, Edinburgh Oncology Unit, University of Edinburgh Cancer Research Centre, Edinburgh, United Kingdom

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Requests for reprints: Sigal Gery, Division of Hematology/Oncology, Cedars-Sinai Medical Center, Davis Building 5066, 8700 Beverly Boulevard, Los Angeles, CA 90048. Phone: 310-423-4609; Fax: 310-423-0225; E-mail: gerys@cshs.org.

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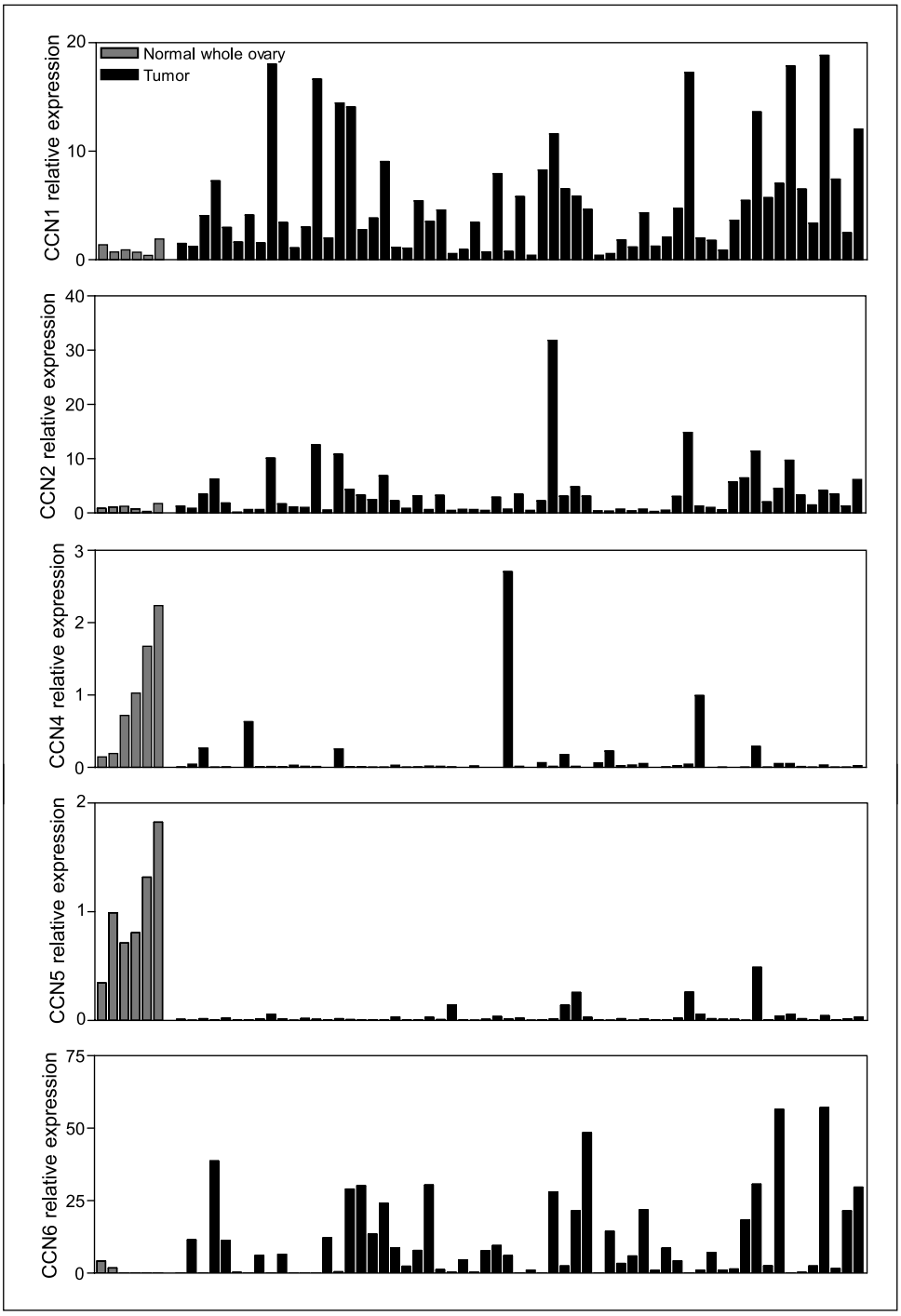

Fig. 1. *CCN* gene expression in ovarian tissues. Relative expression levels of CCN1, CCN2, CCN4, CCN5, and CCN6 are shown in six normal ovarian samples and 59 primary ovarian carcinoma samples. The results are expressed in arbitrary units as a ratio of the target gene transcripts to β-actin transcripts. *Columns,* mean of three measurements of the sample. The relative expression level was normalized so that the mean ratio of the six normal ovarian samples equals a value of 1.

direct binding with the integrin receptor $\alpha_v\beta_3$ (13, 14). Depending on the cellular context, multiple downstream mechanisms, including the p53/p21 (lung), phosphoinositide 3-kinase/Akt (brain and breast), β-catenin-TCF/Lef (brain), and extracellular signal-regulated kinase 1 (ERK1)/ERK2 mitogen-activated protein kinase (breast) signaling pathways, have been proposed to account for CCN1-induced phenotypic changes (15–19).

In the present study, using quantitative real-time reverse transcription-PCR (RT-PCR), we find dysregulation of the various *CCN* genes in a large series of primary ovarian carcinomas, indicating that CCN proteins may be implicated in this disease. We focused our additional studies on CCN1 given its known role in tumor development in other tissues. Our results suggest that CCN1 promotes proliferation of ovarian carcinomas cells and that targeting CCN1 could become a valuable approach in future antitumor therapy.

Materials and Methods

Patient samples. We analyzed tissue from excised primary ovarian carcinomas of 59 women diagnosed with ovarian cancer that were

treated at the University of Edinburgh Cancer Research Centre (Edinburgh, United Kingdom). Collection of patient materials and preparation of RNA for this study has been granted ethical approval by the Cancer and Medicine Ethics Committee of the Lothian University Hospital National Health Services Trust (Edinburgh, United Kingdom). All tumor biopsies were from the primary lesion and were composed of at least 70% epithelial cancer cells as determined by histopathology. Whole ovarian samples, containing epithelial and

Table 1. Relationship between expression levels of *CCN* genes and clinical features in primary ovarian carcinomas

CCN1

Clinical characteristics	CCN1 negative (n = 22)	CCN1 positive (n = 37)	P
Age			
Mean ± SD	66 ± 13.2	64 ± 11.1	0.5656*
Median, Min-Max	66 (45-90)	65 (37-82)	
Stage			
I-II	7	3	0.0247†
III-IV	15	32	
(Missing)	(0)	(2)	
Grade			
1-2	3	9	0.5027
3	18	26	
(Missing)	(1)	(2)	
Differentiation			
Well + Mod	3	9	0.5027
Poor	18	26	
(Missing)	(1)	(2)	
Histopathologic type			
Serous papillary	9	17	0.7093
Others	13	20	

