Frequent Inactivation of a Putative Tumor Suppressor, Angiopoietin-Like Protein 2, in Ovarian Cancer

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Abstract
Angiopoietin-like protein 2 (ANGPTL2) is a secreted protein belonging to the angiopoietin family, the members of which are implicated in various biological processes, although its receptor remains unknown. We identified a homozygous loss of ANGPTL2 (9q33.3) in the course of screening a panel of ovarian cancer (OC) cell lines for genomic copy-number aberrations using in-house array-based comparative genomics hybridization. ANGPTL2 mRNA expression was observed in normal ovarian tissue and immortalized normal ovarian epithelial cells, but was reduced in some OC lines without its homozygous deletion (18 of 23 lines) and restored after treatment with 5-aza-2′-deoxycytidine. The methylation status of sequences around the ANGPTL2 CpG-island with clear promoter activity inversely correlated with expression. ANGPTL2 methylation was frequently observed in primary OC tissues as well. In an immunohistochemical analysis of primary OCs, ANGPTL2 expression was frequently reduced (51 of 100 cases), and inversely correlated with methylation status. Patients with OC showing reduced ANGPTL2 immunoreactivity had significantly worse survival in the earlier stages (stages I and II), but better survival in advanced stages (stages III and IV). The restoration of ANGPTL2 expression or treatment with conditioned medium containing ANGPTL2 inhibited the growth of OC cells originally lacking the expression of this gene, whereas the knockdown of endogenous ANGPTL2 accelerated the growth of OC cells with the expression of ANGPTL2. These results suggest that, at least partly, epigenetic silencing by hypermethylation of the ANGPTL2 promoter leads to a loss of ANGPTL2 function, which may be a factor in the carcinogenesis of OC in a stage-dependent manner. [Cancer Res 2008;68(13):5067–75]

Introduction
Epithelial ovarian cancer (OC) is the most common and lethal gynecologic malignancy and is one of the leading causes of cancer mortality in women because the disease usually presents at an advanced stage, as there are no overt symptoms at early stages (1, 2). Despite the use of primary surgical cytoreduction and systemic administration of paclitaxel-containing and platinum-containing chemotherapy regimens, minimal improvements have been made in overall survival over the past three decades. Therefore, a critical need exists for the identification of molecular markers and targets for diagnosis as well as therapy, which will come from a better understanding of the molecular mechanisms responsible for the tumorigenesis of this disease (3).

Sporadic OCs often show complex, aneuploid karyotypes, with a myriad of nonrandom structural chromosomal abnormalities (4), which may activate oncogenes or inactivate tumor suppressor genes (TSG) during the transformation process. To identify novel candidates for TSGs, homozygously deleted regions within the cancer cell genome are likely to serve as a good landmark (5–9), albeit biallelic loss is a rare event, and other factors, such as point mutations and epigenetic abnormalities (10), may predominantly contribute to functional inactivation. Therefore, high-resolution mapping of homozygous deletions within the entire genome of cancer cells would be of considerable help in the rapid identification of TSGs. Recently, we have applied an in-house bacterial artificial chromosome (BAC)–based array containing 800 BAC clones (MCG Cancer Array-800; ref. 5) to an array-based comparative genomic hybridization (array-CGH) analysis of OC cell lines, and identified connective tissue growth factor (CTGF/CCN2) as a putative ovarian TSG mainly inactivated by DNA methylation from homozygous loss at 9q23 (11). Because (a) there is no doubt that carcinoma is the result of the accumulation of multiple somatic genetic and/or epigenetic alterations resulting in either the activation of oncogenes or the inactivation of TSGs and (b) homozygous loss is usually small, more TSGs involved in the ovarian carcinogenesis will be identified through the genome-wide search for copy-number changes using arrays with a higher resolution, as shown in our previous studies in various other cancers (12–14).