CCN2

Clinical characteristics	CCN2 negative (n = 32)	CCN2 positive (n = 27)	P
Age			
Mean ± SD	66 ± 13.3	63± 9.8	0.302*
Median, Min-Max	66 (37-90)	64 (45-82)	
Stage			
I-II	9	1	0.016†
III-IV	22	25	
(Missing)	(1)	(1)	
Grade			
1-2	4	8	0.149
3	25	19	
(Missing)	(3)	(0)	
Differentiation			
Well + Mod	4	8	0.149
Poor	25	19	
(Missing)	(3)	(0)	
Histopathologic type			
Serous papillary	19	14	0.562
Others	13	13	

Table 1. Relationship between expression levels of *CCN* genes and clinical features in primary ovarian carcinomas (Cont'd)

CCN4

Clinical characteristics	CCN4 negative (n = 51)	CCN4 positive (n = 8)	P
Age			
Mean ± SD	65 ± 12.0	61 ± 11.2	0.3953*
Median, Min-Max	65 (37-90)	61 (45-80)	
Stage			
I-II	8	2	0.5939
III-IV	42	5	
(Missing)	(1)	(1)	
Grade			
1-2	9	3	0.3483
3	39	5	
(Missing)	(3)	(0)	
Differentiation			
Well + Mod	9	3	0.3483
Poor	39	5	
(Missing)	(3)	(0)	
Histopathologic type			
Serous papillary	30	3	0.4458
Others	21	5	

CCN5

Clinical characteristics	CCN5 negative (n = 54)	CCN5 positive (n = 5)	P
Age			
Mean ± SD	64 ± 12.0	66 ± 11.1	0.6943‡
Median, Min-Max	64 (37-90)	65 (51-79)	
Stage			
I-II	9	1	1.0000
III-IV	44	3	
(Missing)	(1)	(1)	
Grade			
1-2	9	3	0.0604
3	42	2	
(Missing)	(3)	(0)	
Differentiation			
Well + Mod	9	3	0.0604†
Poor	42	2	
(Missing)	(3)	(0)	
Histopathologic type			
Serous papillary	23	3	0.6458
Others	31	2	

(*Continued on the following page*)

Table 1. Relationship between expression levels of *CCN* genes and clinical features in primary ovarian carcinomas (Cont'd)

CCN6			
Clinical characteristics	**CCN6 negative (*n* = 37)**	**CCN6 positive (*n* = 22)**	***P***
Age			
Mean ± SD	64 ± 12.5	65 ± 10.8	0.6769*
Median, Min-Max	62, 37-90	66, 45-81	
Stage			
I-II	8	2	0.2953
III-IV	28	19	
(Missing)	(1)	(1)	
Grade			
1-2	6	6	0.5084
3	28	16	
(Missing)	(3)	(0)	
Differentiation			
Well + Mod	6	6	0.5084
Poor	28	16	
(Missing)	(3)	(0)	
Histopathologic type			
Serous papillary	19	14	0.4232
Others	18	8	

Abbreviations: Min-Max, average from the least (min) to the maximum (max); Well + Mod, well differentiated and moderately differentiated.
*t test.
† Statistically significant.
‡ Wilcoxon rank sum test, all other calculations used χ^2 test.

stromal cells, were used as normal controls. Samples were snap-frozen in liquid nitrogen at the time of collection and processed as previously described (20).

Cell lines and cell culture. IOSE-80 and IOSE-144 immortalized ovarian surface epithelium cells are low-passage, normal ovarian surface epithelial cells. The ovarian carcinoma cell lines PEA1 and PEO1 have been previously described (21). The ovarian carcinoma cell lines OVCA420, OVCA429, OVCA432, and OVCA433 were kindly provided by Robert C. Bast Jr. (M.D. Anderson Cancer Center, Houston, TX). The following cell lines were obtained from the American Type Culture Collection: ovarian carcinoma, SKOV3, OV-90, and TOV-112D; breast cancer, MDA-MB-231; normal breast, MCF-12A; and embryonic kidney transformed, 293T. Cells were grown in the recommended medium and conditions. In experiments in which the effects of estrogen were studied, cells were first cultured in phenol red–free medium with charcoal-treated newborn calf serum and then treated with β-estradiol (Sigma Chemical Co.). Experiments that make use of carboplatin (Sigma Chemical) were done in serum-free medium.

Northern blot analysis and quantitative real-time reverse transcription-PCR. Total RNA was extracted from ovarian cancer specimens and cell lines using TRIzol reagent (Invitrogen) or Absolutely RNA RT-PCR Miniprep kit (Stratagene) according to standard protocol. Northern blot analysis and quantitative real-time RT-PCR were done as described previously (8, 10). The following primes and probes were used for real-time RT-PCR—CCN1: 5′-ACTTCATGGTCCCAGTGCTC, 5′-AAATCCGGGTTTCTTTCACA, and 5′-TTACCAATGACAACCCTGAGTGCCG; CCN2: 5′-AGTATGGCACAGTGCAAG, 5′-ATGTCTTCATGCTGGTGCAG, and 5′-TGCGAAGCTGACCTGGAAGAGAACA; NOV: 5′-GATCATTGCTCCTCCTGAGC, 5′-GGTGTGCCACTTACCTGTCC, and 5′-TTGCCTGACCTTCCTGCTTCTCCA; CCN4: 5′-AGCATGCAGAGTGTGCAGAG, 5′-GTGTGTGTAGGCAGGGAGTG, and 5′-TAACTCACTGCCTAGGAGGCTGGCC; CCN5: 5′-ATTAACACGCTGCCTGGTCT, 5′-AGAGATGGGACAAGCAGTCC, and 5′-GCTGGCCAAGGTGTCCAGGG; CCN6: 5′-CCCACACAAAGGGCTGTATT, 5′-GTTCAGCTGCCTCTGTGTGA, and 5′-CATAATGGCCAAGTGTTTCAGCCCA β-actin: 5′-GATCATTGCTCCTCCTGAGC, 5′-ACTCCTGCTTGCTGATCCAC, and 5′-CTCGCTGTCCACCTTCCAGCAGAT.

Statistical analysis. χ^2 test, t test, and Wilcoxon's rank-sum test were used to study the association of each gene with single clinical factors (age, stage, grade, differentiation, and histopathologic type). A logistic regression model was developed to associate the probability of being a positive CCN marker with various clinical features. Stage was dichotomized as stages I/II and III/IV. Backward procedure was used for predictor selection. Classification tree analysis was also carried out to explore the association of gene status with clinical factors. The κ statistic was used to assess the relationship between all pairs of *CCN* genes. The κ value, its SE, and 95% confidence interval were reported.

Immunohistochemistry. Immunohistochemical staining of paraffin-embedded normal ovaries and ovarian cancers was done with anti-CCN1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

Western analysis. Cells were placed in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.5% NP40]; the resulting cell lysates were resolved on 4% to 15% gradient SDS-PAGE and transferred to nitrocellulose membranes (Sigma Chemical). Immunoblots were incubated with various primary antibodies followed by incubation with appropriate antirabbit or antimurine secondary IgG antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). SuperSignal West Pico substrate (Pierce) was used for detection. The following primary antibodies were used: anti-CCN1 and anti-p-ERK from Santa Cruz Biotechnology, anti-p-Akt (Ser^{473}) and anti-XIP from Cell Signaling, and anti-glyceraldehyde-3-phosphate dehydrogenase from Research Diagnostics. Western blots were stripped between hybridizations with stripping buffer [10 mmol/L Tris-HCl (pH 2.3) and 150 mmol/L NaCl].

Conditioned medium preparation. 293T cells were transfected with either empty vector (EV, pcDNA3.1) or CCN1 expression vector (pcDNA3.1-CCN1) using LipofectAMINE 2000 (Invitrogen). One day after transfection, the medium was replaced with serum-free medium and the cells were incubated for an additional 2 days. Conditioned medium was collected and stored at −80°C.

Stable transfections. OVCA433 and SKOV3 cells were transfected with either EV (pcDNA3.1) or CCN1 expression vector (pcDNA3.1-CCN1) using LipofectAMINE 2000 (Invitrogen). Multiple polyclonal (OVCA433) and monoclonal (SKOV3) clones were obtained by selection with G418 (500 μg/mL). Clones were screened for CCN1 expression by Western blot analysis.

Flow cytometric analysis. Fluorescence-activated cell sorting analysis was done using integrin $\alpha_v\beta_3$ antibody (Santa Cruz Biotechnology). Results were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) using CellQuest 2.0 software (Becton Dickinson).

Small interfering RNA. Primers were designed using the web-based small interfering RNA (siRNA) hairpin engine at Cold Spring Harbor Laboratories (http://katahdin.cshl.org:9331/RNAi/). The following sequences were used: CCN1 siRNA, 5′-GGCACCATCAATACACGTACACTGATGCTCAAGCTTCAACATCAGTGCACTGTATTGATGGCGC and control siRNA (lacking the hairpin sequence), 5′-GGCACCATCAATACACGTACACTGATGCTCAAGCTTCAGCACATGTATTGAGGCGC. The siRNA primers together with the U6 promoter were cloned into pCR2.1 and confirmed by sequencing. OVCA433 cells were cotransfected with either CCN1 siRNA or the control siRNA along with pMSCVpuro vector (Clontech) and selected with puromycin. Surviving cells were harvested and used in subsequent experiments.

Cell proliferation, colony formation, cell cycle, and apoptosis assays. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Roche Diagnostics) according to the protocol of the manufacturer. For colony formation, equal number of transfected cells were seeded in six-well plates and cultured with puromycin selection. After 2 weeks, the colonies were stained with 0.1% crystal violet and photographed. For cell cycle analysis, transfected cells were fixed in cold ethanol, stained with 50 μg/mL propidium iodide, and analyzed by FACScan and CELLFit program (Becton Dickinson). Apoptosis analysis was done with Annexin V-FITC apoptosis detection kit I (BD PharMingen) according to the instructions of the manufacturer. Statistical analyses were done using *t* test.

Tumorigenesis assay. SK-CCN1 and SK-EV cells (2×10^6) were injected s.c. on different sides of 14 nude mice. Size of the tumors was measured twice a week. Statistical analysis was done using *t* test.

Results

Expression of CCN genes in primary ovarian carcinomas. CCN expression profiles were measured from six normal whole ovaries and 59 primary ovarian carcinomas by real-time PCR (Fig. 1). The expression levels were expressed as a ratio between either CCN1, CCN2, CCN4, CCN5, or CCN6 and the reference gene *β-actin* to correct for variation in the amounts of RNA. The relative target gene expression was also normalized to a mean value (value = 1) for the six normal WO tissue samples (calibrator). Each of the normalized target values was divided by the calibrator normalized target value to generate the final relative expression levels. Up-regulation in a given tumor was defined as expression levels 2-fold above the highest expression level in the normal tissue group. Correspondingly, down-regulation of a given tumor was defined as expression levels 2-fold below the lowest expression level in the normal tissue group.

Using these criteria, we found that the expressions of CCN1, CCN2, and CCN6 were up-regulated, whereas levels of CCN4 and CCN5 were down-regulated in the ovarian carcinomas. Overexpression of CCN1 was found in 63% of the tumor samples with a range up to 10-fold above the levels found in normal samples. Up-regulation of CCN2 was found in 46% of the ovarian carcinomas and expression levels increased up to 18-fold above normal levels. CCN6 levels were up-regulated in 37% of the tumor samples; however, their values were somewhat scattered. CCN6 was undetectable in 20% of the ovarian carcinomas, whereas a 30- to 57-fold increase was found in 15% of the samples. Levels of CCN4 and, even to a larger extent, CCN5 were

Table 2. Correlations of expression among *CCN* genes in primary ovarian carcinomas

	CCN1		CCN4		CCN5		CCN6	
	−	+	−	+	−	+	−	+
CCN2								
−	22	10	28	4	31	1	23	9
+	0	27	23	4	23	4	14	13
CCN1								
−			19	3	21	1	16	6
+			32	5	33	4	21	16
CCN4								
−					48	3	31	20
+					6	2	6	2
CCN5								
−							34	20
+							3	2

Comparison	κ*	SE	95% Confidence interval	*P*
CCN2 and CCN1	0.67	0.09	0.49-0.85	0.0001
CCN2 and CCN4	0.02	0.10	NS	
CCN2 and CCN5	0.12	0.08	NS	
CCN2 and CCN6	0.20	0.13	NS	
CCN1 and CCN4	−0.00	0.07	NS	
CCN1 and CCN5	0.05	0.05	NS	
CCN1 and CCN6	0.14	0.11	NS	
CCN4 and CCN5	0.23	0.18	NS	
CCN4 and CCN6	−0.08	0.10	NS	
CCN5 and CCN6	0.01	0.09	NS	

Abbreviation: NS, not significant.
*κ Statistics showed significant associations between CCN1 and CCN2. The associations among other genes were not significant.

dramatically down-regulated in the majority of ovarian tumors—86% of specimens for CCN4 and 92% of specimens for CCN5.