In the report presented here, we have employed an in-house BAC array with an average spacing of 0.7 Mb (MCG Whole Genome Array-4500), which has ~5.6-fold higher resolution than MCG Cancer Array-800 (5), to a panel of OC cell lines for genomewide copy-number analysis. During the course of these experiments, we identified a novel homozygous loss at 9q33.3 containing angiopoietin-like protein 2 (ANGPTL2), the expression of which was absent in some OC cell lines without homozygous loss, although it was present in the normal ovary. To clarify the mechanism and the effect on ovarian carcinogenesis of down-regulated ANGPTL2 expression, we further determined the expression and methylation status of ANGPTL2 and their clinicopathologic and functional significance in OC.
Materials and Methods

Cell lines and primary tumors. Twenty-four OC cell lines whose derivation and sources have been previously reported (11) were used. The immortalized normal ovarian epithelial cell line OSE-2a (15), kindly provided by Dr. Hidetaka Katabuchi (Kumamoto University School of Medicine, Kumamoto, Japan), was used as a normal control. All cell lines were maintained in appropriate medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. To prepare a conditioned medium, SAS, an oral squamous cell carcinoma cell line that grows in serum-free DMEM/F12 (1:1), was used.

Primary OC tumor samples were obtained during surgery from 100 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 committee, and were embedded in paraffin for immunohistochemistry. Samples from 45 of these patients were immediately frozen in liquid nitrogen and stored at −80°C until required. DNA of a quality good enough for a methylation analysis was obtained from each of the 45 samples, whereas RNA of a quality good enough for an expression analysis was obtained from only 4 samples. 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 conducted in patients with advanced disease. Surgical staging was based on the International Federation of Gynecology and Obstetrics staging system: stage I, 53 patients; stage II, 11 patients; stage III, 28 patients; and stage IV, 8 patients.

Array-CGH. Array-CGH using a MCG Whole Genome Array-4500 (5) was carried out as described elsewhere (13). Images acquired by a GenePix 4000B (Axon Instruments) were analyzed with GenePix Pro 6.0 software (Axon Instruments). After normalization, average ratios that deviated significantly (>2 SD) from 0 (log2 ratio, <−0.4 and >0.4) were considered abnormal.

PCR. Homozygous deletions were detected by genomic PCR (11, 13). For expression analyses, single-stranded cDNA generated from total RNA was amplified with primers specific for each gene (16). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified at the same time to allow the estimation of the efficiency of cDNA synthesis. For conventional reverse transcription-PCR (RT-PCR), PCR products were electrophoresed, whereas quantitative real-time RT-PCR was conducted with an ABI Prism 7900 sequence detection system (Applied Biosystems). Each assay was conducted in triplicate. All primer sequences are listed in Supplementary Table S1.

Drug treatment. OC cells were cultured with various concentrations of 5-aza-2′-deoxycytidine (5-aza-dCyd) for 5 days and/or 100 ng/mL of trichostatin A (TSA) for the last 12 h.

Methylation analysis. Genomic DNA was treated with sodium bisulfite, and subjected to PCR using primers to amplify regions of interest (Supplementary Table S1). For the combined bisulfite restriction analysis (COBRA), a semiquantitative bisulfite-PCR analysis (17), PCR products were digested with BstUI and electrophoresed. For bisulfite sequencing, PCR products were subcloned and then sequenced. For the methylation-specific PCR (MSP) analysis, sodium bisulfite–treated DNA was subjected to PCR using primer sets specific to the methylated and unmethylated forms of DNA sequences, and PCR products were visualized on 3% agarose gels. DNA from cell lines recognized as unmethylated by bisulfite sequencing was used as negative controls for methylated alleles, whereas DNA from cell lines (data not shown) were the same as those in our previous analysis. For the methylation-specific PCR, DNA sequences, and PCR products were visualized on 3% agarose gels. DNA from cell lines recognized as unmethylated by bisulfite sequencing was used as negative controls for methylated alleles, whereas DNA from cell lines (data not shown) were the same as those in our previous

Results

Array-CGH analysis of OC cell lines. In the array-CGH analysis using an MCG Whole Genome Array-4500, frequently detected copy-number gains and losses within the entire genome of 24 OC cell lines (data not shown) were the same as those in our previous
Compared with the MCG Cancer Array-800, we identified more homozygous deletions (log2 ratio < -2) and high-level amplifications (log2 ratio > 2), which are likely to be landmarks of TSGs and oncogenes, respectively, using the MCG Whole Genome Array-4500: homozygous deletions at 4q, 6q, 8q, 9p, and 9q (Supplementary Table S2), and amplifications at 2q, 11q, and 19q (Supplementary Table S3). All these alterations were confirmed by fluorescence in situ hybridization (Fig. 1A; data not shown). Among them, the homozygous loss at 9q33.3 observed in OVSAHO cells had never been previously documented in OC, prompting us to examine whether genes located within this region are involved in the pathogenesis of OC.