Correlation of clinical features with CCN gene expression. Statistical univariate analysis was done to determine if a possible correlation occurred between expression of the *CCN* genes and clinical variables (Table 1). Among the clinical features, stage was the only factor that showed a significant correlation with the expression of CCN1 ($P = 0.0247$) and CCN2 ($P = 0.016$). Statistical analysis showed no significant association between CCN6 expression and the clinical features in the primary ovarian tumors. Very few samples were either CCN4 or CCN5 positive, and none of the clinical variables showed significant correlation with either gene. A logistic regression model was developed to associate the probability of a sample having a positive CCN marker with the various clinical features. Stage and grade were selected by the model as the significant predictors for being CCN2 positive. Stage was selected as the significant predictor for being CCN1 positive. None of the clinical factors was selected by the model as the significant predictor for being either CCN4 or CCN6 positive. The regression model selected grade as the significant predictor for being CCN5 positive. Finally, stage was selected as the significant predictor for having two or more positive CCN markers. To assess the relationship between all pairs of the five genes, κ statistics was used (Table 2). The analysis showed that a significant association existed between CCN1 and CCN2 ($P < 0.0001$). The associations among the other *CCN* genes were not significant. Univariate analysis was used to investigate the

Table 3. Correlations of *CCN* expression levels with overall survival in primary ovarian carcinomas

Clinical characteristics	No. death*/Total *n*	Median survival months (95% confidence interval)	Log-rank test, *P*
Age			
<65	14/29		
≥65	16/30		
Stage			
I-II	1/10	NA	0.0132†
III-IV	27/47	24.4 (18.4-30.3)	
Grade			
1-2	4/12	48.2 (14.5-48.2)	0.1963
3	24/44	24.4 (13.8-33.4)	
Differentiation			
Well + Mod	4/12	48.2 (14.5-48.2)	0.1963
Poor	24/44	24.4 (13.8-33.4)	
Histopathologic type			
Serous papillary	18/33	24.4 (14.5-33.4)	0.2568
Others	12/26	30.3 (19.8-NA)	
CCN2			
Negative	16/32	27.3 (18.4-NA)	0.7419
Positive	14/27	25.9 (14.5-48.2)	
CCN1			
Negative	8/22	30.3 (24.4-NA)	0.0979
Positive	22/37	20.8 (12.1-33.4)	
CCN4			
Negative	25/51	27.0 (18.4-NA)	0.7305
Positive	5/8	24.3 (1.5-48.2)	
CCN5			
Negative	28/54	25.9 (19.8-33.4)	0.8426
Positive	2/5	NA	
CCN6			
Negative	17/37	27.0 (20.8-NA)	0.1974
Positive	13/22	25.9 (6.2-33.4)	
Total no. positive markers			
0-1	13/27	27.3 (13.8-NA)	0.7136
2 or more	17/32	25.9 (19.8, 48.2)	

NOTE: The median follow-up time was 18.4 months.
Abbreviation: NA, not available.
*Among the 59 patients, 30 died.
† Statistically significant.

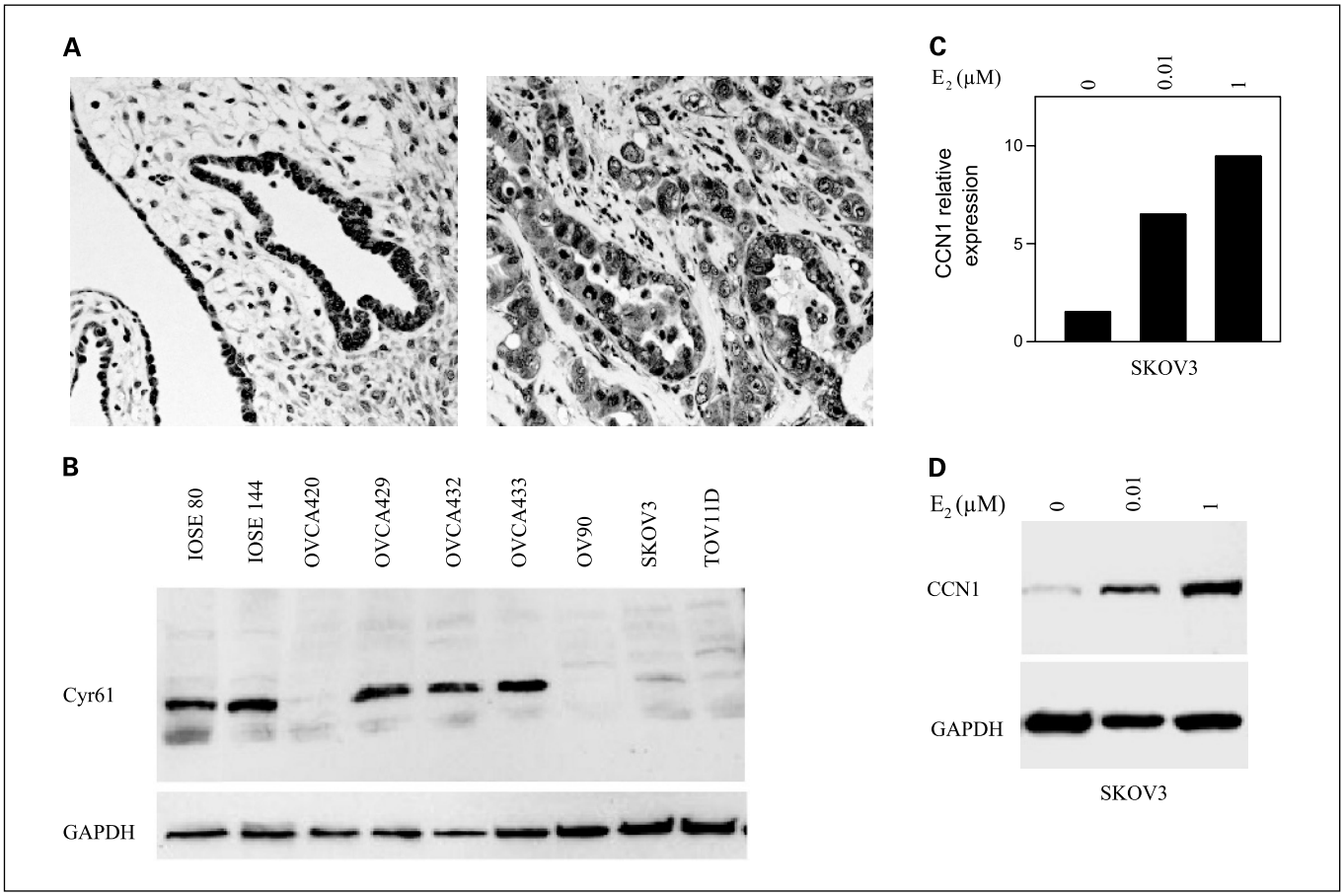

Fig. 2. CCN1 protein expression in normal and ovarian cancer cells. *A,* immunohistochemical staining of CCN1 expression in normal ovary (*left*) and ovarian carcinoma (*right*). Representative images are shown (×400 magnification). *B,* Western blot analysis showing CCN1 (*Cyr61,* cysteine-rich 61) expression in immortalized ovarian surface epithelium (*IOSE*) cells and the indicated ovarian cancer cell lines. Real-time PCR (*C*) and Western blot analysis (*D*) of CCN1 expression in SKOV3 cells treated with β-estradiol (E_2) for 24 hours. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to control for equal loading.

correlation of expression levels of each of the *CCN* genes, and survival of the patients and their clinical characteristics (Table 3). The results showed that stage is the only factor associated with overall survival. Neither individual marker nor the total number of positive markers showed significant association with overall survival.

CCN1 is expressed in normal ovarian epithelial cells and some ovarian cancer cell lines. Our additional studies focused on CCN1. To determine the levels of CCN1 protein expression, we did immunohistochemical analysis on tissue samples of normal ovary and ovarian carcinomas. In normal ovary, strong staining was observed in the epithelial cells (surface and glandular), whereas weak staining was noted in the stromal cells. The ovarian carcinomas (four of four specimens) expressed high levels of CCN1 protein in the epithelial and stromal cells (Fig. 2A). Protein levels were also examined in a series of ovarian cell lines by Western blot analysis. CCN1 expression was found in immortalized ovarian surface epithelium cell lines (IOSE-80 and IOSE-144) and in a subset of ovarian cancer cell lines (OVCA429, OVCA432, and OVCA433; Fig. 2B).

Previous studies showed that CCN1 expression is inducible in breast and uterine cells by estrogen treatment (8–10, 22). To examine whether estrogen has a similar effect on CCN1 expression in ovarian cells, the estrogen-responsive ovarian cancer cell line SKOV3 was treated with estradiol, and CCN1 expression levels were determined by real-time PCR and Western blot analysis. Results showed that estrogen treatment led to a dose-dependent increase in CCN1 mRNA and protein levels (Fig. 2C-D).

CCN1 stimulates proliferation of ovarian cancer cell lines. In earlier studies, we showed that forced expression of CCN1 promoted cell growth in breast as well as brain cancer cell lines (8, 18); on the other hand, it inhibited growth of lung cancer cell lines (15, 17). To test the effect of CCN1 on ovarian cancer cell growth, the ovarian cancer cell lines OVCA429 and OVCA433 were cultured for 2 days in the presence of conditioned medium from cells transfected with either an empty vector or a CCN1 expression vector. CCN1 stimulated the growth rate of both cell lines (OVCA429, 18% and OVCA433, 24%) as measured by MTT assays (Fig. 3A-B).

In breast and brain cancers, CCN1 growth-promoting effects are mediated, in part, via the phosphoinositide 3-kinase/Akt and the ERK/mitogen-activated protein kinase pathways (18, 19). Therefore, we tested whether CCN1 could activate these kinases in ovarian cancer cells. OVCA433 cells were transfected with either a CCN1 expression vector or an

empty control vector. Following a brief antibiotic selection, cells were harvested and phosphorylation of Akt and ERK were measured by Western blot analysis with phospho-specific antibodies. Levels of phosphorylated Akt and phosphorylated ERK were significantly higher in the CCN1-transfected cells compared with control cells (Fig. 3C, where results for two independent polyclones, clones 1 and 2, are shown). The antiapoptotic gene, *XIAP*, is a downstream target of Akt and plays an important role in mediating the effects of Akt on ovarian cancer cell survival (23, 24). Western blot analysis showed a substantial increase in XIAP protein levels in the CCN1-overexpressing OVCA433 cells (Fig. 3C).

To analyze further the consequences of CCN1 expression, we selected an ovarian cancer cell line expressing low levels of CCN1, SKOV3, for additional studies. The SKOV3 cells were stably transfected with either a CCN1 expression vector (SK-CCN1) or a control vector (SK-EV). MTT assays showed that expression of CCN1 stimulated the growth of SKOV3 compared with control cells (Fig. 4A). ERK activation status was significantly higher in the CCN1-overexpressing SKOV3 cells compared with controls as measured by Western blot analysis (Fig. 4B). Akt activity and the levels of XIAP were similar in the SK-CCN1 and the control SK-EV cells (Fig. 4B). In several cell types, including breast, brain, and smooth muscle, CCN1 up-regulates the expression of its own integrin receptor, $\alpha_v\beta_3$ (13, 14, 25). Using flow cytometry, we found higher levels of $\alpha_v\beta_3$ on the cellular surface of SK-CCN1 cells compared with SK-EV cells (Fig. 4C).

CCN1 promotes tumor growth in nude mice. We next examined whether CCN1 promotes ovarian tumor development *in vivo*. SK-CCN1 and the control SK-EV cells were injected s.c. into nude mice and tumor growth was measured twice a week. The CCN1-overexpressing cells, SK-CCN1, developed tumors with significantly ($P < 0.05$) increased tumor growth compared with the tumors from the control SK-EV cells (Fig. 4D).

Suppression of CCN1 expression inhibits ovarian cancer cell growth. We used small interfering RNA (siRNA) to evaluate the role of endogenous CCN1 in cell proliferation. Six siRNA sequences were designed and cloned into the pCR2.1 vector under the control of the U6 promoter. One target sequence (CCN1 siRNA) was efficient in inhibiting CCN1 in 293T cells (data not shown). The ovarian cancer cell line OVCA433, expressing high levels of CCN1, was selected for further studies. The siRNA construct decreased CCN1 expression but not glyceraldehyde-3-phosphate dehydrogenase levels in OVCA433 cells over a 5-day period (Fig. 5A). To examine the effect of CCN1 suppression in ovarian cancer cells, OVCA433 cells were transfected with either the CCN1 siRNA construct or siRNA control vector. Colony formation assays showed that CCN1 siRNA–transfected cells formed 72% fewer colonies compared with the control cells (Fig. 5B). Similarly, the OVCA433 cells transfected with CCN1 siRNA exhibited 32% decrease in cell growth compared with cells transfected with the control vector in liquid culture as measured by MTT assays (Fig. 5C).

We next examined the effects of suppression of expression of CCN1 on the cell cycle of the OVCA433 cells. These cells transfected with the CCN1 siRNA construct (populations 1 and 2) had an increased number of cells (63% and 64%, respectively) in the G_0-G_1 phase, and a decrease