**Identification of target genes involved in homozygous deletion at 9q33.3.** To define the extent of the homozygous loss at 9q33.3 in OVSAHO cells and to identify other OC lines harboring a cryptic homozygous loss in this region, we performed genomic PCR with 12 genes (LMX1B, ZBTB43, ZBTB34, RALGPS1, ANGPTL2, GARNL3, SLC2A8, ZNF79, RPL12, LRSAM1, FAM129B, and STXBP1; Fig. 1B) located around RP11-1M19 according to information archived by genome databases.9,10 We detected a complete loss of ANGPTL2, RALGPS1, LRSAM1, and FAM129B only in OVSAHO cells, whereas LMX1B, ZBTB43, ZBTB34, GARNL3, SLC2A8, ZNF79, RPL12, and STXBP1 were retained in this cell line (Fig. 1C), indicating that the homozygous deletion has a structurally complicated pattern, and its total size is ~0.55 Mb at maximum.

10 http://genome.ucsc.edu/
Loss of ANGPTL2 expression in OC cell lines. Next, we determined the mRNA expression levels of ANGPTL2, RALGPS1, LRSAM1, and FAM129B by RT-PCR in all 24 OC lines, normal ovary, and the OSE-2a cell line. RALGPS1, LRSAM1, and FAM129B were expressed in most of the OC lines at levels similar to or higher than those in normal ovary and/or the OSE-2a cell line (Fig. 1D). On the other hand, ANGPTL2 mRNA was frequently silenced in OC lines without the homozygous deletion (18 of 23, 78%; Fig. 1D), but was expressed in normal ovary and OSE-2a cells, suggesting that this gene is likely to be the most probable target for inactivation through mechanisms other than genomic deletion in OC cells. Because aberrant methylation within the CpG-island around the transcription start site (TSS) of genes is known to be one of the key mechanisms by which TSGs can be silenced (9), and the CpGLOT program \(^\text{11}\) identified the CpG-island around the TSS of ANGPTL2, we focused on ANGPTL2 for further DNA methylation analyses. None of the two lines that had shown a hemizygous loss around ANGPTL2 in array-CGH exhibited a decreased expression of this gene (data not shown).

Methylation of the ANGPTL2 CpG-island in OC cell lines. To show the potential role of methylation within the CpG-island in the silencing of ANGPTL2, we first assessed the methylation status of each CpG site around the ANGPTL2 CpG-island (Fig. 2A) in OC lines with or without ANGPTL2 expression and the OSE-2a cells, by bisulfite sequencing. ANGPTL2 expression was observed in cells either with or without 5-aza-dCyd treatment. Bottom, restored ANGPTL2 expression in HNOA and HTBOA cell lines after treatment with 5-aza-dCyd (5 or 10 \(\mu\)mol/L) for 5 d with or without TSA (100 ng/mL) for the last 12 h. Notably, almost no effect of TSA treatment on ANGPTL2 expression was observed in cells either with or without 5-aza-dCyd treatment. Bottom, restored ANGPTL2 expression in RMUG-L, RMUG-S, W3UF, MCAS, HiAnu, ES-2, and OVIS cell lines after treatment with 5-aza-dCyd, which showed reduced expression of ANGPTL2 mRNA (Fig. 1D) and a methylated pattern (Fig. 1B). D, promoter activity of the ANGPTL2 CpG-rich region around the CpG-island, pGL3 basic empty vectors (mock) and constructs containing one of three different sequences around the highly methylated region of ANGPTL2 (fragments 1–3; 542, 225, and 150 bp in size, respectively, in A) were transfected into HNOA and HTBOA cells. Luciferase activity was normalized vs. an internal control. Columns, means for three separate experiments, each performed in triplicate; bars, SD.

\(^{11}\) http://www.ebi.ac.uk/emboss/cpgplot/
means of bisulfite sequencing. CpG sites around the ANGPTL2 CpG-island tended to be extensively (HNOA, HTBOA, and W3UF) or partially (ES-2, OVISE, and HMKOA) methylated in the non-expressing cell lines, whereas ANGPTL2-expressing OC lines (KK, HUOA, HTOA, and HT) and OSE-2a cells were almost unmethylated (Fig. 2B). We then compared the methylation and expression status of ANGPTL2 in a larger number of OC lines by COBRA covering the region around the CpG-island (Fig. 2A) and MSP designed to target the region around TSS (Fig. 2A), because two BstUI restriction sites for COBRA may fail to detect DNA methylation around TSS. Consistent with the results of bisulfite sequencing, no methylated allele was detected among any of the OC cell lines with ANGPTL2 expression and OSE-2a cells with either method (Fig. 2B; Supplementary Fig. S2). On the other hand, a methylated allele was detected in 10 of 15 OC cell lines lacking ANGPTL2 expression by either method, although some of these cell lines retained an unmethylated allele. Five of the 15 OC cell lines (KF28, KFr13, RMG-I, RMG-II, and HMOA) lacking ANGPTL2 expression were found to have only an unmethylated allele by either method, suggesting that mechanisms other than DNA methylation, including epigenetic silencing of transcription factors regulating ANGPTL2 transcription, or upstream components of the signaling pathway activating ANGPTL2 expression, also contribute to the silencing of ANGPTL2 directly or indirectly.

To investigate whether DNA demethylation could restore the expression of ANGPTL2 mRNA, we treated OC cells lacking ANGPTL2 expression with 5-aza-dCyd. The induction of ANGPTL2 mRNA expression occurred after treatment with 5 or 10 μmol/L of 5-aza-dCyd. The induction of ANGPTL2 mRNA expression occurred after treatment with 5 or 10 μmol/L of 5-aza-dCyd.

Figure 3. Methylation and expression status of ANGPTL2 in primary tumors of OC. A, representative results of a MSP analysis (left) and bisulfite sequencing (right) of the ANGPTL2 promoter region in primary OC tissues. Left, in the MSP analysis, parallel amplification reactions were performed using primers specific for unmethylated (U) or methylated (M) DNA. Right, methylation status of ANGPTL2 determined by bisulfite sequencing in tumor samples. See legend of Fig. 2A for interpretation. B, representative results indicating an inverse correlation between methylation status of ANGPTL2 determined by MSP and mRNA expression status determined by quantitative real-time RT-PCR in four primary tumors. C, representative results of immunohistochemical staining of ANGPTL2 protein in normal human ovarian epithelial cells and primary OC tumors. ANGPTL2 expression is shown in normal ovarian epithelial cells (top left). In primary OC, strong (OVC1T and OV81ST) or very weak (OVS15T and OVE8T) expression of ANGPTL2 was observed (original magnification, ×200). D, Kaplan-Meier curves for overall survival rates of patients at all stages (left), stage I and II (middle), and stage III and IV OC (right). In overall survival, no significant difference was observed between the patients with positive ANGPTL2 expression and those with negative ANGPTL2 expression in all stages (P = 0.600). In stage I and II disease, however, negative ANGPTL2 immunoreactivity in tumor cells was significantly associated with a worse overall survival (P = 0.0432), whereas positive ANGPTL2 immunoreactivity was significantly associated with a worse overall survival in stage III and IV disease (P = 0.0039).
To determine if the aberrant methylation of ANGPTL2 also takes place in primary tumors of OC, we did MSP analysis with primer sets targeting the sequence around the most frequently methylated sites (Fig. 2A) in 45 primary cases. Consistent with the results of bisulfite sequencing and COBRA (Fig. 2A and B), a representative cell line lacking ANGPTL2 expression (HNOA) was methylated, whereas the ANGPTL2-expressing cell line (OSE-2a) was unmethylated (Fig. 3A). We detected ANGPTL2 hypermethylation in 11 of the 45 primary OCs (24%; Supplementary Table S4; Fig. 3A; data not shown). To quantitatively confirm the results of MSP analysis, we performed bisulfite sequencing in some representative cases. Aberrant methylation in a pattern similar to that observed in OC lines lacking ANGPTL2 expression was observed in OC tissues, which showed a methylation pattern in the MSP, whereas tumors with an unmethylated pattern in the MSP showed hypomethylation in bisulfite sequencing (Fig. 3A; data not shown).