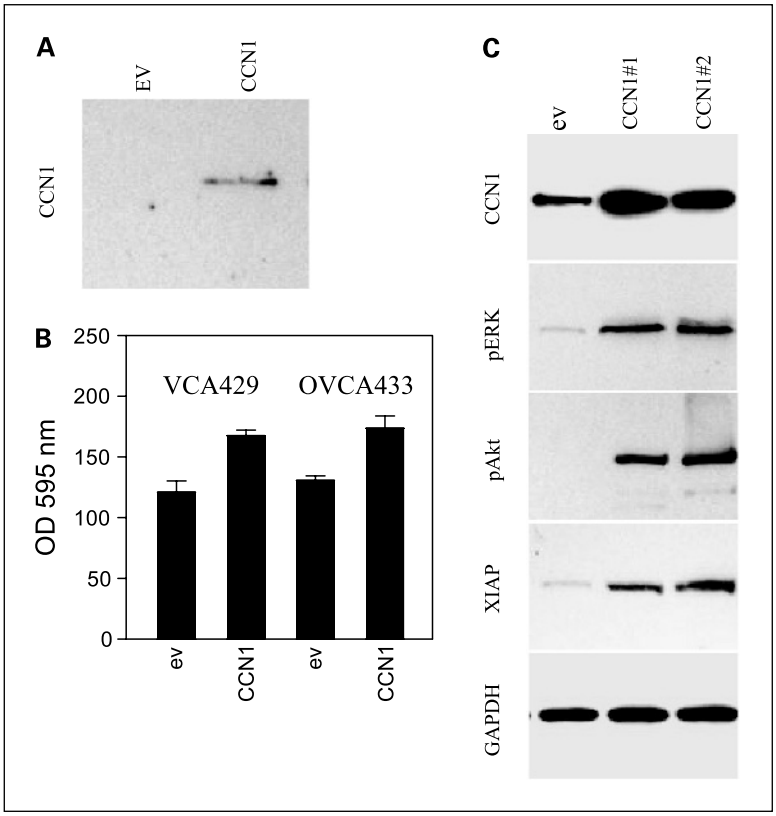

Fig. 3. CCN1-conditioned medium enhances cell growth. *A,* Western blot analysis of CCN1 expression in conditioned medium collected from 293T cells transfected with either pcDNA3.1 empty vector (EV) or pcDNA3.1-CCN1 (*CCN1*). *B,* MTT assay. OVCA429 and OVCA433 cells were incubated with control-conditioned medium (*ev*) or CCN1-conditioned medium (*CCN1*) and cell proliferation was measured by MTT assays. *Columns,* mean of quadruplicate samples; *bars,* SD. The experiment was repeated twice. *C,* OVCA433 cells transfected either with EV or CCN1 expression vector (*CCN1#1* and *CCN1#2*) were analyzed by Western blot for expression of the indicated proteins.

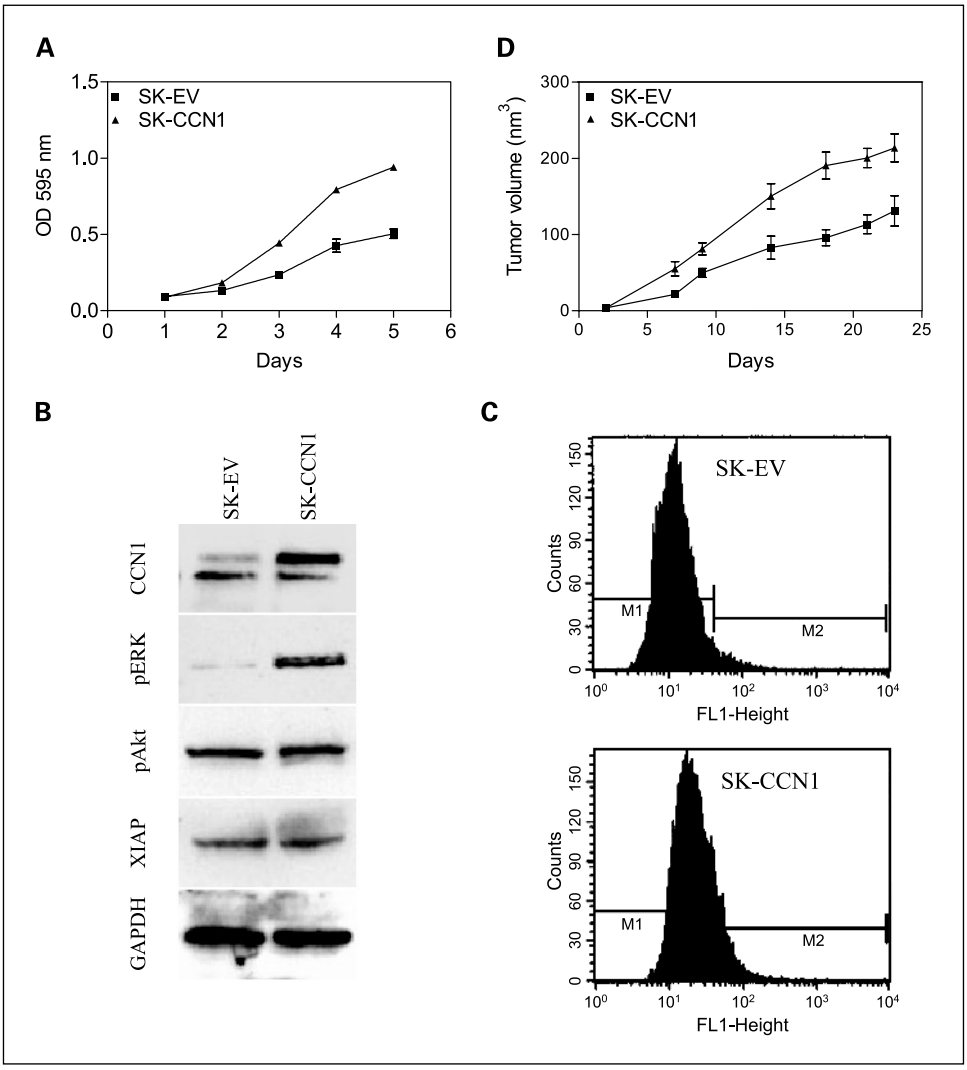

Fig. 4. Stable overexpression of CCN1 enhances growth in liquid culture and tumor formation in nude mice. *A,* growth rates of SKOV3 cells stably transfected with either CCN1 expression vector (*SK-CCN1*) or EV (*SK-EV*) were measured by MTT assays. *B,* Western blot analysis of the indicated proteins in SK-CCN1 and SK-EV cells. *C,* fluorescence-activated cell sorting analysis of $\alpha_v\beta_3$ expression was done using a monoclonal antibody to integrin $\alpha_v\beta_3$. *D,* SK-CCN1 and SK-EV cells (2×10^6) were s.c. injected on different sides of 14 nude mice. Tumor size was measured twice a week.

number of cells (23% and 24%, respectively) in the S phase of the cell cycle compared with control cells transfected with the siRNA control vector (48%, G_0-G_1 phase and 43%, S phase; Fig. 6A). Furthermore, a small increase in the apoptosis rate, measured by Annexin V, was observed in the CCN1 siRNA–transfected cells (7%, populations 1 and 2), in contrast to the low level of apoptosis in the control-transfected cells (1%) cultured under identical conditions (Fig. 6B).

Expression of CCN1 confers resistance to platinum-based drugs. In MCF-7 breast cancer cells, CCN1 increases resistance to several chemotherapeutic drugs (19, 26). Interestingly, we found that ovarian cancer cell lines established from primary ovarian cancer before cisplatin therapy (PEA1 and PEO1) had lower mRNA and protein levels of CCN1 than their match cisplatin-resistant lines, established after the patients' tumors no longer responded to cisplatin (Fig. 7A). MTT assays were used to evaluate whether CCN1 increases resistance to carboplatin cytotoxicity. SKOV3 cells stably transfected with a CCN1 vector had greater cell viability following carboplatin treatment compared with control EV-transfected cells (Fig. 7B-C). The observed increase in cell viability was accompanied by an increase in resistance to carboplatin-induced apoptosis in the CCN1 overexpressing cells (Fig. 7D).