In four cases of OC in which tumor samples were available for triple analyses, i.e., a methylation analysis, real-time RT-PCR, and immunohistochemistry, we compared the expression status of ANGPTL2 with its methylation status (Fig. 3A–C). Methylation-positive tumors tended to express fewer ANGPTL2 mRNA than methylation-negative tumors even in this small number of cases. In addition, methylation-negative tumors showed positive ANGPTL2 immunostaining in >10% of cancer cells, whereas methylation-positive tumors showed positive ANGPTL2 staining in <10% of cancer cells, suggesting that methylation is one of the mechanisms suppressing the mRNA and protein expression of ANGPTL2. In neighboring nonneoplastic epithelia, staining for ANGPTL2 was observed (Fig. 3C).

Association between expression of ANGPTL2 protein and clinicopathologic characteristics in primary cases. To clarify the clinical significance of ANGPTL2 in OC, the expression level of ANGPTL2 protein in 100 primary OC tissues was evaluated by immunohistochemistry. Negative and positive immunoreactivities of ANGPTL2 (Supplementary Fig. S1) were found in 51 (51%) and 49 (49%) of 100 cases, respectively. The relationship between the expression of ANGPTL2 protein and the clinicopathologic characteristics in primary cases is summarized in Table 1. In 45 cases from which high-quality DNA was available for a MSP, the methylation status of ANGPTL2 was inversely correlated with the expression of ANGPTL2 protein ($P = 0.0402$).

ANGPTL2 protein expression in each sample was not associated with age, histologic subtype, tumor staging, the age of patients, the results of surgery, or peritoneal cytology, although data were not fully available for some of those variables. Methylation status in each sample was not associated with these characteristics either (Supplementary Table S4). In overall survival (Fig. 3D), no significant difference was observed between the patients with negative and positive ANGPTL2 at all stages. In stage I and II disease, however, negative ANGPTL2 immunoreactivity in tumor cells was significantly associated with a worse overall survival ($P = 0.0432$), whereas positive ANGPTL2 immunoreactivity was significantly associated with a worse overall survival in stage III and IV disease ($P = 0.0039$).

### Table 1. Correlation between clinical background and expression of ANGPTL2 protein

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NOTE: Statistically significant values are in boldface type.


*ANGPTL2 protein expression was evaluated by immunohistochemical analysis as described in Materials and Methods.

$^a$ No information was available in five cases.

$^b$ No high-quality DNA for methylation analysis was available in 55 cases.

5-aza-dCyd in HNOA and HTBOA cell lines (Fig. 2C). In addition, treatment with TSA had no effect on the ANGPTL2 mRNA expression with or without 5-aza-dCyd in those cell lines, suggesting that DNA methylation is of primary importance for epigenetic silencing in OC cell lines. Restoration of ANGPTL2 expression by 5-aza-dCyd was also observed in other cell lines, such as RMUG-L, RMUG-S, W3UF, MCAS, HIOAnu, ES-2, and OVISe, lacking expression and showing methylation of ANGPTL2 in the COBRA and/or MSP analyses (Fig. 2C). Among ANGPTL2 nonexpressing cell lines showing no methylation pattern in both COBRA and MSP analyses, almost no restoration of ANGPTL2 expression was observed in RMG-I, RMG-II, and KFR13 cell lines, whereas a little restoration was observed in HNOA and KF28 cell lines by 5-aza-dCyd alone (Supplementary Fig. S3). TSA alone did not affect the expression of ANGPTL2 in those lines, but induced expression in combination with 5-aza-dCyd in some cell lines (Supplementary Fig. S3).