Discussion

Using quantitative real-time RT-PCR, we found up-regulation of CCN1, CCN2, and CCN6 in a series of 59 ovarian cancers, whereas levels of CCN4 and CCN5 were markedly lower compared with normal whole ovarian tissue. Statistical analysis showed that CCN1 and CCN2 expression levels correlated with stage, suggesting that they are important in ovarian cancer progression. We did not find significant association between *CCN* gene expression and survival. This suggests that CCN mRNA levels are not sufficient to determine the clinical outcome of ovarian cancer. Nevertheless, CCN proteins may constitute potential therapeutic targets in patients expressing high levels of these genes. Further studies are warranted to evaluate the prognostic utility of CCN expression, possibly in combination with other molecular markers, in this disease. In recent years, mounting evidence showed that the angiogenic

factor CCN1 is involved in many aspects of cell proliferation, differentiation, and tumorigenesis (3–5, 12). Overexpression of CCN1 has been reported in gliomas, breast, pancreas, and pediatric cancers (4, 18). These findings and our real-time RT-PCR results motivated us to examine further the function of CCN1 in ovarian cancers.

Imunohistochemistry showed that CCN1 protein is expressed at high levels in normal surface epithelial cells and ovarian cancer cells. Normal immortalized ovarian surface epithelium and some ovarian cancer cell lines also expressed high levels of CCN1. In addition, we showed that estrogen increased CCN1 mRNA and protein levels in estrogen-responsive ovarian cancer cells. Using several experimental models, we showed that CCN1 is a positive regulator of ovarian cancer cell growth. At the molecular level, forced expression of CCN1 was associated with higher levels of Akt and ERK phosphorylation. We concurrently found that levels of integrin $\alpha_v\beta_3$, the CCN1 receptor, were induced in the SKOV3-CCN1–transfected cells. Recent studies showed that in breast cancer cells, CCN1 up-regulates the levels $\alpha_v\beta_3$ and this, in turn, leads to activation of phosphoinositide 3-kinase/Akt and ERK/mitogen-activated protein kinase signaling cascades. We previously showed that overexpression of CCN1 is involved in the development of gliomas through activation of integrin-linked kinase, an important upstream regulator of Akt. Interestingly, α_v integrin subunits are frequently expressed in ovarian carcinoma (27) and $\alpha_v\beta_3$ was shown to promote proliferation of ovarian cancer cells by activating integrin-linked kinase (28). Collectively, these data support a model in which CCN1 promotes cell growth, at least in part, by increasing the levels of $\alpha_v\beta_3$ and subsequently activating the $\alpha_v\beta_3$ downstream signaling pathways, integrin-linked kinase/phosphoinositide 3-kinase/Akt, and ERK/mitogen-activated protein kinase in a variety of cancers.

Resistance to chemotherapy, resulting in part from an inability of the cells to undergo apoptosis, is one of the major causes for treatment failure in ovarian cancer. We found markedly higher levels of CCN1 mRNA and protein in cisplatin-resistant ovarian cancer lines compared with cell lines established before the patient received cisplatin chemotherapy. Furthermore, we showed that SKOV3 ovarian cancer cells overexpressing CCN1 acquired resistance to apoptosis induced by carboplatin. CCN1 was recently shown to confer resistance to a number of chemotherapeutic agents in MCF-7 breast cancer cells (19, 26). These findings suggest that CCN1 is a potentially important therapeutic and chemopreventive target in ovarian and breast cancers.

In summary, our studies found abnormal expression of *CCN* genes in a large number of ovarian tumors. Although the CCN proteins have a well-recognized role in oncogenic

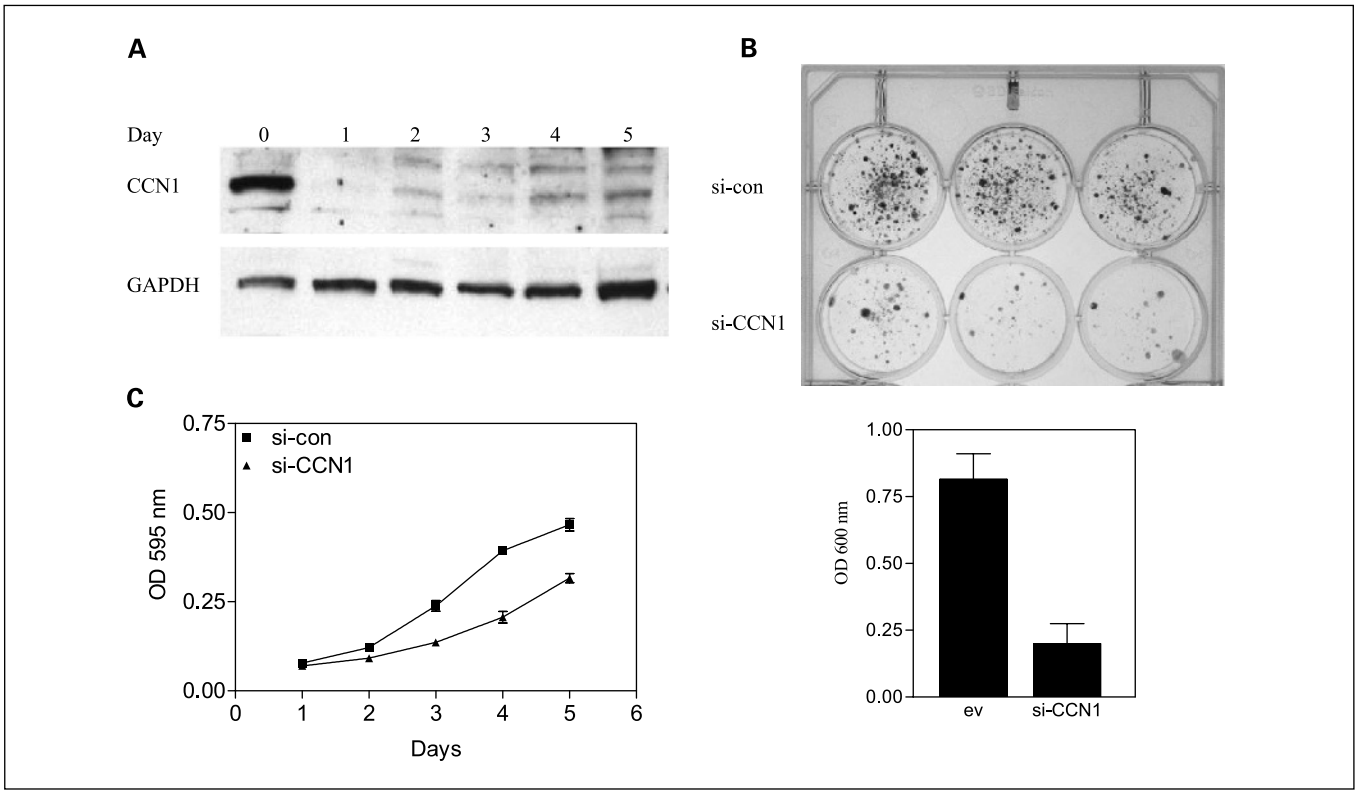

Fig. 5. Suppression of CCN1 expression inhibits proliferation of ovarian cancer cells. OVCA433 cells were cotransfected with a small interference RNA construct targeting CCN1 along with a vector containing the puromycin resistance gene; the cells were selected with puromycin. *A,* Western blot. Cells were harvested daily and analyzed for CCN1 expression. GAPDH was used to control for equal loading and siRNA specificity. *B,* colony formation assay. Transfected cells were grown for 2 weeks in the presence of puromycin. Colonies were fixed, stained, and photographed. Colonies were dissolved and absorbance (*OD*) was measured at 600 nm. *Columns,* means of three independent experiments; *bars,* SD. *C,* MTT assay. Transfected cells were used to measure growth in liquid culture by MTT. *Points,* mean of quadruplicate samples; *bars,* SD. The experiment was repeated thrice. *si-con,* control siRNA; *si-CCN1,* CCN1 siRNA.

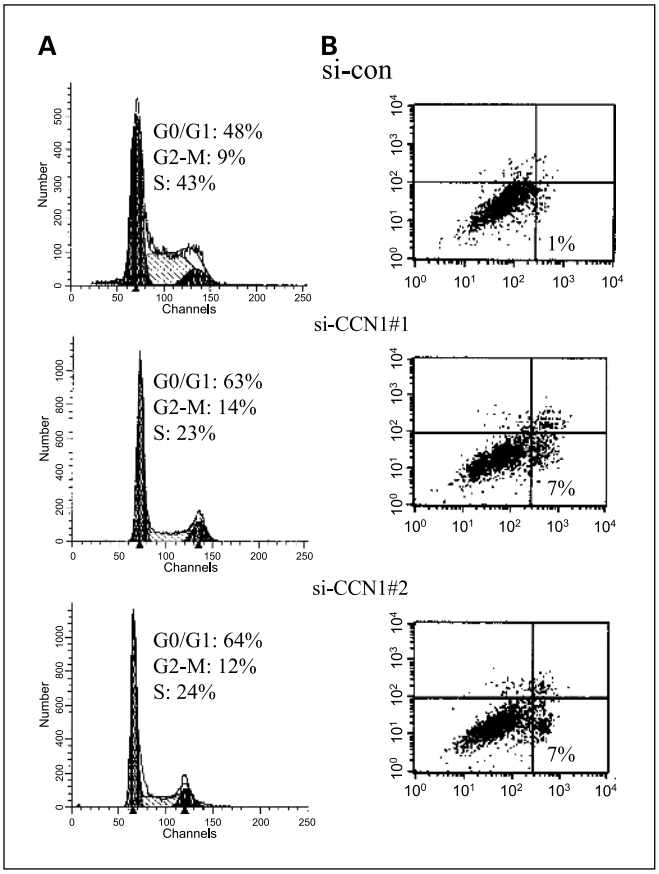

Fig. 6. Depletion of CCN1 induces growth arrest and apoptosis in ovarian cancer cells. OVCA433 cells were transfected with a vector containing the puromycin resistance gene and either CCN1 siRNA or control siRNA and then selected with puromycin for 3 days. *A,* fluorescence-activated cell sorting analysis. Cell cycle was analyzed using propidium iodide staining. *B,* apoptosis analysis. Cells were stained with Annexin V-FITC and propidium iodide. *si-CCN1#1* and *si-CCN1#2,* CCN1 siRNA populations 1 and 2.

transformation in a number of tissues, this is, to our knowledge, the first report suggesting their involvement in ovarian carcinomas. In the ovary, the formation and regression of the corpus luteum are associated with extensive changes in the vascular network comparable with the rapid angiogenesis observed during tumor formation. The presence of the angiogenic factor CCN1 in normal ovarian epithelial cells and ovarian tumors, as well as its regulation by estrogen and the finding that overexpression of this gene promotes ovarian cancer cell growth, suggests that it is involved both in normal ovarian function and ovarian carcinogenesis.

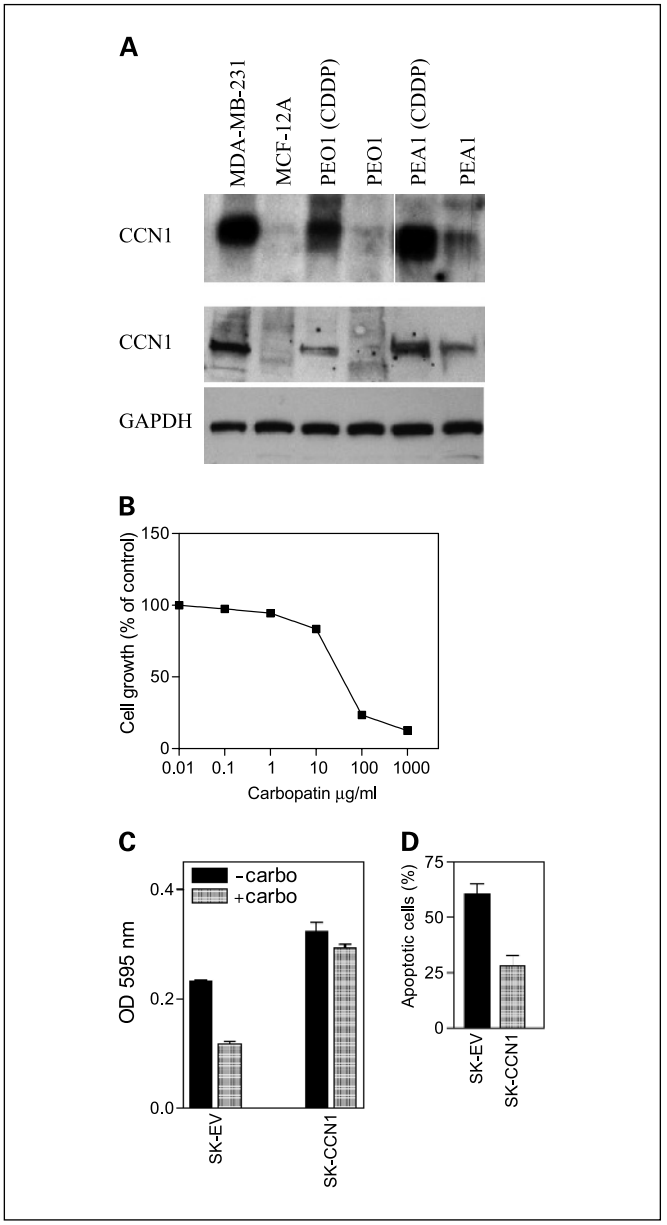

Fig. 7. CCN1 overexpression confers resistance to carboplatin. *A,* Northern blot (*top*) and Western blot (*bottom*) showing CCN1 expression in MDA-MB-231 (breast cancer cell line that served as a positive control), MCF-12A (normal breast cell line that served as a negative control), PEO1 and PEA1 (ovarian cancer cell lines), and PEO1 (CDDP) and PEA1 (CDDP) (cisplatin-resistance ovarian cancer cell lines). *B,* dose-response curve was generated using MTT assays. *C* and *D,* SKOV3 cells stably transfected with either CCN1 expression vector (SK-CCN1) or empty vector (SK-EV) were treated with 150 μg/mL carboplatin. Cell viability was measured by MTT assays (*C*); apoptosis analysis was done with Annexin V-FITC and propidium iodide (*D*).

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