Promoter activity of the sequence around the ANGPTL2 CpG-island. Because the sequence around the ANGPTL2 CpG-island seems to be a target for methylation-mediated gene silencing, we tested three fragments designed according to the results of bisulfite sequencing for promoter activity (fragments 1–3 in Fig. 2A). All three fragments showed a remarkable increase in transcriptional activity in HNOA and HTBOA lines (Fig. 2D), suggesting that the region around the ANGPTL2 CpG-island, especially the sequence around TSS, contains critical sequences for basal gene expression and may be a target for methylation.
Suppression of cell growth induced by ANGPTL2 in OC cells.

To investigate if restoration of ANGPTL2 expression would suppress the growth of ANGPTL2-nonexpressing OC cells, we performed a colony formation assay using an expression construct of the full-coding sequence of ANGPTL2 in HNOA, KFr13, and OVSAHO cell lines (Fig. 4A). Two weeks after transient transfection and subsequent selection of drug-resistant colonies, the number of larger colonies produced by ANGPTL2-transfected cells decreased compared with those of cells transfected with empty vector.

To avoid nonspecific toxicity on OC cells from the forced expression of ANGPTL2, we next assessed the effect of the ANGPTL2 protein treatment on the growth of OC cells. Instead of using purified ANGPTL2, as reported by Zhang and colleagues (18), we used Myc-tagged and His-tagged ANGPTL2 protein–containing conditioned medium prepared from epitope-tagged ANGPTL2 expression vector–transfected SAS cells, which grow in serum-free medium; conditioned medium from mock-transfected cells served as a negative control. The culture supernatant contained secreted ANGPTL2.

To avoid nonspecific toxicity on OC cells from the forced expression of ANGPTL2, we next assessed the effect of the ANGPTL2 protein treatment on the growth of OC cells. Instead of using purified ANGPTL2, as reported by Zhang and colleagues (18), we used Myc-tagged and His-tagged ANGPTL2 protein–containing conditioned medium prepared from epitope-tagged ANGPTL2 expression vector–transfected SAS cells, which grow in serum-free medium; conditioned medium from mock-transfected cells served as a negative control. The culture supernatant contained secreted ANGPTL2.

Figure 4. A, effects of restoration of ANGPTL2 expression on growth of OC cell lines lacking expression of this gene using colony-formation assay. Cells were transiently transfected with a Myc-tagged and His-tagged construct containing ANGPTL2 (pcDNA3.1-ANGPTL2-Myc-His), or empty vector (mock), and selected for 2 wk 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 pcDNA3.1-ANGPTL2-Myc-His expressed Myc-tagged and His-tagged ANGPTL2. Top right, 2 wk after transfection and subsequent selection of drug-resistant colonies, the number of larger colonies produced by ANGPTL2-transfected cells decreased compared with those of cells transfected with empty vector.

B, production of Myc-tagged and His-tagged ANGPTL2 by transfected SAS cells. Western blotting of pooled and concentrated conditioned medium of SAS cells transfected with pcDNA3.1-ANGPTL2-Myc-His encoding Myc-tagged and His-tagged ANGPTL2 (lane 1) or pcDNA3.1-mock vector (lane 2), probed with ANGPTL2-specific antibody. Two major bands, one at the position expected for Myc-tagged and His-tagged ANGPTL2, and the other a smaller peptide, which may be produced through partial proteolysis (18).

C, effects of human ANGPTL2 on growth of OC cells. OC cell lines lacking expression of ANGPTL2 were treated with RPMI 1640 containing 0.2% serum and 1% conditioned medium, which was obtained from the culture of pcDNA3.1-ANGPTL2-Myc-His- or the control pcDNA3.1-mock-transfected SAS cell line lacking expression of ANGPTL2 under serum-free conditions, and concentrated (1:100). Top, cell viability was determined by WST assay for 72 h. The percentage of absorbance relative to mock conditioned medium–treated cells at each indicated time (%). Columns, means of three separate experiments, each performed in triplicate; bars, SD. Statistical analysis used the Mann-Whitney U test: *, P < 0.05 vs. control. Bottom, representative results of the population in each phase of the cell cycle assessed by FACS 48 h after treatment. A similar result was obtained in the HNOA cell line (data not shown).

D, effect of knockdown of endogenous ANGPTL2 on growth of OC cells. ANGPTL2-specific siRNA (50 nmol/L) or a control siRNA for the luciferase gene (Luc-siRNA) was transfected into OC cell lines expressing (HTOA and HT) or lacking (OVSAHO) ANGPTL2, and the number of viable cells after transfection was assessed at the indicated times by WST assay. The percentage of absorbance relative to Luc-siRNA–treated cells at each indicated time (%). Western blot confirmed the knockdown of endogenous ANGPTL2 protein expression in HTOA and HT cell lines for 96 h. Because OVSAHO cells lack ANGPTL2 expression through homozygous deletion, its protein expression was not detected. Columns, means for triplicate experiments, each performed in triplicate; bars, SD. Statistical analysis used the Mann-Whitney U test: *, P < 0.05 vs. control.
epitope-tagged ANGPTL2 protein migrating with the expected size with some cleaved forms (Fig. 4B). Treatment with the conditioned medium containing ANGPTL2 for 72 hours reduced cell viability in the HNOA, KFr13, and OVS/AHO cell lines compared with control conditioned medium from mock-transfected cells (Fig. 4C, top). Upon treatment with conditioned medium containing ANGPTL2, the number of cells decreased with time, most notably, in the HNOA and KFr13 lines. The growth-suppressive effect of conditioned medium containing ANGPTL2 on those OC cell lines decreased by partial but significant depletion of ANGPTL2 (Supplementary Fig. S4). In the FACS analysis of OVS/AHO cells, treatment with the conditioned medium containing ANGPTL2 resulted in an accumulation of cells in G0-G1 phase but no increase in sub-G1 phase cells compared with control conditioned medium, whereas in HNOA and KFr13 cells, treatment with the conditioned medium containing ANGPTL2 resulted in an accumulation of cells in sub-G1 phase (Fig. 4C, bottom), indicating that ANGPTL2 induced cell death in the HNOA and KFr13 lines, and suggesting that effects of ANGPTL2 on OC cells depend on the cell type, although its receptors and downstream intracellular signaling pathways remain unknown. 

To confirm the growth-suppressive effect of ANGPTL2, endogenously expressed ANGPTL2 was knocked down through the introduction of ANGPTL2-specific siRNA (ANGPTL2-siRNA) into the HTOA and HT cell lines (Fig. 4D). Transfection of ANGPTL2-siRNA showed small but significant growth-accelerating effects on those cells compared with that of Luc-siRNA, whereas the introduction of ANGPTL2-siRNA into OVS/AHO cells with a homozygous deletion of ANGPTL2 had no effect on cell growth compared with that of Luc-siRNA, suggesting that the growth-promoting effect of ANGPTL2-siRNA on HTOA and HT cells is unlikely to be caused by its nonspecific/off-target effects.

Discussion

In this study, we identified ANGPTL2 as one of the targets for inactivation in OC from the homozygous loss at 9q33.3, although the possible involvement of other target genes within this region remains unclear. ANGPTL2 was frequently silenced in OC cell lines through DNA methylation within sequences around the CpG-island exhibiting promoter activity. In primary OCs, lower ANGPTL2 protein levels were frequently observed, and the clinical significance of ANGPTL2 expression might differ among disease stages. In addition, the ectopic expression of ANGPTL2 or treatment with conditioned medium containing ANGPTL2 inhibited the growth of ANGPTL2-nonexpressing cells, whereas knockdown of ANGPTL2 accelerated the growth of ANGPTL2-expressing cells, suggesting that ANGPTL2 is likely to work as a functional TSG for OC in a stage-dependent manner.

ANGPTL2 is located at 9q33.3. Although deletion or loss of heterozygosity around 9q33-q34 in OC has been reported previously (19–21), candidates for TSGs within this region have never been identified. The frequent silencing of ANGPTL2 in cell lines and primary tumors of OC suggests that this gene is one of the targets for 9q33-q34 deletion in this disease. In our array-CGH analysis of 24 cell lines, however, only three lines (12.5%) showed a deletion pattern around ANGPTL2: one had a homozygous deletion and two had a hemizygous deletion. In addition, two lines having a hemizygous deletion expressed ANGPTL2, suggesting that the silencing of this gene was not simply caused by the deletion of one allele and some other mechanisms, including methylation, in the retained allele. It is possible that additional TSGs other than ANGPTL2 may exist as targets for deletion around 9q33-q34.

The CpG sites around the ANGPTL2 CpG-island, whose methylation was associated with the silencing of this gene in some OC cells, showed clear promoter activity. The OC cell lines and immortalized normal ovarian epithelial cell line expressing ANGPTL2 showed an almost unmethylated pattern around the ANGPTL2 CpG-island. In addition, demethylation through treatment with 5-aza-dCyd in OC lines with the ANGPTL2 methylation restored its expression. Therefore, methylation around the ANGPTL2 CpG-island may contribute to the silencing of this gene. However, some OC cell lines showed reduced ANGPTL2 expression without DNA methylation, and the effect of 5-aza-dCyd alone on ANGPTL2 expression was different among those cell lines. In addition, reduced ANGPTL2 protein expression was observed more frequently than ANGPTL2 methylation in primary OCs, suggesting that epigenetic modifications other than DNA methylation in the ANGPTL2 gene and/or unknown transcriptional regulatory mechanisms also contribute to the silencing of ANGPTL2. Further analyses will be needed to clarify all the mechanisms for ANGPTL2's inactivation and the functional significance of each mechanism in OC.

There are seven known members of the ANGPTL family that share limited sequence homology with angiopoietins (22). Similar to angiopoietins, each ANGPTL protein contains an NH2-terminal coiled-coil domain and a COOH-terminal fibrinogen-like domain. Unlike angiopoietins, they do not bind to the Tie-2 or Tie-1 receptor (22), and their receptors and downstream signal transduction pathways remain unknown, suggesting that ANGPTLs may have different biological functions than angiopoietins. Although the antiproliferative and antistromal effects of ANGPTL4 (23, 24), a well characterized member of this family, and DNA methylation of this gene (25) have been reported in some cancers, the expression and methylation status of ANGPTL2 in cancer and their contribution to carcinogenesis have never been previously reported. Although Zhang and colleagues (18) reported very recently that ANGPTL2 and ANGPTL3 stimulate the ex vivo expansion of hematopoietic stem cells, their physiologic and pathologic functions remain to be discovered. Notably, we observed clear growth-suppressive effects, such as cell death and G0-G1 arrest of ANGPTL2-containing conditioned medium on OC cells, although the effects differed among the OC lines. The growth-suppressive effects of conditioned medium containing His-tagged ANGPTL2 protein significantly decreased after its partial depletion from conditioned medium using Ni-charged resin. These results suggest that (a) the growth-suppression of OC cells is not a nonspecific toxic effect of the conditioned medium, and (b) the receptors and/or downstream signaling pathways of ANGPTL2 and/or their cross-talk with other signaling pathways, which are still unknown, might differ among cell and/or tissue lineages.

Because the expression status of ANGPTL2 significantly correlates with the survival of patients with OC in a stage-dependent manner, it was suggested that ANGPTL2 shows various biological functions in different stages and functions at least partly as a conditional tumor suppressor for OC. Therefore, the evaluation of the ANGPTL2 expression status with disease stage might be useful for predicting the progression or aggressiveness of this disease, although the question of how tumors in different stages are able to determine the action of ANGPTL2 in carcinogenesis deserves further investigation. We previously showed that CTGF, another bioactive cytokine, also shows conditional tumor-suppressor
activity for OC, although survival analysis in both early and advanced type OC did not reach a level of significance between CTGF-negative and positive cases (11). Because CTGF is one of the transcriptional targets for the transforming growth factor-β (TGFB) signaling pathway, we have speculated that CTGF expression in the advanced stage may be induced by TGFB in unmethylated tumors (11). It has been suggested that the expression of ANGPTL2 may also be regulated not only by methylation status but also by some upstream signaling pathways, including TGFB-signaling pathway exerting both tumor-suppressive activity and invasive/metastatic activities through epithelial-mesenchymal transition on epithelial cells, in a progression-dependent manner in OC. Alternatively, receptors and molecules in downstream signaling pathways for ANGPTL2 expressed in tumor cells or other cells, including endothelial cells, might be different among disease stages, resulting in different biological functions in a stage-dependent manner.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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